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Conservation in the first internal transcribed spacer region (ITS1) in *Hematodinium* species infecting crustacean hosts found in the UK and Newfoundland

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ABSTRACT: Parasitic dinoflagellates in the genus *Hematodinium* infect a number of decapod crustaceans in waters off the UK, including the Norway lobster *Nephrops norvegicus* and the edible crab *Cancer pagurus*. This study investigated sequence variability in the first internal transcribed spacer (ITS1) region of the ribosomal RNA complex of *Hematodinium* spp. infecting *N. norvegicus*, *C. pagurus*, and *Pagurus bernhardus* from 4 locations in the UK and from the *Hematodinium* sp. infecting *Chionoecetes opilio* from the province of Newfoundland and Labrador, Canada. Phylogenetic analysis of the *Hematodinium* ITS1 sequences from *N. norvegicus*, *C. pagurus*, *P. bernhardus* and *C. opilio* suggest that these crustaceans are infected with the same species of *Hematodinium*. Length variability of the ITS1 region was observed (324 to 345 bp) and attributed to 4 variable microsatellite regions $(CATG)_n$, $(GCC)_nTCCGC(TG)_n$, $(TA)_n$, and $(GAA)_n(GGA)_n$ within the sequenced ITS1 fragment. The observed variation may be due to co-infection of the host crustacean with several different strains of *Hematodinium* or differences among copies of ITS1 region within the genome of a single parasite cell. The *Hematodinium* ITS1 sequence from *N. norvegicus*, *C. pagurus*, *P. bernhardus* and *C. opilio* isolates was sufficiently conserved in primer binding regions targeted by previous molecular diagnostic assays; therefore, we suggest that this assay could be used to screen for *Hematodinium* infections in these crustacean hosts.

KEY WORDS: *Hematodinium* · Conservation of ITS1 · Crustacean hosts · Parasite

INTRODUCTION

Several marine crustaceans are parasitized by dinoflagellates of the genus *Hematodinium*. The type species, *Hematodinium perezi*, was first found to infect the shore crab *Carcinus maenas* and the harbour crab *Liocarcinus depurator* and was described by Chatton & Poisson (1931). It has since been tentatively recorded from the blue crab *Callinectes sapidus* (Newman & Johnson 1975). A second species, *H. australis*, was described from the sand crab *Portunus pelagicus* by Hudson & Shields (1994). *Hematodinium*-like dinoflagellates are found to infect many commercially important crustaceans (see Stentiford & Shields 2005 for review) including the edible crab *Cancer pagurus* (Latrouite et al. 1988), the Norway lobster *Nephrops norvegicus* (Field et al. 1992), the velvet swimming crab *Necora puber* (Wilhelm & Mialhe 1996), the tanner crab *Chionoecetes bairdi* (Meyers et al. 1987), the snow crab *Chionoecetes opilio* (Taylor & Khan 1995) and recently the grooved tanner crab *Chionoecetes tanneri* (Bower et al. 2003). *Hematodinium*-like infections have caused significant annual economic losses to several national and international fisheries. These losses are due to morbidity (e.g. bitter crab disease) or mortality caused by *Hematodinium* spp.

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In waters surrounding the UK *Hematodinium*-like infections have been reported in *Nephrops norvegicus* from the Clyde and Irish seas (Field et al. 1992, 1998, Briggs & McAliskey 2002) and in *Cancer pagurus* from the English Channel (Stentiford et al. 2002) and from the Atlantic Ocean (Anonymous 2004). Both *N. norvegicus* and *C. pagurus* support large and valuable fisheries in UK waters; however, it is unknown whether different species of *Hematodinium* parasites infect each of these different hosts or whether one species infects multiple hosts.

PCR-based diagnostics to detect *Hematodinium* spp. have been developed for detecting infections in several host species (Hudson & Adlard 1994, Gruebl et al. 2002, Small et al. 2006, 2007). In the first 2 studies the primer binding regions were in the 18S and 5.8S ribosomal RNA genes and are almost certainly genus specific, not species specific, due to the conserved nature of these genes. Small et al. (2006) developed a potentially species-specific set of PCR primers to target the *Hematodinium* sp. infecting *Nephrops norvegicus* using the conserved 18S rRNA gene and variable first internal transcribed spacer region (ITS) of the rRNA gene complex. This PCR assay also detected infections in *Cancer pagurus* from the English Channel tentatively suggesting that this is either the same species or a closely related species to that infecting *N. norvegicus*.

