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PCR techniques applied to *Hematodinium* spp. and *Hematodinium*-like dinoflagellates in decapod crustaceans

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ABSTRACT: The amount of DNA recovered from 1×10^6 cells of *Hematodinium*-like dinoflagellates from *Chionoecetes bairdi* was 75 ng. PCR (polymerase chain reaction) amplification products were created from the host and *Hematodinium* spp. Amplified products of 680 base pairs (bp) were produced from *Hematodinium*-like dinoflagellates from *C. bairdi* for DNA concentrations of 100, 50, 20, 10 and 1 ng μl^{-1} This amplified fragment was produced only from host tissue infected with the parasite. The lowest detectable limit of 1 ng of DNA from *Hematodinium*-like sp. from *C. bairdi* corresponded to 1.0×10^4 cells. Diagnostic bands of 680 bp were produced from *Hematodinium* spp. and *Hematodinium*-like spp. isolated from the following hosts: *Chionoecetes opilio, Callinectes sapidus, Nephrops norvegicus* and *Portunus pelagicus*. Host DNA (*C. bairdi*) produced a faint band of 1300 bp for concentrations of 100, 50 and 20 ng μl^{-1}.

KEY WORDS: PCR · Dinoflagellate · *Hematodinium* · Decapod

INTRODUCTION

Hematodinium spp. are parasitic dinoflagellates that infect the hemolymph of predominantly decapod crustaceans in many parts of the world. *Hematodinium* spp. have been reported from several commercially important host species, including the portunid blue crab *Callinectes sapidus* (Newman & Johnson 1975), the cancer crabs *Cancer irroratus* and *C. borealis* (MacLean & Ruddell 1978), the majid Tanner crabs *Chionoecetes bairdi* and *C. opilio* (Meyers et al. 1987, Meyers 1990), the Norway lobster *Nephrops norvegicus* (Field et al. 1992), the portunid sand crab *Portunus pelagicus* (Shields 1992, Hudson & Shields 1994) and the portunid mud crab *Scylla serrata* (Hudson & Lester 1994). *Hematodinium*-like dinoflagellates cause Bitter Crab Disease (BCD) in *C. bairdi* and *C. opilio* (Meyers et al. 1987, Meyers 1990) and 'watery flesh' in *N. norvegicus* (Field et al. 1992); both of these conditions make their flesh unmarketable.

Gross signs of high intensity *Hematodinium* spp. infections are characterized by a milky-white appearance of the hemolymph and/or an abnormal colouration of the body (Meyers et al. 1987, Field et al. 1992, Hudson & Shields 1994). Milder infections can be diagnosed by microscopical examination. In this study we attempt to diagnose the presence of *Hematodinium* spp. using the polymerase chain reaction (PCR), and to determine empirically the minimum level of infection that can be detected by this method.

MATERIALS AND METHODS

Sample collection. Sand crabs *Portunus pelagicus* were collected in March 1992 from Moreton Bay, Queensland, Australia. Norway lobsters *Nephrops norvegicus* were collected in January 1993 from the Firth of Clyde on the west coast of Scotland, UK. Blue crabs *Callinectes sapidus* were collected in January and November 1993 from the Rappalamoda River, Chesapeake Bay, Virginia, USA. Tanner crabs *Chionoecetes bairdi* and *C. opilio* were collected in February 1993 near Juneau and Dutch Harbor, Alaska, USA, respectively. *Hematodinium australis* from *Portunus pelagicus* were frozen at -60 °C. Hemolymph samples

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from *C. bairdi*, *C. opilio* and *N. norvegicus* containing *Hematodinium*-like dinoflagellates were collected via syringe and fixed directly in 100% ethanol. *Hematodinium*-infected testes from *C. sapidus* were fixed directly in 100% ethanol and were shaken to produce the sediment that contains the *Hematodinium* sp. cells. Uninfected testes from *C. bairdi* were fixed directly in 100% ethanol. *Hematodinium* spp. were identified using light and electron microscopy (Meyers et al. 1987, Field et al. 1992, Shields 1992, Hudson & Lester 1994, Hudson & Shields 1994).

