Vol. 49: 61–70, 2002 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published April 24

Development of an 18S rRNA gene-targeted PCR-based diagnostic for the blue crab parasite *Hematodinium* sp.

Tomas Gruebl, Marc E. Frischer, Michael Sheppard, Monika Neumann, Andreas N. Maurer, Richard F. Lee*

Skidaway Institute of Oceanography, 10 Ocean Science Circle, Savannah, Georgia 31411, USA

ABSTRACT: The 18S rRNA gene from *Hematodinum* sp., a parasitic dinoflagellate that infects blue crabs, was amplified, cloned, and sequenced. The sequence showed a high similarity (95% at the nucleotide level) to sequences obtained from other dinoflagellate species, including both free-living and symbiotic species. Sequence similarity was much lower when compared with parasites of other marine invertebrates with similar life histories and with the 18S rRNA gene from the blue crab. Based on comparison of sequence alignments between *Hematodinium*, other dinoflagellate species, protozoan pathogens of oysters, and blue crab 18S rRNA gene sequences, 2 sets of PCR primers that specifically amplified fragments of the *Hematodinium* 18S rRNA gene were developed and tested. One of these primer sets (Hemat-F-1487 and Hemat-R-1654) amplified a 187 bp fragment that could be used routinely as a diagnostic test for the presence of *Hematodinium* in hemolymph from blue crabs. This fragment was consistently amplified from genomic DNA extracted from hemolymph of *Hematodinium* infected blue crabs. Comparison between the PCR technique and standard histological examination indicated that the PCR technique was reliable and provided 1000 times more sensitivity than the histological methods. The sensitivity of the PCR diagnostic was estimated to be one parasite cell among 300 000 crab hemocytes. Preliminary studies using the PCR diagnostic technique suggest that *Hematodinium* sp. is absent in crabs collected from waters with low salinity (5 to 10 ppt), but common in crabs from higher salinity environments in estuarine waters from southeastern Georgia (USA).

KEY WORDS: Blue crab · PCR · *Hermatodinium* · 18S ribosomal RNA

INTRODUCTION

Hematodium perezi is a parasitic dinoflagellate that was first reported in 1931 in crabs along the French coast (Chatton & Poisson 1931). It proliferates in the crab's hemolymph, consuming hemolymph constituents, and can be highly pathogenic. Crabs infected with *Hematodinium* sp. have been discovered in Australia, Alaska, Scotland, Canada and the eastern United States (Newman & Johnson 1975, Meyers et al. 1987, 1990, Field et al. 1992, Messick & Sinderman 1992, Shields 1992, Hudson & Shields 1994, Messick 1994, Taylor & Khan 1995, Shields & Squyars 2000). Infected blue crabs *Callinectes sapidus* have been found in estuaries along the east and gulf coasts of the United States with very high incidences of *Hematodinium* sp. in coastal bays of Maryland and Virginia (Newman & Johnson 1975, Messick 1994, Messick et al. 1999, Shields & Squyars 2000). Recently we found very high prevalence and intensities of *Hematodinium* sp. in blue crabs from salt marsh estuaries in coastal Georgia (Lee 2000). The lifecycle of *Hematodinium* sp. in blue crabs involves several different stages, including dinospores, prespores, trophonts and plasmodia (Messick 1994, Shields 1994). Two orders of dinoflagellates which parasitize crustaceans are Blastodinida and Syn-

*Corresponding author. E-mail: dick@skio.peachnet.edu

dinida with *Hematodinium* sp. belonging to the order Syndinida (Shields 1994).