The ribosomal RNA gene complex is present as tandemly repeated clusters of highly conserved genes encoding the 18S, 5.8S and 28S rRNA genes, which are separated by variable spacer sequences (Long & Dawid 1980). Sequence variation in the ITS regions has been used to distinguish among species and strains of many other dinoflagellates and parasites, including *Perkinsus marinus* (Goggin 1994, Reece et al. 1997, Brown et al. 2004), *Pfiesteria shumwayae* (Litaker et al. 2003), trichomonad protozoans (Felleisen 1997), *Entamoeba* spp. (Som et al. 2000) and *Hematodinium* spp. (Hudson & Adlard 1996). However, Small et al. (2006, 2007) observed that partial ITS1 region sequences from the *Hematodinium* spp. infecting *Nephrops norvegicus* and *Callinectes sapidus* were only 77 and 82% similar, respectively, to the same sequences in Hudson & Adlard's (1996) study, revealing an uncertainty over the sequence data and species hypothesis contained within that report.

In the current study DNA sequence variation of 16 *Hematodinium* spp. isolates from 5 geographically distinct areas, encompassing 4 host crustacean species (Table 1) was examined at the ITS1 rRNA locus to investigate similarities and differences among *Hematodinium* spp. infecting various hosts and assess the potential application of the PCR assay developed by Small et al. (2006) to detect infections in other crustaceans.

MATERIALS AND METHODS

Sample collection. Fourteen field isolates of *Hematodinium* spp. were obtained from 3 crustacean species. In addition, 2 *in vitro* culture samples of *Hematodinium* spp. were also obtained (see Table 1 for details). Infected haemolymph, hepatopancreas, gill tissue and cell culture samples were collected and preserved in 100% ethanol before DNA extraction. All isolates originated from crustaceans found in geographically separate locations surrounding the UK (Fig. 1), except for 2 samples of *Hematodinium* sp. from *Chionoecetes opilio* from Conception Bay, Newfoundland and Labrador, Canada.

DNA extraction and amplification. DNA was extracted from preserved tissues using a Qiagen DNeasy Tissue Kit following the manufacturer's protocol (Qiagen). The 3′ end of the 18S, complete ITS1 and the 5′ end of the 5.8S rDNA genes were amplified using the forward primer (5′-GTT-CCC-CTT-GAA-GGA-GGA-ATT-C-3′) and reverse primer (5′-CGC-ATT-TCG-CTG-CGT-TCT-TC-3′) previously described by Hudson & Adlard (1994). The amplification conditions used were those described previously by Hudson & Adlard (1994). Amplification products (5 µl) were electrophoresed on a 1.5% (w/v) agarose gel, stained with ethidium bromide and viewed under a UV light source.

Table 1. *Hematodinium* spp. Source and number of *Hematodinium* samples used in this study

Sample name	No. of individuals sampled	Host species	Sample location	rRNA GenBank sequences
Nn1–3	3	*Nephrops norvegicus*	Clyde Sea, UK	EF031966–EF031974
Nn4–6	3	*Nephrops norvegicus*	Irish Sea, UK	EF031975–EF031983
Cp1–3	3	*Cancer pagurus*	English channel, UK	EF031984–EF031992
Cp4–6	3	*Cancer pagurus*	NE Atlantic	EF031993–EF032001
Co1–2	2	*Chionoecetes opilio*	Conception Bay, Newfoundland	EF032002–EF032007
IvNn1	1	*Nephrops norvegicus*	Clyde Sea, UK	EF032008–EF032010
IvPb1	1	*Pagurus bernhardus*	Clyde Sea, UK	EF032011–EF032013

