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Molecular detection of *Hematodinium* spp. in Norway lobster *Nephrops norvegicus* and other crustaceans

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ABSTRACT: The Norway lobster *Nephrops norvegicus* (L.) from the coastal waters of Scotland is seasonally infected by a parasitic dinoflagellate of the genus *Hematodinium*. Methods used to detect infection include a morphological index (pleopod diagnosis) and several immunoassays. The present study describes the development and application of a set of *Hematodinium*-specific polymerase chain reaction (PCR) primers and DNA probes based on *Hematodinium* ribosomal DNA (rDNA). In the PCR assay, a diagnostic band of 380 bp was consistently amplified from total genomic DNA isolated from *Hematodinium*-infected *N. norvegicus*. The sensitivity of the assay was 1 ng DNA, which is equivalent to 0.6 parasites. The primer pair also detected *Hematodinium* DNA in preparations of the amphipod *Orchomene nanus*, indicating that the amphipod may be infected with the same *Hematodinium* sp. infecting *N. norvegicus*. DNA probes detected *Hematodinium* parasites in heart, hepatopancreas and gill tissues from *N. norvegicus*, and hepatopancreas and gill tissues from *Carcinus maenas*, confirming *Hematodinium* infection in the latter.

KEY WORDS: Hematodinium · Parasite · Norway lobster · Molecular detection

INTRODUCTION

Infection of the Norway lobster *Nephrops norvegicus* by a parasitic dinoflagellate of the genus *Hematodinium* has been described from a number of locations around the West Coast of Scotland and the Irish Sea (Field et al. 1992, 1998, Briggs & McAliskey 1996, 2002, Appleton et al. 1997). In UK waters, the edible crab *Cancer pagurus* has also recently been found to harbour *Hematodinium* spp. (Stentiford et al. 2002). Parasitic *Hematodinium* species have previously been reported infecting several decapod crustaceans including *Callinectes sapidus* (Newman & Johnson 1975, Messick 1994, Messick & Shields 2000), *Cancer pagurus* (Latrouite et al. 1988), *Chionoecetes bairdi* (Meyers et al. 1987, Love et al. 1993), *Chionoecetes opilio* (Taylor & Khan 1995), *Necora puber* (Wilhelm & Mialhe 1996), *Ovalipes ocellatus* (MacLean & Ruddell 1978) and *Portunus pelagicus* (Hudson & Shields 1994). In each of the above examples, infection by *Hematodinium* species caused serious mortalities in the host decapod population.

Previous methods to diagnose infection of *Nephrops norvegicus* by *Hematodinium* species include an assessment of the external colouration of the carapace and appendages of infected lobsters. However this diagnostic method lacks sensitivity and only advanced infections can be reliably identified (Stentiford et al. 2001a). The pleopods of infected lobsters can be examined under low power light microscopy for the aggregation of parasites in the vasculature (Field & Appleton 1995). The severity of infection was classified on a 5-point scale, from apparently uninfected to an advanced infection. The pleopod method is reliable as a

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field method for identifying advanced infections, but cannot detect tissue-based and low-level haemolymph infection. Immunological methods including an indirect fluorescent antibody technique (IFAT), Western blot and enzyme linked immunosorbent assay (ELISA) have all been developed using a polyclonal rabbit antiserum raised against a mixed *in vitro* culture of vegetative forms of *Hematodinium* from *N. norvegicus* (Field & Appleton 1996, Stentiford et al. 2001b, Small et al. 2002). However, the polyclonal antibody cross-reacts with epitopes found on other protozoan parasites (Bushek et al. 2002) and may not recognise *in vivo* life cycle stages of *Hematodinium* species that were absent from the original innoculum or present only in other hosts. Because of the above concerns and the need for more sensitive and specific diagnostic methods, a molecular approach to parasite detection was undertaken.

DNA-based diagnostic methods utilising the polymerase chain reaction (PCR) and *in situ* hybridisation (ISH) assays have facilitated diagnosis of many marine pathogens of shellfish (for review see Cunningham 2002). Nuclear ribosomal DNA (rDNA) is widely acknowledged as a useful target for the definition of genetic markers informative at several levels (Gasser & Zhu 1999). The rate of evolution varies between different regions of rDNA, resulting in target sequences ranging from highly conserved to highly variable (Hillis & Dixon 1991). PCR primers used to diagnose *Hematodinium* infection in crustacean hosts have previously been reported (Hudson & Adlard 1994, Gruebl et al. 2002). However, both primer sets used in those studies were located in the conserved 18S and 5.8S regions of the rDNA complex and are not specific for individual *Hematodinium* species. Variable regions of rDNA from other dinoflagellates have been identified allowing species-specific PCR assays to be developed (Litaker et al. 2003). We describe herein the development and application of PCR and ISH assays for detection of *Hematodinium* species infection in *Nephrops norvegicus* and other crustaceans.

MATERIALS AND METHODS

Sample collection and preparation. Norway lobsters *Nephrops norvegicus* and other crustaceans used in this study (unless indicated otherwise) were caught by otter bottom-trawl (70 mm mesh size) south of Little Cumbrae in the Clyde Sea Area, Scotland, UK. Haemolymph samples were taken from the base of the fifth pereiopod, using a sterile 1 ml disposable syringe and 25-gauge needle, and were frozen at –20°C. Samples were assayed for the presence of *Hematodinium* by the ELISA method of Small et al. (2002). Infected and uninfected haemolymph samples were retained for DNA isolation. For lobsters having a low-level infection (as indicated by ELISA), a subsample of the same haemolymph was used to estimate parasite cell numbers (ml haemolymph^{-1}) using an improved Neubauer counting chamber.

Tissue samples from infected and uninfected lobsters were fixed in Davidson's seawater fixative (20 ml formalin [40% v/v], 10 ml glycerol, 10 ml glacial acetic acid, 30 ml 100% ethanol, 30 ml seawater) for 24 h, dehydrated in an ethanol series and embedded in paraffin wax. Several other samples of haemolymph and tissue from crustacean and dinoflagellate species commonly found in UK waters were also retained and prepared for DNA extraction (see Table 1). Davidson's fixed-tissue sections from a shore crab (*Carcinus maenas*) collected from the English Channel with a probable *Hematodinium* infection were obtained from Dr. G. Stentiford (CEFAS Weymouth Laboratory). *H. perezi* cells from *in vitro* culture (Small 2004) were preserved in 100% ethanol prior to DNA extraction.