The method presently used to assay for *Hematodinium* sp. involves fixing, staining of hemolymph or tissues, and identification by light microscopy. Since *Hematodinium* sp. tends to concentrate in the hemolymph, aliquots of hemolymph are generally examined to determine the prevalence and intensity of infection (Messick 1994). The intensity detection limit using this method is approximately 0.3% (1 parasite cell among 300 hemocytes). Thus, a diagnostic method that is more general, sensitive, and rapid than the presently used histological method should allow for the detection of low concentrations or perhaps even of resting stages of *Hematodinium* in blue crabs and other as of yet unidentified hosts for the parasite. Ribosomal RNA has been used extensively as a target for group and species-specific PCR primer sets and hybridization probes for bacteria, metazoans and plants, including the Dinophyceae (Sanders et al. 1997, Abouheif et al. 1998, Carlos et al. 1999, Woese 2000). In this study we describe the development of *Hematodinium*-specific primers targeted to the 18S rRNA gene and their use in assaying for *Hematodinium* in hemolymph from blue crabs.

MATERIALS AND METHODS

Collection and preparation of hemolymph. Blue crabs *Callinectes sapidus* were collected from estuaries in Georgia (USA) by trawling or trapping. Crabs were bled at the hemal sinus with a 1 ml syringe. Fifty microliters of 0.5 M EDTA was added per milliliter of hemolymph to prevent clotting. To determine if crabs were infected with *Hematodinium* sp., hemolymph samples were applied to poly-L-lysine-coated microscope slides as described by Messick (1995), fixed in Bouin's fluid, and stained with Mayer's hematoxylin and eosin (Luna 1968). Fixed and stained slides were examined at 1000× with a Nikon Eclipse 6400 microscope equipped with a Nikon 100× 1.30 NA oil objective. *Hematodinium* sp. was identified based on morphologic similarities to blue crab *Hematodinium* sp. on slides authenicated by G. Messick (NOAA, Oxford, MD, USA). Infection intensity was determined by counting the number of *Hematodinium* cells among 300 hemolymph cells and dividing number of infected by total number of cells.

Amplification, cloning and sequencing of 18S rRNA gene. The cells from the hemolymph of highly infected crabs (infection intensity ranged from 90 to 98%) and from uninfected crabs were collected by centrifugation (12 000 × *g*) for 10 min. Cell preparations from highly infected crabs were primarily *Hematodinium* with only a few crab hemocytes observed. Genomic DNA was extracted from these cell preparations by the procedures described by Frischer et al. (2000), which included addition of sodium dodecyl sulfate, proteinase K, and RNase A, followed by extraction with saturated phenol, chloroform and isoamyl alcohol. DNA was precipitated in cold ethanol. Final DNA concentration and purity were estimated by spectrophotometry (Sambrook et al. 1989).

The 18S rRNA gene from infected crab hemolymph was amplified from purified genomic DNA using a universal oligonucleotide primer (Univ-F-15) and a *Hematodinium* specific primer (Hemat-R-1654). PCR primer sequences and reaction conditions are shown in Table 1.

Table 1. 18S rRNA targeted PCR primer sets and reaction conditions used in this study

Specificity	Forward primer (5′–3′)	Reverse primer (5′–3′)	Expected frag. size (bp)	Reaction conditions[a]	Amplification parameters[b]
Generic eukaroytic[c]	Univ-F-15 ctc cca gta gtc ata tgc	Univ-R-1765 acc ttg tta cga ctt tac	1760	Standard	Standard Annealing temp. 53°C
Generic dinoflagellate	Dino-F-1108 cgc aag gct gaa act	Dino-R-1275 aca aat cac tcc acc aac	184	Standard	Standard Annealing temp. 50°C
Hematodinium-specific	Univ-F-15 ctc cca gta gtc ata tgc	Hemat-R-1654 ggc tgc cgt ccg aat tat tca c	1682	Standard	Standard Annealing temp. 54°C
Hematodinium-specific	Hemat-F-1487 cct ggc tcg ata gag ttg	Hemat-R-1654 ggc tgc cgt ccg aat tat tca c	187	Standard	Standard Annealing temp. 56°C

[a]Standard PCR reaction conditions: 40–400 µg genomic DNA; 30 ng each forward and reverse primers (primer stock concentration 100 ng μl^{-1}); Taq Master Mix Buffer (Qiagen)*; double-distilled sterile water to 25 µl

[b]Standard PCR amplification conditions: initial template denaturation (94°C for 10 min); 30 amplification cycles (94°C—15 s; annealing—15 s; 72°C—30 s). Following the completion of amplification cycles, a final extension step (72°C—10 min) is completed and samples are stored at 4°C until analysis

[c]Primer set will not amplify *Hematodinium* sp.