DNA extraction and purification. Initial *Hematodinium* spp. cell counts were determined using a hemocytometer Preparation of *Hematodinium* spp. required centrifugation of 250 µl of ethanol-fixed infected hemolymph at 1600 × *g* for 3 min; the pelleted cells were retained. Approximately 4 mm^3 of ethanol-fixed testes was used as the source of host DNA. DNA extraction for parasite cells and host testes required suspension in 500 µl of extraction buffer (50 mM Tris, 5 mM EDTA, 100 mM NaCl, pH 8), 200 µl of 10% SDS and 20 µl of proteinase-K (10 µg ml^{-1}) and incubation for 18 to 24 h at 56°C. Samples were centrifuged at 1600 × *g* for 15 min and the lysates transferred to clean tubes. DNA was purified using phenol/chloroform and was then precipitated in cold ethanol (100% EtOH, 0.2 M NaCl). Dried DNA was resuspended in 100 µl of sterile deionised water. The DNA concentrations were determined using a spectrophotometer for both host and *Hematodinium*-like dinoflagellates from *Chionoecetes bairdi*.

DNA amplification. The first internal transcribed spacer (ITS1) of ribosomal DNA and flanking 3' end of the small subunit (SSU) was amplified by the PCR using oligonucleotide primers A (forward primer: 5' GTT CCC CTT GAA CGA GGA ATT C) and B (reverse primer: 5' CGC ATT TCG CTG CGT TCT TC). Primer A was located approximately 230 base pairs (bp) upstream of the 3' end of the SSU/ITS1 boundary, while primer B was located in the 5.8S region. Primers were designed on conserved regions after alignment of published sequences available through GenBank. PCR amplifications were performed on known DNA concentrations of *Chionoecetes bairdi* and its *Hematodinium*-like dinoflagellates (Table 1) and on *Hematodinium* spp. from *C. opilio*, *Callinectes sapidus*, *Portunus pelagicus* and *Nephrops norvegicus*. PCR reactions were performed in 50 µl total reaction volume by adding 5 µl of 10× reaction buffer [final concentration 67 mM Tris-HCl, pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 1 mM β-mercaptoethanol, 6.7 µM EDTA, 0.15% Triton X-100, 200 µg μl^{-1} gelatin], 5 µl $MgCl_2$ (final concentration 2 mM), 3 µl each of dNTP's (final concentration 200 µM), 2 µl each of forward and reverse primers (final concentration 2 ng μl^{-1}), volume of target DNA to approximately 50 ng, 3 units of Taq polymerase (Bresatec) and sterile deionized water to a final volume of 50 µl. The reactions were mixed briefly and overlaid with 30 µl of mineral oil. Cycling parameters were as follows: denaturation at 94°C for 60 s, primer annealing at 55°C for 30 s, chain extension at 72°C for 90 s, with a total of 30 cycles; then a final extension cycle of 94°C for 60 s, 55°C for 30 s and 72°C for 7 min. Each amplification product was electrophoresed through a 1.5% agarose gel (ethidium bromide stain, bromophenol blue marker dye) for 30 min at 100 V. The gel was visualized under UV light and photographed.

RESULTS AND DISCUSSION

The purity of the *Hematodinium*-like dinoflagellates from the hemolymph of *Chionoecetes bairdi* was 99% after microscopical examination. The amount of DNA recovered from *Hematodinium*-like dinoflagellates from *C. bairdi* was 75.0 µg from 1.0 × 10^6 cells. This amount was higher than that recovered by Eaton et al. (1991), i.e. 30 µg of DNA from 1.0 × 10^6 cells of *Hematodinium*-like dinoflagellates from the same host.

Results of PCR amplification showed that both host and parasite were amplified using the primers described, but the product of 680 bp in length was unique to *Hematodinium* sp. Furthermore, the intensity of products derived from host DNA was much lower than that from the parasite. Amplification of *Hematodinium*-like dinoflagellates from *Chionoecetes bairdi* resulted in a diagnostic band at DNA concentrations of at least 1 ng μl^{-1} (Fig. 1). The intensities of these bands were

Table 1. DNA concentrations/combinations for *Chionoecetes bairdi* (host) and *Hematodinium*-like dinoflagellates for PCR reactions to determine sensitivity

Sample	Host conc. (ng μl^{-1})	Parasite conc. (ng μl^{-1})
Control	0	0
1	0	100
2	0	50
3	0	20
4	0	10
5	0	1
6	0	0.5
7	0	0.1
8	0	0.01
9	100	0
10	50	0
11	20	0
12	10	0
13	1	0
14	50	50