To amplify parasite DNA for oligonucleotide primer design, 1×10^5 parasites from an *in vitro* culture of *Hematodinium* sp. isolated from the Norway lobster (Appleton & Vickerman 1998) were collected by centrifugation ($1000 \times g$ for 4 min at 4°C), the resultant supernatant was removed, and the sedimented cells processed for DNA extraction (see next subsection). The same centrifugation procedure was also carried out for the free-living dinoflagellates *Alexandrium tamarense* (Culture Collection of Algae and Protozoa, CCAP 1119/5), *Gymnodinium catenatum* (CCAP 1117/6) (both 1×10^5 cells), *H. perezi* and a *Mesanophrys*-like parasitic ciliate (5×10^4 cells) prior to DNA extraction.

To examine a possible secondary host for the *Hematodinium* sp. infecting *Nephrops norvegicus*, amphipods *Orchomene nanus* were captured in baited traps from the Hunterston Channel in the Clyde Sea area in August 2001, March 2003 and August 2003, according to the method of Moore & Wong (1995). Previous studies had indicated that there was a high prevalence of *Hematodinium* sp. infection of Norway lobsters at this location (J. Atkinson pers. comm.). Individual *O. nanus* were held for 3 d in seawater at 12°C prior to preservation in 100% ethanol or Davidson's seawater fixative (only the March 2003 and August 2003 samples). Genomic DNA samples were prepared from each amphipod and assayed by PCR for the presence of *Hematodinium* spp.

DNA extraction. Genomic DNA was extracted from 100 µl haemolymph samples, 100 mg tissue samples, the *in vitro Hematodinium* sp., ciliate and dinoflagellate pellets, and from individual whole *Orchomene nanus* according to standard procedures (Sambrook et al. 1989). Briefly, samples were homogenised/

resuspended in 250 µl extraction buffer (50 mM Tris, 5 mM EDTA, 100 mM NaCl, pH 8), 100 µl of 10% SDS (w/v) and Proteinase K (0.28 ng μl^{-1}) and incubated at 56°C for 18 to 24 h. All DNAs were purified by single-step standard phenol/chloroform (1:1) extraction, precipitated in 550 µl 100% ethanol using 20 µl 5 M NaCl, and resuspended in 50 µl sterile deionised water. DNA concentrations and purity were estimated by measuring the 260/280 optical density ratios using a spectrophotometer (Gene Quant II, Pharmacia Biotech), and adjusted to between 0.1 and 100 ng for experimental use.

Primary amplification, cloning and sequencing. The first internal transcribed spacer (ITS1) and flanking 3′ region of the 18S rDNA complex were amplified independently from 2 *Hematodinium* genomic DNA templates (*in vitro* culture and infected haemolymph), using the forward primer 5′ GTT CCC CTT GAA CGA GGA ATT C 3′ and reverse primer 5′ CGC ATT TCG CTG CGT TCT TC 3′. Primer sequences and amplification conditions were as described by Hudson & Adlard (1994). Amplification products were run on 1.5% (w/v) agarose gels, stained with ethidium bromide, and viewed under UV illumination. Gel images were obtained using a gel documentation system (Appligene). Each amplification product of approximately 680 bp was excised from the agarose gel and purified by the use of a QIA-quick gel-extraction kit (Qiagen). Purified amplification products were ligated into the pGEMT-Easy plasmid vector (Promega) and used to transform *Escherichia coli* (Strain JM 109) by heat shock according to the manufacturer's instructions. Transformed cells were plated onto Luria-Bertani (LB) agar (bacto-agar [1.5% w/v] in LB medium [NaCl, 10 g l^{-1}; bacto-tryptone, 10 g l^{-1}; yeast extract, 5 g l^{-1}, pH 7.5]), containing ampicillin (50 µg ml^{-1}), isopropyl-beta-D-thiogalactopyranoside (IPTG, 40 µg ml^{-1}) and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal, 100 µg ml^{-1}), and grown overnight at 37°C. Positive transformations were identified by blue/white selection and selected colonies were grown overnight in LB medium containing ampicillin (50 µg ml^{-1}). Recombinant plasmids were purified using a miniprep kit (Qiagen) according to the manufacturer's instructions. Plasmid DNA concentrations and purity were estimated by measuring the 260/280 optical density ratios. Ligation of correct products was confirmed by restriction enzyme digestion (*Eco*RI) and analysis of products on agarose gels. Bi-directional sequencing of 1 clone from a single PCR reaction from each template was performed by MWG-AG Biotech.

Primer and probe design. The nucleotide sequences obtained from initial amplifications were aligned using the software programmes ClustalX 1.81 (Thompson et al. 1994) and BoxShade 3.21 (www.ch.embnet.org/software/BOX_form.html). Sequences were compared for similarity to those of other dinoflagellates by Basic Local Alignment Tool searches (BLAST; Altschul et al. 1990) in GenBank. Suitable priming regions for PCR exhibiting specificity for the *Hematodinium* sp. from *Nephrops norvegicus* were identified from the sequence alignment (see Fig. 1) and by comparison with previously published *Hematodinium* sp. sequences (Hudson & Adlard 1996). We designed 4 oligonucleotides, designated 18S F1, 18S F2, 18S R1, and ITS R1 (see Table 2), for PCR and the synthesis of PCR-generated DNA probes.