*The Qiagen Taq Master Mix Buffer System contains 10 U Taq DNA polymerase/100 µl, 6 mM $MgCl_2$, and 800 µM dNTP mix

The sequence of Hemat-R-1654 primer was designed and based on the partial sequence of the small subunit (SSU) rDNA gene of *Hematodinium perezi* reported by Hudson & Adlard (1994,1996). Primers were synthesized using an ABI DNA/RNA synthesizer (model 394) by the Molecular Genetics Facility at the University of Georgia. Amplification was facilitated using the Qiagen Taq Master Mix System (Qiagen) as described in Table 1. PCR was performed using a GenAmp PCR system 2400 thermal cycler (Perkin Elmer). PCR amplification products were routinely visualized and sized after electrophoretic separation on 1% agarose gels. Gels were stained with Gel Star (BMS).

To facilitate sequencing, amplified 18S rRNA gene PCR products were cloned into the pCR 2.1-TOPO cloning vector using a TOPO™ Cloning Kit, Version J (Invitrogen) following the manufacturer's instructions. Recombinant plasmids were purified from *Esherichia coli* using the High Pure Plasmid Isolation Kit (Boehringer Mannheim) following the manufacturer's instructions. Plasmid concentrations were determined by spectrophotometry (absorption at 260 nm). Cloned rDNA inserts were sequenced by the Molecular Genetics Instrumentation Facility at the University of Georgia using an ABI automated DNA sequencer. Sequencing reactions were facilitated using the ABI Big Dye prism dideoxy sequencing dye terminator kit following the manufacturer's protocols. Sequence analysis was facilitated using ABI analysis software version 3.3. (ABI). A total of 6 sequencing primers (Table 2) were utilized such that the complete gene sequence was determined in the forward (primers M13-20F[18]; 18S-570F; 18S-1138F) and reverse (primers M13-48R[24]; 18S-570R; 18S-1138R) directions. Sequences from individual sequencing reactions, excluding all primer sequences, were assembled using the assembly and editing features of the DNAsis software package Version 7.00 (Hitachi Software Engineering).

Design of *Hematodinium*-specific primers. The sequence of the 18S rRNA gene from *Hematodinium* was aligned and compared with the 18S rRNA gene of the blue crab, several other closely related dinoflagellates, and other protozoan parasites of invertebrates available from GenBank. Sequences were initially aligned using the CLUSTAL W Version 1.7 multiple sequence alignment algorithm (Thompson et al. 1994). Alignments were viewed and edited using the Genetic Database Editor (GDE; Smith et al. 1992). Sequence strings of 15 to 25 bp unique to *Hematodinium* were identified using the FIND VARIABLE REGIONS algorithm available in GDE loaded with the aligned 18S rRNA dinoflagellate and protozoan parasite database. Optimal probe target sites (14 to 25 base pairs) were initially identified based on the criteria of exhibiting at least 2 nucleotide differences from other aligned sequences. Following the initial identification of suitable sequences, PCR primer sets were designed that exhibited a minimum propensity for the formation of primer dimers and self-hybridization, that positioned nucleotide positions unique to *Hematodinium* in optimal locations for enhancing primer specificity, and that would amplify the desired fragment size range. Primer design tools available in the DNAsis software package and the Primer Premier Version 5.00 package (Premier Biosoft International) were used to facilitate optimal primer design. Oligonucleotides were synthesized at the Molecular Genetics Instrumentation Facility at the University of Georgia.