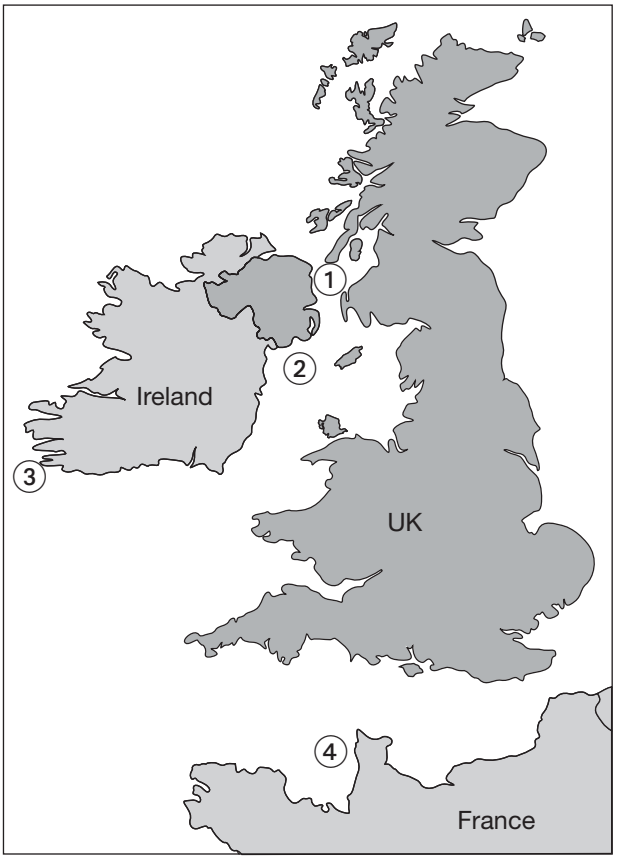

Fig. 1. *Hematodinium* spp. Locations in UK and Ireland where parasite infected crustaceans were sampled. 1, Clyde Sea area; 2, Irish Sea; 3, northeast Atlantic Ocean; 4, English Channel

Cloning and sequencing. PCR amplification products of approximately 680 bp were excised from agarose gels and purified using a QIA-quick gel extraction kit (Qiagen). Fragments were cloned into the plasmid pCR4-TOPO (Invitrogen) and transformed into *E. coli* using a TOPO TA Cloning Kit (Invitrogen) following the manufacturer's protocols. Transformed bacterial colonies were screened for inserts using a heat-lysis method and subsequent PCR assay employing the M13 forward and reverse primer pairs supplied in the cloning kit. Briefly, bacterial colonies were picked from the agar plates using a sterile wooden toothpick and then inoculated into 10 µl of sterile deionised water. The bacterial cell suspensions were heated for 4 min at 95°C and 0.5 µl of the lysis preparation was used as a template in a PCR screen for successful transformation. The amplification reaction mixtures contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, each primer at 1.0 µM, 0.32 U *Taq* DNA polymerase (Invitrogen), 0.2 mg ml^{-1} BSA and sterile deionized water to a final volume of 12.5 µl. Thermocycling conditions were as follows: an initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, primer annealing at 54°C for 30 s and extension at 72°C for 1 min with the final cycle incorporating a 5 min extension step at 72°C. Following amplification a 5 µl aliquot of each PCR product was electrophoresed on a 2% (w/v) agarose gel and visualized as above. PCR products from clones containing the correct insert size were treated with shrimp alkaline phosphatase (SAP) and exonuclease I (*Exo* I) (Amersham Biosciences) to degrade unincorporated nucleotides and single stranded DNA (primers) remaining after PCR. Five microliters of the M13 PCR product were combined with 0.5 units of SAP and 5.0 units of *Exo* I and incubated at 37°C for 30 min, 80°C for 15 min and 15°C for 5 s.

Plasmid inserts were sequenced bi-directionally using the Big Dye Terminator kit (Applied Biosystems) with M13 sequencing primers using 1/8th of the recommended Big Dye volume ratio dictated in the manufacturer's protocols. Each 5 µl reaction contained 0.0625 µl Big Dye, 0.96875 µl 5× buffer, 1.6 pmol of primer and 10 ng of *Exo* I/SAP treated PCR product. Thermocycling parameters were as follows: 96°C for 1 min, then 25 cycles of 96°C for 10 s, 50°C for 5 s, 60°C for 4 min followed by a final incubation at 4°C. The sequencing reaction products were precipitated using ethanol/sodium acetate (ABI User Bulletin, April 11, 2002). Precipitated sequencing reactions were resuspended in 20 µl of Hi-Di formamide (Applied Biosystems) and 10 µl of each was electrophoresed on an ABI 3100 or 3130 Prism genetic analyzer (Applied Biosystems). Three DNA clones from each sample were sequenced bidirectionally.