PCR assay sensitivity and specificity. The sensitivity of the *Hematodinium*-specific primers 18S F2 and ITS R1 for use in total genomic DNA sample screening by PCR was assessed by serial dilution of a genomic DNA sample from an infected lobster. The number of *Hematodinium* sp. cells in the infected haemolymph sample was estimated using an improved Neubauer haemocytometer, and total DNA was extracted by the previously described methods. The amplification reaction mixtures contained 0.1 to 100 ng genomic DNA, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100 (v/v), 1.5 mM $MgCl_2$, 100 µM dNTPs, 10 pmol each primer, 1 unit of *Taq* polymerase (Promega), and sterile deionised water to a final volume of 20 µl. Reactions were overlaid with 10 µl of mineral oil. Thermal cycling conditions were as follows: denaturation at 94°C for 30 s; primer annealing at 57°C for 1 min; chain extension at 72°C for 1 min; repeated for 35 cycles, with a final cycle incorporating a 7 min extension step at 72°C. A 5 µl aliquot of each PCR reaction was checked for amplification products by 1.5% (w/v) agarose gel electrophoresis and ethidium bromide staining. Images were captured using a gel documentation system (Appligene).

The PCR primers were tested for specificity against genomic DNA samples (100 ng) isolated from *Hematodinium*-infected and uninfected *Nephrops norvegicus*, *H. perezi*, *Cancer pagurus*, *Callinectes sapidus*, several other crustacean species common to the UK (detailed in Table 1), a *Mesanophrys*-like ciliate found infecting *N. norvegicus*, and the free-living dinoflagellates *Gymnodinium catenatum* and *Alexandrium tamarense*. Reaction conditions were as described above.

As part of ongoing studies, samples of *Hematodinium*-infected haemolymph were obtained from *Nephrops norvegicus* collected from the Clyde Sea Area, the Fladen and North Minch fishing grounds in Scotland, the Irish Sea and the Swedish Skagerrak fishing grounds. Total genomic DNA from 100 µl haemolymph samples was extracted and the PCR assay performed as described above. Total genomic DNA was also extracted from individual amphipods (*Orchomene nanus*) and the PCR assay was carried out as described above.

DNA probe synthesis and *in situ* hybridisation. DNA probes were synthesised by incorporation of digoxygenin-11-dUTP (DIG, Roche) during PCR using primer sets 18S F2/ITS R1 and 18S F1/18S R1. PCR was carried out using 100 ng of genomic DNA extracted from parasite cells from an *in vitro* culture of *Hematodinium* sp. Locations and sequences of the primers used to synthesise the probes are given in Table 2. Reaction conditions were followed as suggested by the manufacturer, with annealing temperatures of 52 and 56°C for primers 18S F2/ITS R1 and 18S F1/18S R1, respectively. Incorporation of DIG was indicated by an increase in molecular mass when amplification products were visualised on ethidium bromide-stained agarose gels. The labelled PCR product was gel-extracted and purified using a QIAquick gel-extraction kit (Qiagen). Probe concentration was estimated by side-by-side comparison of a diluted series of the probes and a DIG-labelled control of known concentration in a spot test on nylon membrane following the manufacturer's protocols.

Paraffin-embedded tissue sections from *Hematodinium* sp.-infected *Nephrops norvegicus* and *Carcinus maenas* were cut at 6 µm thickness, placed on salinised slides, and baked for 45 min at 60°C. Sections were de-paraffinised (xylene, 2 min), rehydrated in an ethanol series (1 min each ethanol grade, 100, 90 and 70% v/v), washed in distilled water, and permeabilised with 10 to 50 µg ml^{-1} Proteinase K in TNE buffer (50 mM Tris-HCl, 10 mM NaCl, 1 mM EDTA, pH 7.4) for 30 min at 37°C in a humid chamber. Proteolysis was inactivated by two 1 min washes in 20 ml PBS (phosphate-buffered saline) followed by equilibration in 20 ml 2× SSC (saline sodium citrate). Samples were prehybridised in 500 µl prehybridisation buffer (2 × SSC [20 × SSC = 3 M NaCl, 0.3 M Na-citrate, pH 7.0], 50 % [v/v] formamide, 5 × Denhardt's solution and 100 µg ml^{-1} herring sperm DNA) in a humid chamber for 60 min at 37°C. The prehybridisation buffer was replaced with 50 to 100 µl hybridisation buffer (2 × SSC, 50% [v/v] formamide, 5 × Denhardt's solution, 100 µg ml^{-1} herring sperm DNA and 1% [v/v] dextran sulphate) containing 0.1 ng $µl^{-1}$ heat-denatured DIG-labelled probe. After applying glass coverslips, sections were placed on a heating block at 95°C for 5 min to denature the target DNA, then immediately put on ice for 5 min and allowed to hybridise overnight in a humid chamber at 42°C. Post-hybridisation washes included 2 × SSC at room temperature, twice for 5 min, and 0.1 × SSC at 42°C, once for 10 min, followed by equilibration in 20 ml maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5). Sections were blocked with 500 µl blocking buffer (maleic acid buffer plus 1% [w/v] blocking reagent: Roche) at 37°C for 15 min followed by incubation for 1 h at 37°C with 500 µl of dilute anti-DIG-alkaline phosphatase antibody (Roche) diluted 1:1000 in blocking buffer. Unbound antibody was removed by two 5 min washes in 20 ml washing buffer (maleic acid buffer plus 0.3% [v/v] Tween 20) followed by one 5 min wash in 20 ml detection buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5); 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (NBT/BCIP) was diluted (1/50 dilution of stock solution) in detection buffer and 200 µl was added to sections and incubated at room temperature (22°C) in the dark for 2 to 6 h. The reaction was stopped with a 20 ml TE buffer wash (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Slides were washed in double-distilled H_2O and counterstained with 1% (w/v) eosin for 1 min, followed by ethanol dehydration (1 min each ethanol grade, 100, 90 and 70% v/v), and mounted in aqueous mounting medium (histomount). Hybridisation conditions were optimised by varying the concentration of Proteinase K (10 to 50 µg ml^{-1}) and length of incubation (15 to 60 min), and the concentration of DIG-labelled DNA probes (0.1 to 1 ng $µl^{-1}$ heat-denatured DIG-labelled probe). Negative controls included samples treated without the addition of DIG-labelled probe as well as the use of uninfected tissue sections.