Table 2. Sequencing primers used in this study

Name	Sequence (5′ – 3′)	Direction
M13-20-F (18)	TGT AAA ACG ACG GCC AGT	Forward
18S-570F	GCC AGC AGC CGC GGT	Forward
18S-1138F	GAA ACT TAA AGG AAT	Forward
M13-48R (24)	AGC GCA TAA CAA TTT CAC ACA GGA	Reverse
18S-570R	ACC GCG GCT GCT GGC	Reverse
18S-1138R	ATT CCT TTA AGT TTC	Reverse

Empiric testing of primer specificity. For the purpose of experimentally testing the specificity of the PCR primers designed in this study, genomic DNA was extracted from hemolymph from histologically authenticated *Hematodinium*-infected and uninfected blue crabs, from cultures of 8 dinoflagellate species, and from the protozoan pathogens of oysters *Perkinsus marinus* and *Haplosporidium* sp. DNA was purified using the DNeasy™ Tissue Kit (Qiagen) following the manufacturer's instructions for whole-nucleated blood. Cultures of the dinoflagellates *Heterocapsa pygmaea* (CCMP1322), *Gymnodinium simplex* (CCMP420), *Symbiodinium microadriaticum* (CCMP827), *Katodinium rotundatum* (CCMP1542), *Gyrodinium galatheanum* (CCMP2064), *Pfiesteria piscicida* (CCMP 1928), and an unidentified 'Pfiesteria like' dinoflagellate (CCMP1873) were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton. *Gymnodinium nelsoni* was provided by G. Paffenhöffer (Skidaway Institute of Oceanography [SkIO]), *Perkinsus marinus* (meront stage) by F.-L. Shu (Virginia Institute of Marine Science, VIMS), and oysters infected with *Haplosporidium nelsoni* by Lisa Ragone (VIMS) and Susan Ford (Haskin Shellfish Research Laboratory, Rutgers University, New Brunswick, NJ, USA). Amplification of 18S rRNA gene fragments from each of these organisms was attempted using the *Hematodinium*-specific primer set

Hemat-F-1487 and Hemat-R-1654 and either the generic 18S rRNA gene-targeted universal primer set Univ-F-15 and Univ-R-1765 or the generic dinoflagellate primer set Dino-F-1108 and Dino-R-1275. These primers amplify a fragment of the 18S rRNA gene from most dinoflagellates, but are not specific for dinoflagellates. Primer sequences and PCR reaction conditions are shown in Table 1.

Sensitivity of the PCR primer assay. The sensitivity of the PCR primer assay was determined by amplifying serial dilutions (10^1 to 10^6 dilution) of isolated genomic DNA from 1 ml of hemolymph collected from a lightly infected blue crab. Hemolymph from this crab contained 300 000 *Hematodinium* cells ml^{-1} as estimated histologically. Purified DNA from hemolymph was diluted in distilled water.

Detection of *Hematodinium* in native blue crabs. One ml of hemolymph was obtained from crabs collected from the Skidaway River estuary (20 to 30 ppt salinity) and the Ogeechee River estuary (5 ppt salinity). *Hematodinium* sp. primers Hemat-F-1487 and Hemat-R-1654 were utilized to detect the presence of *Hematodinium* by PCR as outlined in Table 1 utilizing 0.1 to 0.3 µg of genomic DNA purified from each crab as starting template. Genomic DNA was isolated from hemolymph cells of blue crabs as described above using the DNeasy™ Tissue Kit. Ten crabs from each site were analyzed. The presence of the correct-sized amplification product (187 bp) was evidence of *Hematodinium*. The identity of the amplified 187 bp fragment was confirmed by sequencing of representative samples.