Analysis of sequence data. The *Hematodinium* rRNA sequences were aligned using the CLUSTAL-W algorithm in the MacVector DNA sequence analysis package (Accelrys) using gap penalties of 8 for insertions and 3 for extensions, in both pairwise and multiple alignment phases, then edited visually. Primer sequences were removed from the 5′ and 3′ end of all sequences generated. To accurately assess sequence variation between samples within the complete ITS1 region the boundaries between the 3′ end of the 18S rRNA gene, the ITS1 region and the 5′end of the 5.8S rRNA gene were identified using data from S1 nuclease mapping of *Prorocentrum minimum* (Maroteaux et al. 1985) and rRNA gene sequence data from *Syndinium turbo* (Skovgaard et al. 2005). The 3′ end of the 18S rRNA gene was defined by the sequence 5′-CCT- GCG-GAA-GGA-TCA-TTC-3′ and the 5′ end of the 5.8S rRNA gene was defined by 5′-ATT-TTA-GCG- ATG-AAT-GCC-3′. The partial 18S and 5.8S rRNA gene sequences were removed from the alignment. The ITS1 region sequences were aligned with *Hematodinium* spp. ITS1 sequences in GenBank (DQ084245, DQ084246 and DQ925227 to DQ925236)

using the same gap penalties (above) and edited visually. MEGA3 (Kumar et al. 2004) was used to calculate pairwise distances (uncorrected genetic distance '*p*' values) between clones and isolate groups (same host species and geographical location).

PCR detection. PCR primers (18S F2 and ITS R1) previously designed to amplify rDNA from the *Hematodinium* sp. infecting *Nephrops norvegicus* (Small et al. 2006) were tested to evaluate whether they could be used to detect the *Hematodinium* spp. present in other crustacean samples. This was done by analysis of the primer binding locations in the 18S rRNA and ITS1 region sequences and testing by means of standard PCR. Amplification reaction mixtures contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 mM $MgCl_2$, 0.2 mM dNTPs, each primer at 5.0 µM, 0.4 U *Taq* DNA polymerase (Invitrogen), 50 to 100 ng DNA template and sterile deionized water to a final volume of 20 µl. Thermocycling conditions were as follows: an initial denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 56°C for 30 s, chain extension at 72°C for 90 s, with the final cycle incorporating a 5 min extension step at 72°C. Following amplification a 5 µl aliquot of each PCR reaction was electrophoresed on a 2% agarose gel and visualized as above.

RESULTS

rRNA sequence data

The expected ~680 bp 18S-ITS1-5.8S fragments were amplified from all DNA samples. These were cloned, sequenced and deposited in GenBank (see Table 1). The partial 18S and 5.8S rRNA genes were conserved in all isolates sequenced (4 single nucleotide polymorphisms (SNPs) in the 18S rRNA gene sequences and none in the 5.8S region). Alignment of the sequences generated in this study with partial 18S and partial ITS1 region sequences in GenBank for the *Hematodinium* sp. from *Nephrops norvegicus* (Accession Nos. DQ084245 and DQ084246) indicated that all sequences obtained originated from *Hematodinium* and not from the host or other contaminant.

The complete ITS1 region sequences from *Hematodinium* spp. infecting *Nephrops norvegicus, Cancer pagurus, Pagurus bernhardus* and *Chionoecetes opilio* were very similar to each other (>98% similarity) and varied in length between 324 and 345 bp (Table 2). Apart from sequences generated from the *in vitro* culture from infected *P. bernhardus*, from which all clones had an ITS1 region length of 330 bp, the ITS1 region varied even between *Hematodinium* samples from the same location and crustacean host. The sequence alignment revealed the presence of 4 different microsatellites responsible for the length variation of the ITS1 region (Table 3). The insertion of an adenine at the 72 bp position of the ITS1 alignment was observed in all clone sequences from the *P. bernhardus* isolate only and led to the extension (n = 3) of a tetranucleotide microsatellite motif. In multiple instances clone sequences generated from the same sample showed variation in the number of microsatellites. For example, the *Hematodinium* ITS1 region from the second infected *N. norvegicus* sample from the Clyde Sea area (Nn2) had a TG motif (at 103 bp of the alignment) repeated 11, 12 and 13 times, respectively, in the 3 clones sequenced. Eighteen different SNPs (at different locations) were detected between the 48 ITS1

Table 2. *Hematodinium* spp. Size of partial 18S rRNA gene, complete ITS1 region, and partial 5.8S rRNA gene region for the organism studied

Hematodinium spp. from host (no. of clones)	Partial 18S (bp)	ITS1 (bp)	Partial 5.8S (bp)
Nephrops norvegicus (18)	217	336–345	35
Cancer pagurus (18)	217	329–333	35
Chionoecetes opilio (6)	217	324–333	35
Nephrops norvegicus in vitro culture (3)	217	339–345	35
Pagurus bernhardus in vitro culture (3)	217	330	35