Table 1. Use of *Hematodinium*-specific PCR primer set 18S F2 and ITS1 R1 against other dinoflagellate, ciliate and decapod crustacean DNA samples. +: single amplification product of 380 bp; –: no amplification product or amplification product of incorrect size

Genomic DNA template	PCR diagnosis
Alexandrium tamarense CCAP 1119/5	–
Gymnodinium catenatum CCAP 1117/6	–
Mesanophrys-like ciliate	–
Carcinus maenas	–
Necora puber	–
Cancer pagurus	–
Maja squinado	–
Liocarcinus depurator	–
Pagurus bernhardus	–
Callinectes sapidus	–
Hematodinium-infected *Nephrops norvegicus*	+
Hematodinium-infected *Cancer pagurus*	+
Hematodinium-infected *Callinectes sapidus*	–

RESULTS

Hematodinium rDNA sequences

A single 680 bp amplification fragment was produced when template DNA samples from the *in vitro* culture were used in conjunction with the nucleotide primers previously described by Hudson & Adlard

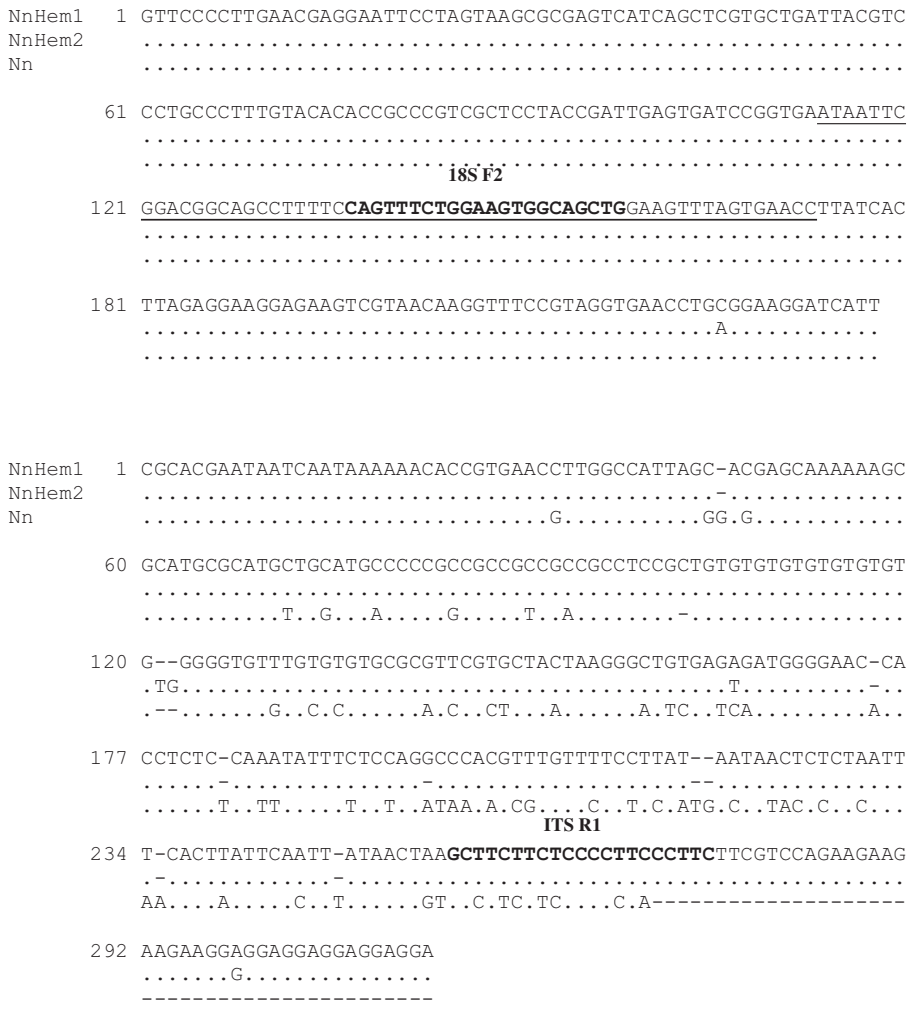

Fig. 1. *Hematodinium* spp. Alignments of nucleotide sequences consisting of the 3′ end of the 18S (upper alignment) and 5′ end of the first internal transcribed spacer (ITS1) region (lower alignment) of the ribosomal DNA gene complex from 2 isolates sequenced as part of this study and the *Nephrops norvegicus Hematodinium* sp. sequence from Hudson & Adlard (1996) (Nn). Isolate NnHem1: *Hematodinium* sp. from a continuous *in vitro* culture first isolated in 1992 (GenBank Accession No. DQ084245); NnHem2: *Hematodinium* sp.-infected *N. norvegicus* haemolymph sample from 2000 (GenBank Accession No. DQ084246). Nucleotide region underlined in 18S sequence indicates V9 domain; boldface print indicates PCR primers 18S F2 and ITS R1; dots: conserved nucleotides; dashes: missing nucleotides

(1994). However, multiple-reaction products were produced using template DNA from infected lobster haemolymph, reiterating the need for the development of molecular diagnostics with increased specificity. The 680 bp products from both PCR amplifications were successfully cloned into the plasmid vector, sequenced and aligned with the *Nephrops norvegicus Hematodinium* sp. sequence from Hudson & Adlard (1996) (Fig. 1). The 3′ end of the 18S gene was almost totally conserved between all isolates, the only variation being 1 nucleotide at Position 236 bp. Comparison of these sequences with sequences held at GenBank using BLAST confirmed that the 3′ end of the 18S gene has a high level of identity (95%) with other dinoflagellate 18S rDNA gene sequences. Both sequences were deposited in GenBank (Accession Nos. DQ084245 and DQ084246)

The 5′ region of ITS1 showed only a small number of nucleotide variations between the isolates sequenced in this study (98% identical); however, conserved regions were identified where primers could be designed to anneal (Fig.1). Sequence similarity of the published ITS1 region of the *Hematodinium* species from *Nephrops norvegicus* (Hudson & Adlard 1996) to the isolates used in this study and another (H. J. Small unpubl. data) was much lower (77%), justifying the present efforts to obtain sequence information for *N. norvegicus Hematodinium* sp. isolates.