RESULTS

Molecular identification of *Hematodinium*

Initially, a large fragment (1682 bp) of the *Hematodinium* 18S rRNA gene was PCR-amplified and sequenced from the hemolymph of a *Hematodinium*-parasitized blue crab collected from Wassaw Sound, Georgia, in October 1999 (infection intensity: 17%). The sequence of the PCR fragment derived from the infected hemolymph was aligned with the 18S rRNA sequence of the blue crab *Callinectes sapidus* (GenBank accession number M34360 with recent corrections provided by L. G. Abele at Florida State University [pers. comm.]) and a 236 bp partial 18S rRNA gene sequence from *Hematodinium perezi* previously reported by Hudson & Allard (1994). Based on these comparisons, the fragment amplified from the parasitized Georgia blue crab was identified as a *Hematodinium* species, submitted to GenBank and assigned the accession number AF286023. Comparison of the *Hematodinium* 18S rRNA gene sequence to those available in GenBank indicates highest sequence similarity (95%) with the free-living dinoflagelate *Gymnodinium* cf. *mikimoti* (GenBank accession number AF009216), although several other dinoflagellate species, including several unidentified symbiotic species, have a comparable level of sequence similarity. These results are consistent with the morphologically derived evolution history of *Hematodinum* that places this species among the Dinophyceae and confirms that the parasite associated with blue crabs in Georgia is *Hematodinium* (Shields 1994).

Primer design

Based on comparison of sequence alignments of the 18S rRNA gene from *Hematodinium*, the blue crab, other dinoflagellates, and 2 oyster parasites, it was possible to identify several short sequence stretches that were sufficiently unique to *Hematodinium* so that they could be targeted as *Hematodinium*-specific oligonucleotide PCR primers. Initially, 18 potential primer targets were identified on the basis of these sequence comparisons, each of which exhibited at least 3 nucleotide mismatches with the 18S rRNA gene sequence of the blue crab and at least 1 nucleotide mismatch with other available sequences. From this collection of potential primer target sites, 2 primer sets conforming to all primer design criteria were identified, synthesized, and tested for their specificity and sensitivity in a PCR format. Sequence alignments for these primer sets are shown in Fig. 1. The first primer set amplified a 1682 bp fragment and consisted of the generic eukaryotic-targeted primer Univ-F-15 (Frischer et al. 2000) and the *Hematodinium*-specific primer (Hemat-F-1654). The second primer set amplified a 187 bp fragment and consisted of 2 *Hematodinium*-specific primers Hemat-F-1487 and Hemat-R-1654. The sequences of all primers utilized as PCR primer sets in this study are shown in Table 1.

Primer specificity

The specificity of each primer set was confirmed by using them to amplify a PCR product from genomic DNA purified from *Hematodinium*-infected and uninfected blue crabs, 8 closely related dinoflagellate species, and the oyster parasites *Perkinsus marinus* and *Haplosporidium nelsoni*. Each genomic DNA preparation was also amplified with either the generic 18S rRNA targeted primer set (Univ-F-15 and Univ-R-1765) or the generic dinoflagellate 18S rRNA targeted primer set (Dino-F-1108 and Dino-R-1275) to confirm that the ge-