Table 3. *Hematodinium* spp. ITS1 region microsatellites responsible for length variation. See Table 1 for sample sources

Sample	ITS1 region alignment position			
	66 $(CATG)_n$	83 $(GCC)_nTCCGC(TG)_n$	254 $(TA)_n$	294 $(GAA)_n(GGA)_n$
Nn1	$(CATG)_1$	$(GCC)_3TCCGC(TG)_{8-9}$	$(TA)_2$	$(GAA)_{4-5}(GGA)_6$
Nn2	$(CATG)_1$	$(GCC)_3TCCGC(TG)_{11-13}$	$(TA)_2$	$(GAA)_4(GGA)_{5-6}$
Nn3	$(CATG)_1$	$(GCC)_{3-5}TCCGC(TG)_{8-9}$	$(TA)_2$	$(GAA)_{4-5}(GGA)_{5-6}$
Nn4	$(CATG)_1$	$(GCC)_{3-5}TCCGC(TG)_8$	$(TA)_2$	$(GAA)_{4-5}(GGA)_6$
Nn5	$(CATG)_1$	$(GCC)_3TCCGC(TG)_{8-9}$	$(TA)_2$	$(GAA)_{4-5}(GGA)_{5-6}$
Nn6	$(CATG)_1$	$(GCC)_5TCCGC(TG)_9$	$(TA)_2$	$(GAA)_4(GGA)_6$
Cp1	$(CATG)_1$	$(GCC)_3TCCGC(TG)_{8-9}$	$(TA)_2$	$(GAA)_3(GGA)_5$
Cp2	$(CATG)_1$	$(GCC)_3TCCGC(TG)_9$	$(TA)_2$	$(GAA)_3(GGA)_5$
Cp3	$(CATG)_1$	$(GCC)_3TCCGC(TG)_{8-9}$	$(TA)_2$	$(GAA)_3(GGA)_5$
Cp4	$(CATG)_1$	$(GCC)_3TCCGC(TG)_{7-9}$	$(TA)_2$	$(GAA)_3(GGA)_5$
Cp5	$(CATG)_1$	$(GCC)_3TCCGC(TG)_{7-9}$	$(TA)_2$	$(GAA)_3(GGA)_5$
Cp6	$(CATG)_1$	$(GCC)_3TCCGC(TG)_{7-9}$	$(TA)_2$	$(GAA)_3(GGA)_5$
Co1	$(CATG)_1$	$(GCC)_3TCCGC(TG)_{5-6}$	$(TA)_{2-3}$	$(GAA)_{3-4}(GGA)_{4-6}$
Co2	$(CATG)_1$	$(GCC)_3TCCGC(TG)_6$	$(TA)_3$	$(GAA)_{3-4}(GGA)_5$
IvNn1	$(CATG)_1$	$(GCC)_{3-5}TCCGC(TG)_9$	$(TA)_2$	$(GAA)_4(GGA)_6$
IvPb1	$(CATG)_3$	$(GCC)_3TCCGC(TG)_6$	$(TA)_3$	$(GAA)_3(GGA)_5$

clone sequences analyzed, with approximately 90% of these being polymorphism transitions and 10% transversions. Generally, the polymorphisms were distributed evenly throughout the different ITS1 clones such that each sequence differed from the others by 1 or 2 bp.

ITS1 region sequences from the same group (same host species and geographical location) ranged from 98.6 to 99.7% similarity (Table 4). Identical ITS1 region sequences were found in multiple DNA clones from many isolates. The ITS1 region sequences from the *Hematodinium* spp. from *Nephrops norvegicus*, *Cancer pagurus*, and *Pagurus bernhardus* showed 99.0 to 100% similarity to each other, while the ITS1 region sequences from the *Hematodinium* sp. from *Chionoecetes opilio* showed >98% similarity to the other *Hematodinium* spp. sequences from UK crustacean hosts. All ITS1 region sequences from the *Hematodinium* sp. from *Callinectes sapidus* were 68.7 to 71.0% similar to the *Hematodinium* spp. sequences from the other crustacean hosts.