Table 2. Oligonucleotide primer sequences and annealing positions, designed to bind to *Hematodinium* 18S and ITS1 regions of the rDNA gene complex for use in PCR assays and construction of DNA probes

Primer	Sequence 5′-3′	Position	Purpose
18S F1	GTTCCCCTTGAAGGAGGAATTC	216–238 bp upstream 18S/ITS1 boundary	Probe 2
18S F2	CAGTTTCTGGAAGTGGCAGCTG	80–102 bp upstream 18S/ITS1 boundary	PCR and Probe 1
18S R1	AGCTGCCACTTCCAGAAACT	81–101 bp upstream 18S/ITS1 boundary	Probe 2
ITS R1	GAAGGGAAGGGGAGAAGAAGC	256–277 bp downstream 18S/ITS1 boundary	PCR and Probe 1

PCR primer design, sensitivity and specificity

A new forward primer was synthesised (18S F2) which was specific to an area within the variable V9 domain of the 3′ end of the 18S gene (80 to 102 bp upstream of the 18S/ITS1 boundary). The V9 domain has previously been shown to be highly conserved between *Hematodinium* species compared with other dinoflagellates (Hudson & Adlard 1996). A new reverse primer was also synthesised (ITS R1), specific to an area within the ITS1 sequence 256 to 277 bp downstream of the 18S/ITS1 boundary. The positions and sequences of primer sets are shown in Table 2 & Fig. 1. Amplification of DNA from *Hematodinium*-infected *Nephrops norvegicus* haemolymph using the primer pair 18S F2 and ITS R1 led to the production of a diagnostic band of 380 bp from 1 ng or more DNA (Fig. 2). This band was not produced using a sample of 100 ng DNA from uninfected haemolymph. Based on initial cell counts of the parasite numbers in the haemolymph, 1 ng genomic DNA is equivalent to 0.6 parasite cells. The *Hematodinium*-specific PCR primer pair did not generate a PCR product of appropriate size when using genomic DNA preparations from *H. perezi*, a *Mesanophrys*-like ciliate found infecting *N. norvegicus*, 2 toxin-producing dinoflagellate species, and a number of other crustacean species (Table 1). However, the primer pair did generate an appropriate reaction product when the PCR assay was performed with genomic DNA templates prepared from *Hematodinium*-infected *N. norvegicus* haemolymph and *Hematodinium*-infected *Cancer pagurus* hepatopancreas tissue.

Detection of *Hematodinium* in *Nephrops norvegicus* and *Orchomene nanus*

Hematodinium infections in haemolymph samples of *Nephrops norvegicus* from the Clyde Sea Area, North Minch and Fladen were detectable by PCR (Fig. 3). *Hematodinium* infections were also detected in *N. norvegicus* haemolymph samples from the Irish Sea and the Swedish Skagerrak (data not shown). Sequencing of reaction products confirmed that only *Hematodinium* DNA was amplified. Variation in PCR product intensity was observed between haemolymph samples from the different locations, presumably re-

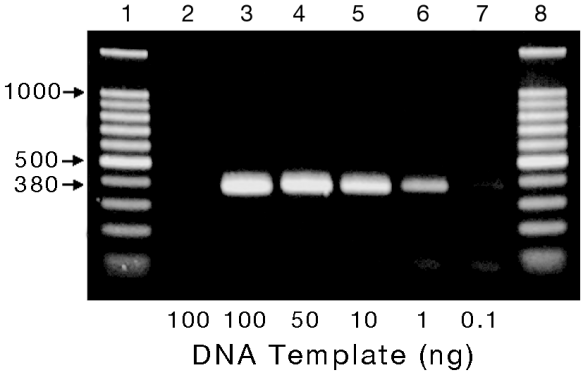

Fig. 2. Sensitivity of PCR assay for detection of *Hematodinium* infecting *Nephrops norvegicus*. Lane 2: 100 ng μl^{-1} *N. norvegicus* host DNA control; Lanes 3 to 7: infected haemolymph DNA template concentrations, 100, 50, 10, 1, 0.1 ng μl^{-1}; Lanes 1 and 8: 100 bp molecular weight marker

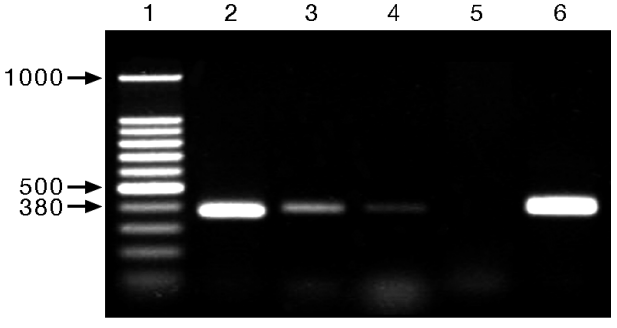

Fig. 3. Verification of PCR assay on *Hematodinium*-infected *Nephrops norvegicus* haemolymph samples from different geographical locations. Lane 1: 100 bp molecular weight marker; Lanes 2 to 4: 100 ng μl^{-1} total genomic DNA from haemolymph of infected *N. norvegicus* collected from Clyde Sea, Fladen and North Minch, respectively; Lane 5: *N. norvegicus* host DNA control (100 ng μl^{-1}); Lane 6: *Hematodinium* DNA control from *in vitro* culture (50 ng μl^{-1})