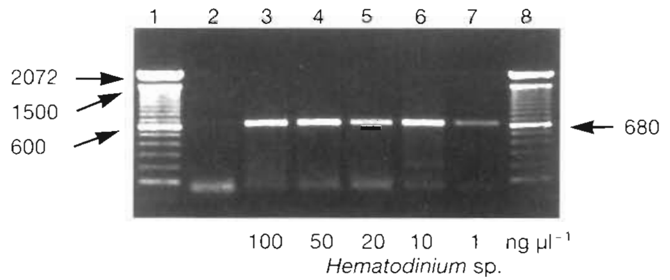

Fig. 1. PCR products derived from *Hematodinium*-like dinoflagellate from *Chionoecetes bairdi*. Lanes 1 & 8: 100 bp molecular weight ladders; lane 2: *C. bairdi* DNA concentration 100 ng μl^{-1}; lanes 3 to 7: *Hematodinium*-like dinoflagellate DNA concentrations, 100, 50, 20, 10 and 1 ng μl^{-1}, respectively

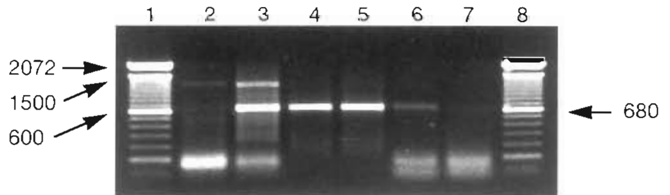

Fig. 2. PCR products derived from *Hematodinium* spp. Lanes 1 & 8: 100 bp molecular weight ladders; lane 2: *Chionoecetes bairdi* DNA concentration 100 ng μl^{-1}; lane 3: 50 ng μl^{-1} DNA from *Chionoecetes bairdi* and 50 ng μl^{-1} DNA from *Hematodinium*-like dinoflagellate; lanes 4 to 7: unknown DNA concentrations of *Hematodinium* spp. from *C. opilio*, *Callinectes sapidus*, *Nephrops norvegicus* and *Portunus pelagicus*, respectively

similar for DNA concentrations of 100, 50, 20 and 10 ng μl^{-1}, but decreased significantly below these levels. Faint bands for the host *C. bairdi* were produced of 1300 bp (Figs. 1 & 2, Lane 2) generated from DNA concentrations of 100, 50 and 20 ng μl^{-1} but not observed at lower concentrations. The host/parasite combination produced the *Hematodinium* sp. band of 680 bp and a band of 1300 bp for *C. bairdi*. The control (no DNA) PCR produced no bands. Short bands of <100 bp are non-specific products and excess primer and can be removed by purification of the PCR products. These bands were not removed to ensure that a valid representation of the amplified fragments resulted.

Hematodinium spp. from the 3 other crab hosts and the lobster host also produced bands of 680 bp (Fig. 2). However, the amplified product of *H. australis* from *Portunus pelagicus* was very faint, probably an indication of degraded DNA from the original sample rather than low levels because the initial cell count was 1.0×10^6 cells ml^{-1}.

The PCR amplification using primers A and B can detect the presence of *Hematodinium* spp. from all hosts tested in this study, at a DNA amount of at least 1 ng. Based on cell counts, 1 ng of DNA is equivalent to 1.3×10^4 cells. The diagnosis of non-host DNA is confirmed by the presence of a band of 680 bp in length. The limiting factor is the amount of DNA present which can vary as a result of the extraction technique. For example, Eaton et al. (1991) required 3.3×10^4 cells of *Hematodinium*-like dinoflagellates from *Chionoecetes bairdi* to extract 1 ng of DNA.

The production of an amplified product from the host/parasite DNA mix (Fig. 2) was significant because hemolymph from a potentially infected host can be sampled, DNA extracted and the diagnostic PCR performed without isolation of the parasite. The host band of 1300 bp acts as a positive control to verify the reaction parameters. It should be stressed that the primers used for this study are located in conserved regions and as such are not specific to *Hematodinium* spp. or other dinoflagellates. It is the length of the amplified product that is diagnostic, not simply the creation of a product. A positive test should be treated as diagnostic for the presence of non-host DNA; but, currently, microscopic examination should be undertaken prior to actual diagnosis of *Hematodinium* sp. However, in our opinion the power of PCR-based diagnostic tests is beyond question. Our continuing research is aimed at identifying regions of nucleotide sequence that are specific to 'species' or 'isolates' of *Hematodinium* spp. and *Hematodinium*-like dinoflagellates. This will provide sensitive and specific diagnosis of these organisms. Nevertheless, in order to determine nucleotide sequence and design more specific primers, the genome of *Hematodinium* spp. and *Hematodinium*-like dinoflagellates must be first amplified and then sequenced. In this paper, we have described primers that will amplify the *Hematodinium* spp. and *Hematodinium*-like ribosomal DNA, even in the presence of host contaminants. This is an ongoing problem with DNA studies of all histozoic protozoa. Futhermore, sequence patterns in appropriate regions will provide a plethora of new 'characters' from which to infer phylogenetic relationships within this enigmatic group.

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