PCR detection

Analysis of the primer binding sites (18S F2 and ITS R1, Small et al. 2006a) in the *Hematodinium* spp. 18S rRNA gene and ITS1 region sequences revealed the conserved nature of the primer target regions in the *Hematodinium* sequences. Of the 48 sequences generated in this study there was 1 single base pair mismatch at the forward primer binding site (80 to 102 bp upstream of the 18S/ITS1 boundary) in one 18S rRNA gene sequence. This was caused by a transition at the 5th nucleotide position of the forward primer sequence. Analysis of the ITS1 region sequence alignment revealed one mismatch in the reverse primer binding region (256 to 277 bp downstream of the 18S/ITS1 boundary) in 1 clone sequence, caused by a transition at the 5th nucleotide position in the primer binding site.

Amplification of DNA samples extracted from infected *Nephrops norvegicus* from the Clyde and Irish seas using the primer pair 18S F2 and ITS R1 produced a single reaction product of ~380 bp. The primers also amplified the 380 bp diagnostic *Hematodinium* DNA fragment from infected *Cancer pagurus* from the English Channel and Atlantic Ocean from the *in vitro Hematodinium* spp. cultures isolated from infected *Pagurus bernhardus* and *N. norvegicus* and from infected *Chionoecetes opilio* from Conception Bay, on the island of Newfoundland (Table 5). The PCR primer pair did not generate any PCR product when tested against genomic DNA preparations from *Hematodinium* spp. infecting blue crabs, harbour crabs, or sand crabs *Portunus trituberculatus* (Table 5).

Table 4. *Hematodinium* spp. Range of sequence similarities and pairwise distances (uncorrected genetic distance '*p*' values) observed among rRNA ITS1 region sequences obtained from the isolates analyzed in this study and those from GenBank (from the *Hematodinium* sp. infecting *Callinectes sapidus*, DQ925227–DQ925236). The range (up to 100%) of observed similarity (within same host species and geographical location = group) is given across the diagonal. Sequence similarity ranges between groups are given above the diagonal and ranges for raw distance values are given below the diagonal

Host and sample name	Nn1–3	Nn4–6	Cp1–3	Cp4–6	Co1–2	IvNn1	IvPb1	DQ084245–DQ084246	DQ925227–DQ925236
Nephrops norvegicus (Clyde Sea, Nn1–3)	>99.0%	99.0–100.0%	99.0–100.0%	99.0–100.0%	98.2–100%	99.0–100.0%	99.0–100.0%	99.0–99.7%	68.7–70.3%
Nephrops norvegicus (Irish Sea, Nn4–6)	0.000–0.01	>99.3%	99.3–100.0%	99.3–100.0%	98.6–100.0%	99.3–100.0%	99.3–100.0%	99.3–99.7%	68.7–70.3%
Cancer pagurus (English Ch., Cp1–3)	0.000–0.01	0.000–0.007	>99.7%	99.3–100.0%	98.6–100.0%	99.3–100.0%	99.3–100.0%	99.3–99.7%	69.4–70.3%
Cancer pagurus (NE Atlantic, Cp4–6)	0.000–0.01	0.000–0.007	0.000–0.007	>99.7%	98.6–100.0%	99.3–100.0%	99.3–100.0%	99.3–99.7%	69.4–70.3%
Chionoecetes opilio (Newfoundland, Co1–2)	0.000–0.018	0.000–0.014	0.000–0.014	0.000– 0.014	>98.6%	98.6–100.0%	98.6–100.0%	98.6–99.7%	68.7–70.5%
Nephrops norvegicus (*In vitro*, IvNn1)	0.000–0.01	0.000–0.007	0.000–0.007	0.000–0.007	0.000–0.014	>99.3%	99.3–100.0%	99.3–99.7%	68.7–70.3%
Pagurus bernhardus (*In vitro*, IvPb1)	0.000–0.01	0.000–0.007	0.000–0.007	0.000–0.007	0.000–0.014	0.000–0.007	>99.7%	99.3–99.7%	69.4–70.3%
Nephrops norvegicus (DQ084245–DQ084246)	0.003–0.01	0.003–0.007	0.003–0.007	0.003–0.007	0.003–0.014	0.003–0.007	0.003–0.007	99.3%	69.4–71.0%
Callinectes sapidus (DQ925227–DQ925236)	0.297–0.313	0.297–0.313	0.297–0.306	0.297–0.306	0.295–0.313	0.297–0.313	0.297–0.306	0.290–0.306	>99.1%

Table 5. *Hematodinium* spp. Specificity of a previously developed PCR assay (Small et al. 2006) to detect parasitic infection in other crustacean hosts