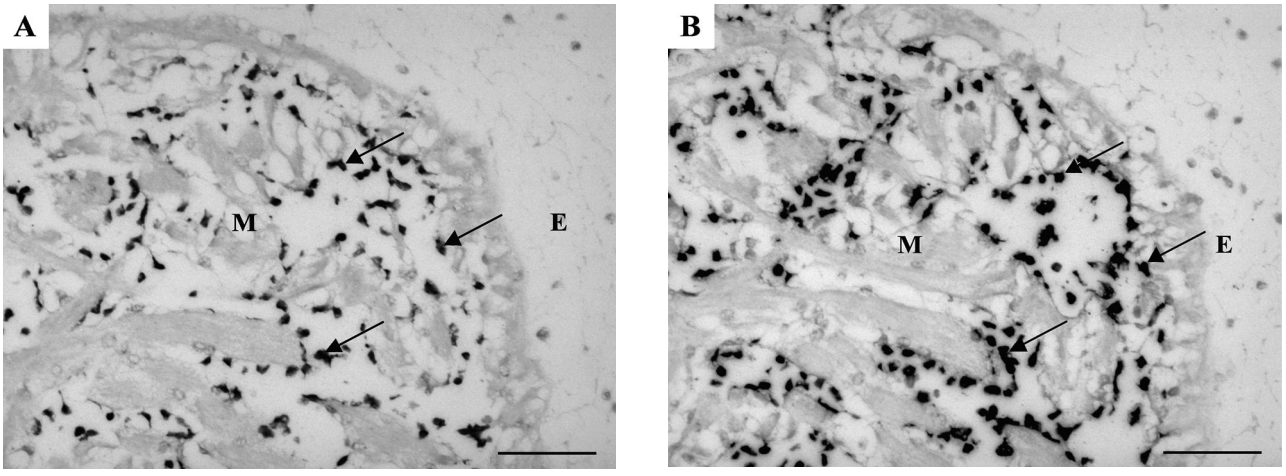

Fig. 4. *Nephrops norvegicus*. Hybridisation of (A) Probe 1 and (B) Probe 2 with *Hematodinium* parasites (arrows) in myocardial heart-tissue sections of the lobster. M: myocardium; E: epicardium. Scale bars = 100 µm

flecting differences in parasite loading of the samples (as rDNA sequences were identical over the oligonucleotide primer regions used: H. J. Small unpubl. data).

PCR screening of *Orchomene nanus* DNA samples from August 2000 indicated that 5 of 13 amphipods were potentially infected with *Hematodinium* sp. However, when screening was repeated with larger samples (n = 40) obtained in March 2003 and August 2003, no indication of infection was observed.

In situ hybridisation

DNA Probes 1 and 2 hybridised to parasite cells present in paraffin-embedded myocardial heart tissue sections, prepared from *Hematodinium* sp.-infected *Nephrops norvegicus* (Fig. 4). There was negligible background hybridisation observed for both DNA probes used. A marked increase in signal intensity was observed when using Probe 2 compared to Probe 1 against parasites in heart tissues. Alteration of DIG-labelled Probe 1 concentration and incubation time did not enhance hybridisation and, as a result, signal intensity. Because of this, only Probe 2 was used in further hybridisation studies. No signal was observed for negative-control uninfected samples or preparations where DNA Probe 2 was absent from hybridisation reactions (Fig. 5). Probe 2 hybridised clearly with parasite cells in the haemal space of the hepatopancreas and gill filaments from infected *N. norvegicus* (Fig. 6). Probe 2 also hybridised well with presumptive *Hematodinium* cells present in haemal spaces of the hepatopancreas and gill filament tissue sections from *Carcinus maenas* (Fig. 7).

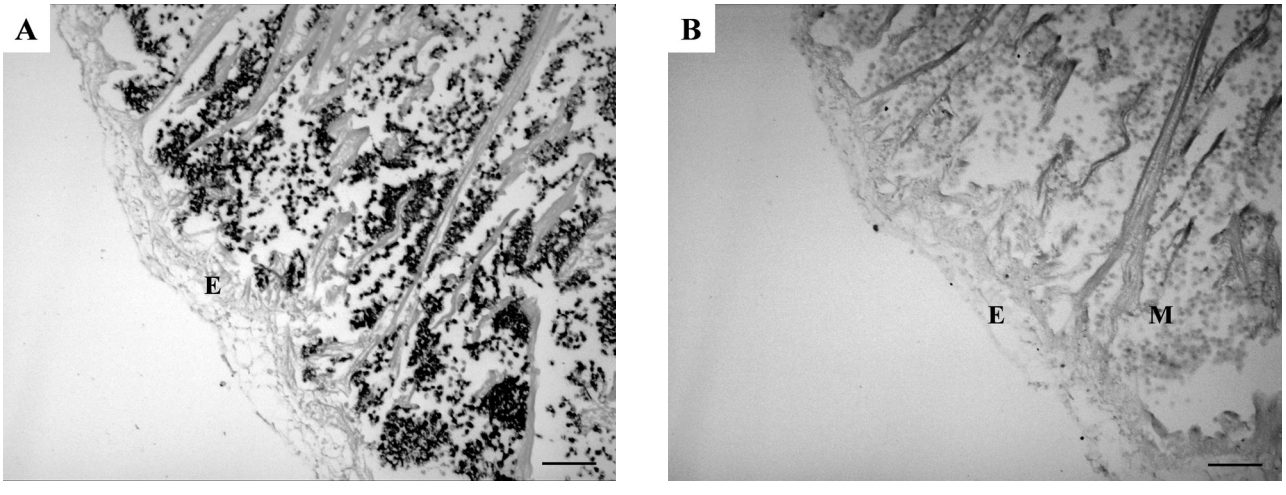

Fig. 5. *Nephrops norvegicus*. *In situ* hybridisation control reactions on *Hematodinium*-infected *N. norvegicus* heart-tissue sections when Probe 2 is (A) present and (B) absent from hybridisation buffer. M: myocardium; E: epicardium. Scale bars = 100 µm

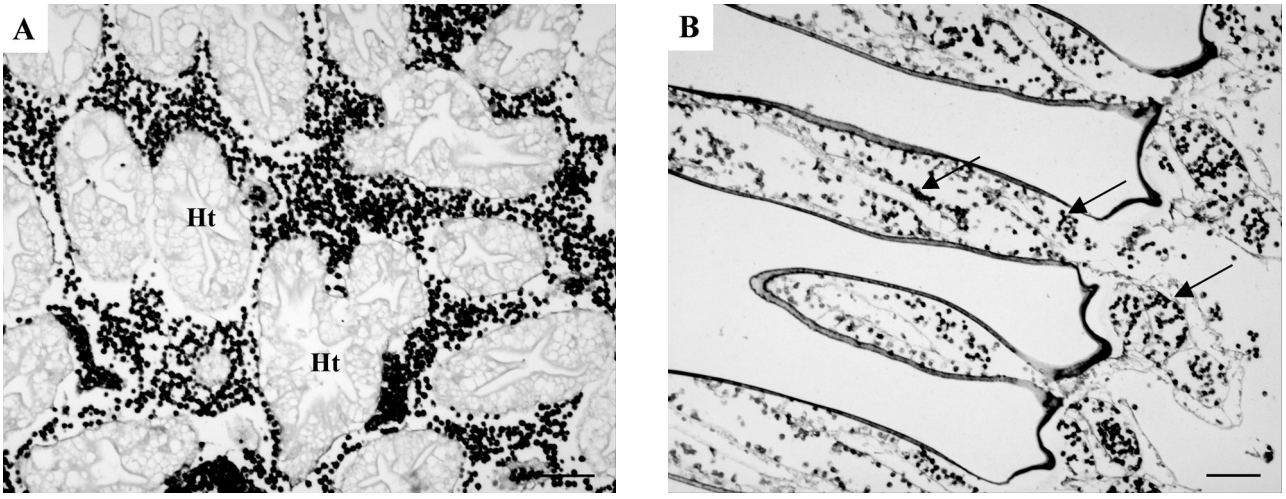

Fig. 6. *Nephrops norvegicus*. Hybridisation of Probe 2 with *Hematodinium* cells in (A) haemal space of hepatopancreas and (B) gills of *N. norvegicus* tissue sections. Ht: hepatopancreatic tubule; arrows indicate parasites. Scale bars = 100 µm

DISCUSSION

Methods developed for the diagnosis of *Hematodinium* spp. infection in the Norway lobster include the examination of lobsters for signs of gross infection by carapace discolouration, monitoring aggregation of parasites in the pleopods, and several immunoassays. In this study, molecular probes were developed and applied for the diagnosis of *Hematodinium* infection in *Nephrops norvegicus* and other crustaceans. The rDNA gene locus was chosen as a target region for the design of molecular probes based on Hudson & Adlard's (1996) finding that there was a significant degree of sequence variation in this gene locus between *Hematodinium* species. However, the rDNA sequences obtained were never deposited in GenBank or any other database; hence, amplification and sequencing of *Hematodinium* sp. isolates from *N. norvegicus* were carried out as part of this study. Cloning of the 3′ end of the 18S and 5′ end of the ITS1 rDNA genes from *Hematodinium* species infecting *N. norvegicus* revealed that the 3′ end of the 18S gene was conserved (apart from 1 polymorphic nucleotide site) between the 2 isolates used in the study, but the ITS1 sequences showed a number of nucleotide variations (5 over 315 bp). Of considerable importance is the finding that Hudson & Adlard's (1996) sequence of the ITS1 rDNA region was only 77% identical to the sequences obtained as part of this study and another (H. J. Small unpubl. data), and cautions against interpretation and use of any *Hematodinium* sp. sequences published as part of that study. Sequencing of other rDNA genes (and others such as actin) from *Hematodinium* isolates from their various hosts is essential for the design of molecular diagnostics, and required to accurately delineate between species and strains of this economically important parasite.

The primer set 18S F2 and ITS R1 efficiently amplified parasite DNA in the presence of host DNA, resulting in the production of a diagnostic band of 380 bp from genomic DNA samples of at least 1 ng, equivalent to 0.6 parasite cells per sample. This is a considerable improvement in sensitivity compared to the ELISA (which requires about 5×10^4 parasites ml^{-1}: Small et al. 2002) The inhibitory effect of host DNA/PCR inhibitors or method of DNA extraction from samples has not been investigated, but warrants further consideration as several diagnostic assays for other shellfish pathogens have shown that these factors can effect the sensitivity of PCR assays (Kleeman & Adlard 2000, Audemard et al. 2004).

The PCR assay was further validated by amplification of parasite DNA from samples of *Hematodinium* sp.-infected *Nephrops norvegicus* haemolymph from geographically separate waters. The differences in PCR product intensity (Fig. 3) probably represent different levels of infection. This may also correlate with a different seasonal pattern of infection from the Clyde Sea Area, as the host moult period has been implicated in *Hematodinium* infection seasonality (Field et al. 1992) and *N. norvegicus* moulting is thought to vary between geographical location in UK waters (J. Atkinson pers. comm.). The primers used did not produce any amplification signal when DNA templates prepared from several other crustacean species were used, indicating that these primers can be used to investigate whether the *Hematodinium* species infecting *N. norvegicus* also occurs in these crustaceans.

In situ hybridisation studies using the DIG-labelled DNA Probe 1, constructed using the same primers