Hematodinium infected host samples	PCR diagnosis
Nephrops norvegicus – Clyde Sea Area, UK (n = 6), Irish Sea, UK (n = 8)	+
Cancer pagurus – English Channel, UK (n = 4), 6 Atlantic Ocean (n = 6)	+
Pagurus bernhardus – Clyde Sea, UK (n = 1)	+
Chionoecetes opilio – Conception Bay, Newfoundland, Canada (n = 6)	+
Liocarcinus depurator – English Channel, UK (n = 4)	–
Callinectes sapidus – Virginia, USA (n = 6)	–
Portunus trituberculatus – Zhejiang Province, China (n = 3)	–

DISCUSSION

This study represents the first detailed analysis of the complete ITS1 rRNA region of *Hematodinium* spp. infecting several host species (*Nephrops norvegicus, Cancer pagurus, Chionoecetes opilio* and *Pagurus bernhardus*) from different geographic locations. Sequence analysis revealed that the ITS1 region from the *Hematodinium* species infecting *N. norvegicus, C. pagurus, P. bernhardus* and *C. opilio* were very similar; therefore, we suggest that these hosts are infected with the same species of parasite. Four microsatellites were found within the ITS1 region and account for the length variation observed. In addition, the primers previously designed by Small et al. (2006) to detect *Hematodinium* infections in *N. norvegicus* also detected the parasite infecting *C. pagurus* and *C. opilio* and amplified the DNA isolated from an *in vitro* culture of *Hematodinium* sp. from *P. bernhardus*. Consequently, this PCR-based diagnostic assay could be used to screen these crustacean species for the parasite.

Molecular sequence information has been used to supplement the morphological taxonomy of many marine parasites. Examples include the phylogenetic position of *Marteilia refringens* (Berthe et al. 2000), *Pfiesteria shumwayae* (Litaker et al. 2005), and the phylogenetic synonymization of *Perkinsus chesapeaki* and *P. andrewsi* (Burreson et al. 2005). Ribosomal DNA sequence variation has previously been used to discriminate between toxic and non-toxic strains of *Alexandrium tamarense* and *Pseudonitzschia* spp. (Scholin et al. 1994, Higman et al. 2001) and the geographic origin and identity of virulent *Perkinsus marinus* strains (Robledo et al. 1999, Reece et al. 2001). A PCR-based restriction fragment length polymorphism assay (PCR-RFLP) based on the ITS1 region has been used to differentiate between *Hematodinium* infections in *Callinectes sapidus*, *Liocarcinus depurator* and *Portunus trituberculatus* (Small et al. 2007) suggesting that this region can be used to identify different *Hematodinium* species. The use of the ITS1 region to differentiate between *Hematodinium* spp. is further supported by the large genetic distances observed in this study between the *Hematodinium* sp. from *C. sapidus* compared with *Hematodinium* spp. from *Nephrops norvegicus, Cancer pagurus* and *Chionoecetes opilio* (Table 4).

In this study 18 single nucleotide polymorphisms were found in the ITS1 region. The number of nucleotide substitutions (independent of microsatellites), 0.33×10^{-2} to 1.25×10^{-2} per bp, is larger than the reported *Taq* polymerase error rate (1.1×10^{-4}) (Tindall & Kunkel 1988). This is most likely due to the amplification and sequencing of different ITS1 region repeat motifs, as each SNP was only observed in single clone sequences similar to the observed SNPs in the ITS1 region from the *Hematodinium* sp. infecting *Callinectes sapidus* (Small et al. 2007). Based on the ITS1 region data, it appears that *Nephrops norvegicus*, *Cancer pagurus*, *Chionoecetes opilio* and *Pagurus bernhardus* are all infected with the same species of *Hematodinium*; this indicates that this parasite species is a host generalist with a broad geographic range.

Significant length variation in the ITS1 region (due to microsatellites) was observed in the clone sequences from all *Hematodinium* spp. ITS1 region sequences generated in this study. This is, therefore, the first report of microsatellites in *Hematodinium* spp. These short sequence repeats are extremely abundant in eukaryotic genomes and are believed to arise through 'slippage' events during DNA replication (Levinson & Gutman 1987, Pupko & Graur 1999). Microsatellites in the ITS regions have been found in other parasitic taxa including *Schistosoma* (Van Herwerden et al. 1998), *Trypanosoma* (Beltrame-Botelho et al. 2005) and *Metastrongylus* (Conole et al. 2001). In this study the ITS1 sequences from each isolate were examined by PCR amplification and sequencing of 3 clones. Apart from the *Pagurus bernhardus* isolate, in which all 3 clone sequences were the same length, the majority of the other isolates had different numbers of repeats between the 3 clones from the same sample. This may be due to infection of the crustacean host with multiple strains of *Hematodinium* with variable repeat numbers or due to variable ITS1 repeats within the genome of the parasite, as has been observed in similar organisms (Litaker et al. 2003, Brown et al. 2004). All of the ITS1 region sequences from the *Hematodinium* sp. from *Cancer pagurus* had fewer repeats of 2 microsatellites ($[GCC]_n$ and $[GAA]_n[GGA]_n$) when compared with those from the *Hematodinium* sp. from *Nephrops norvegicus*. Although the sample size was low the

reduced genetic diversity may indicate that a founder event occurred and that this *Hematodinium* sp. was introduced to *C. pagurus* from *N. norvegicus*, as both host species can be found in the same area. The generation and genetic analysis of clonal cultures of *Hematodinium* or the genetic analysis of single parasite cells is required to investigate the sequence and length variability of this region.

Morphologically, the *Hematodinium* species from *Nephrops norvegicus* and *Cancer pagurus* from the Clyde Sea and English Channel, respectively, are similar and present in both hosts as uni-, bi- and multinucleate forms of comparable size. Parasite cells have similar condensed chromatin profiles, lipid droplets, trichocysts, mitochondria and other organelles (Field et al. 1992, Stentiford et al. 2002). The pathology of infection is similar in both hosts with several tissues being infiltrated by large numbers of parasites (Field & Appleton 1995), however, a subtle difference exists in the pathologies of infection. The claw muscle of *N. norvegicus* is heavily infiltrated by the parasite but the Z-lines of the sarcomeres remain intact (Stentiford et al. 2000). In contrast, the sarcomeres from claw muscle fibres of infected *C. pagurus* are severely disorganised in the region of the Z-line (Stentiford et al. 2002). Given these infections are presumed to be caused by the same species of *Hematodinium* the above differences in pathology may indicate a difference in parasite virulence or host susceptibility and warrants further study.

The *Hematodinium* specific primer set developed to detect the *Hematodinium* sp. infecting *Nephrops norvegicus* from the Clyde Sea area in Scotland (Small et al. 2006) binds to the rRNA gene sequences of the *Hematodinium* spp. isolates infecting *N. norvegicus* from the Irish Sea, *Cancer pagurus* from the English Channel and Atlantic Ocean, *Pagurus bernhardus* from the Clyde Sea, and *Chionoecetes opilio* from Conception Bay, Newfoundland. This observation was confirmed by performing the PCR assay on DNA samples from these host crustaceans with known infections. The successful amplification of *Hematodinium* rDNA from these samples illustrates that this assay may be used to screen these host species for the parasite. Small et al. (2006) also designed 2 *in situ* DNA probes (using the DIG detection system) for *Hematodinium* spp., the first binding to both the 3′ end of the 18S rRNA gene and the ITS1 region (probe 1) while the second bound only to the 3′ end of the 18S rRNA gene (probe 2). The authors noted that the *in situ* signal from probe 1 was weaker than probe 2 and attributed this to the increased length of probe 1 versus probe 2 and also that the ITS region is unstable after being excised from the final rRNA product. The results from this study, however, indicate that the microsatellite variation discovered within the ITS1 region repeats may also contribute to a reduction in probe binding efficiency. Consequently, probe 1 would not have the highest binding capacity when compared with the conserved 18S rRNA gene sequence that probe 2 was based upon.

In summary, ITS1 region sequences from *Hematodinium* spp. infecting *Nephrops norvegicus, Cancer pagurus, Pagurus bernhardus* and *Chionoecetes opilio* suggest that these hosts are all infected with the same species of *Hematodinium*. This is an intriguing finding because large distances separate the ranges of the Norway lobster, edible crab and snow crab hosts. Four microsatellites are responsible for length variation in the ITS1 region in this species of *Hematodinium* and these microsatellites may be indicative of different strains of this parasite species. Finally, the conserved domains in the 18S and ITS1 region (previously targeted in a PCR assay, Small et al. 2006) allow the use of this PCR-based assay for the rapid detection of this *Hematodinium* species in *N. norvegicus, Cancer pagurus, P. bernhardus* and *Chionoecetes opilio*.

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