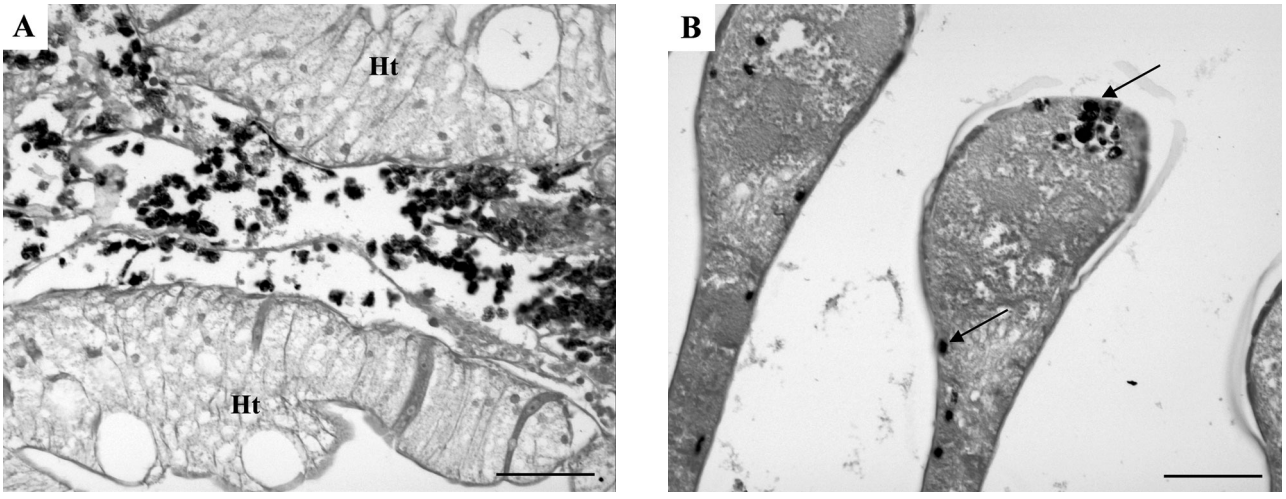

Fig. 7. *Carcinus maenas*. Hybridisation of Probe 2 with *Hematodinium* cells in (A) haemal space of hepatopancreas and (B) gill tips of *C. maenas* tissue sections. Ht: hepatopancreatic tubule; arrows indicate parasites. Scale bars = 10 µm. (Material supplied by Dr. G. Stentiford, CEFAS Weymouth Laboratory)

used for the PCR assay which span the 18S and ITS1 rDNA regions, localised individual parasites in lobster tissues. However, the signal from the probe was weak and could not be improved by either incubating the section with a higher concentration of probe or by increasing the incubation time. In contrast, Probe 2 constructed using Primers 18S F1 and 18S R1, which amplify conserved 18S rDNA only, gave a very intense signal when hybridising to parasite cells within paraffin-embedded sections using the same reaction conditions as Probe 1. Kleeman et al. (2002b) reported variations in sensitivity and signal intensity between different 18S/ITS1-based DIG-labelled probes for *Marteilia sydneyi* and *M. refringens*. This suggests that the observed difference in signal intensity between *Hematodinium* Probes 1 and 2 may reflect the availability of target sequence; ITS regions are excised from the mRNA in the cell cytoplasm prior to ribosomal construction and so are not available for probe hybridisation. Alternatively, the shorter length of Probe 2 may assist in increased binding to target sequences and result in an increased signal. Probe 2, based on 18S rDNA, efficiently hybridised to parasites in the hepatopancreas and gill tissues of *Nephrops norvegicus* and *Carcinus maenas.* The results presented suggest that Probe 2 is able to detect *Hematodinium* species in at least 2 different hosts and is likely to be genus-specific owing to the conserved nature of the 18S rDNA. Consequently, it could be used to confirm and investigate latent *Hematodinium* infection in a wide range of crustacean hosts.

Several life history stages of *Hematodinium* sp. from *Nephrops norvegicus* have been described from *in vitro* cultures (Appleton & Vickerman 1998), but of these only a few forms have been observed during natural infection in the lobster. It was suggested by Appleton & Vickerman (1998) that ingestion of *Hematodinium* dinospores takes place during suspension-feeding by the lobster, and that initiation of infection takes place after penetration of the gut wall by the dinospores. Others have suggested that cuticle tissues of crustaceans damaged during moulting may be the sites of parasite entry (Eaton et al. 1991). Molecular techniques have been used to identify the portal of entry of PKX (phylum Myxozoa) in salmonids (Morris et al. 2000), and to detect different life cycle stages of *Marteilia sydneyi* (Kleeman et al. 2002a). The DNA probes developed for *Hematodinium* will likely be useful for addressing similar issues. They will be valuable in monitoring low-level infections in naturally and experimentally infected lobsters, and also in the identification of life history stages previously unseen.

One issue that we have addressed is the mechanism of transmission of *Hematodinium* spp. The lack of success in transmission experiments with cultured *Hematodinium* and infected haemolymph (Vickerman 1994) indicates that an undiscovered intermediate host may be required for completion of the parasite life cycle and its ability to infect *Nephrops norvegicus* (Appleton & Vickerman 1998). This hypothesis has also been raised for a number of aquatic pathogens, including *Marteilia refringens*, which infects copepods and oysters (Audemard et al. 2002) and the myxosporean *Ceratomyxa shasta*, which infects an annelid worm and salmonids (Bartholomew et al. 1997).

Several amphipods predate or scavenge dead crustaceans (Templeman 1954, Scarratt 1965), and have previously been reported to be infected by dinoflagellates (Johnson 1986). Messick & Shields (2000) also

suggested that several amphipods collected during prevalence studies for *Hematodinium perezi* in *Callinectes sapidus* were potentially infected with this parasite. Thus, the PCR primers developed in the present study were used to investigate *Orchomene nanus* as a possible secondary host or transmission vector for *Hematodinium* spp. This amphipod species is a generalist scavenger, with a preference for crustacean carrion (Moore & Wong 1995), and as such probably feeds on dead *Nephrops norvegicus* with large numbers of parasites present. Analysis of samples of *O. nanus* collected from the Clyde Sea Area and assayed by PCR revealed that in August 2001, 5 of 13 amphipods were positive for the presence of *Hematodinium* spp. Sequencing of a PCR product from the amphipod screen confirmed that it was *Hematodinium* DNA that was amplified, with 98% homology in the ITS1 region to other *Hematodinium* isolates from the Norway lobster (H. J. Small unpubl. data). Considering the size of *Hematodinium* dinospores (11 to 20 µm long, 4.5 to 11.5 µm wide), they could be ingested by the amphipod and result in the positive PCR signal from the gut contents, or from infection of different tissues. However, the possibility of parasites adhering to the exterior surface of the amphipod cannot be ruled out. Unfortunately, no individual amphipods were retained for *in situ* hybridisation studies to localise the parasite in these samples. When amphipod sampling and the PCR assay were repeated in March 2003 and August 2003, no *Hematodinium* DNA could be detected. Thus, the determination of the prevalence of *Hematodinium* in *O. nanus*, its location, and the importance, or not, of *O. nanus* in the life cycle of *Hematodinium* spp. requires further experimental research.

In conclusion, the combined use of the PCR primers and DNA Probe 2 will prove valuable in confirming *Hematodinium* species infection in crustaceans and elucidating the life cycle of *Hematodinium* spp. in *Nephrops norvegicus* and other hosts.

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