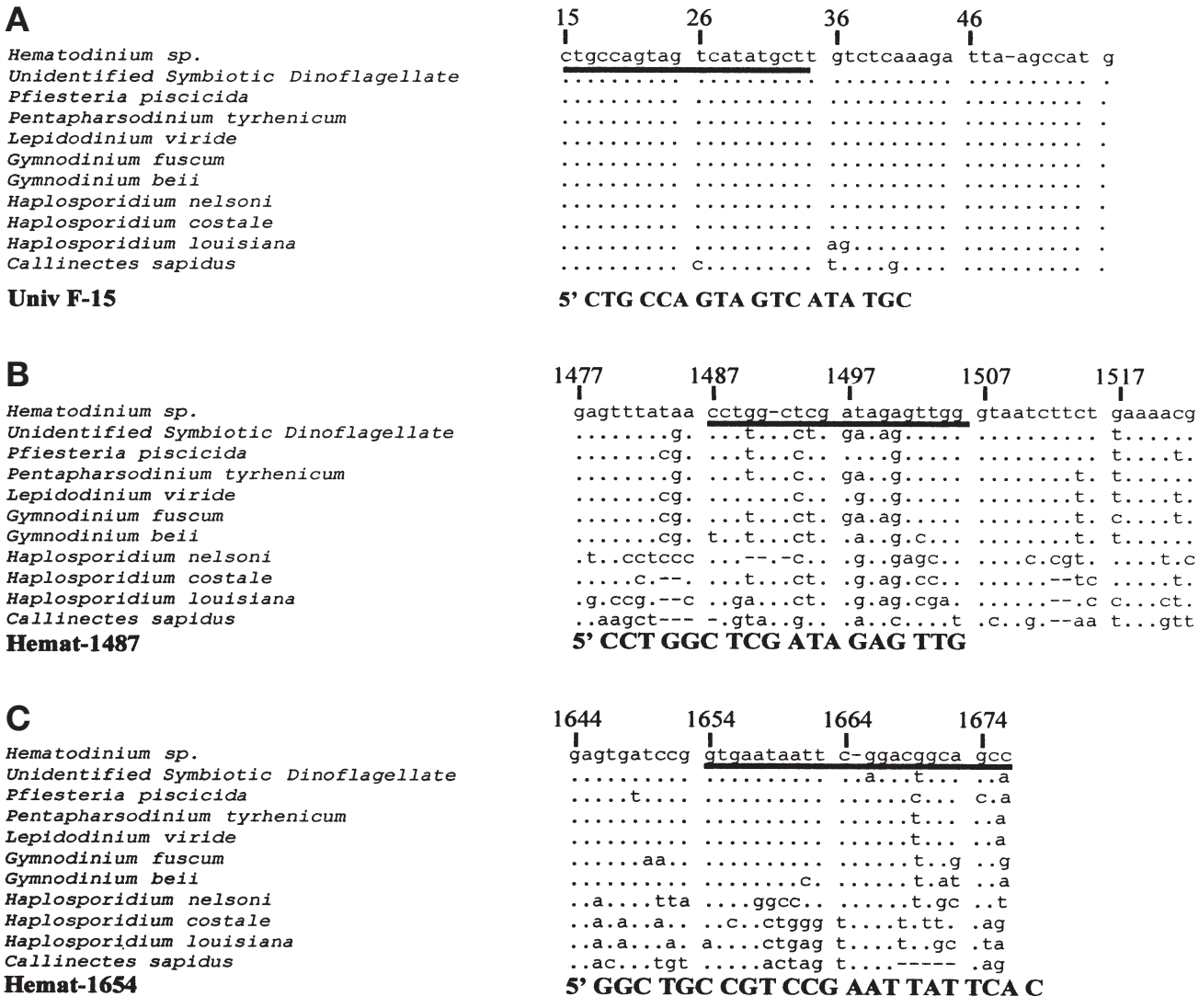

Fig. 1. Target regions in the 18S rRNA gene for (A) generic primer Univ F-15; (B) *Hematodinium*-specific primer Hemat-1487; (C) *Hematodinium*-specific primer Hemat-1654. Probe target regions are underlined and primer sequences are provided underneath each panel. Dots (.) indicate regions of exact nucleotide matches with the *Hematodinium* 18S rRNA gene sequence

nomic DNA used as template for each reaction was amenable to PCR amplification. The results of specificity studies with histologically verified uninfected and infected blue crab hemolymph are shown in Fig. 2. Both *Hematodinium*-specific primer sets amplified the expected size products from positive controls and from DNA isolated from infected crabs, but did not amplify this product from either the uninfected crab or from *P. marinus*. The results of specificity testing against the other dinoflagellate and protozoan oyster pathogens using the generic 18S rRNA targeted primer set Dino-F-1108 and Dino-R-1275 and the *Hematodinium*-specific primer set Hemat-F-1487 and Hemat-R-1654 are summarized in Table 3. As observed in initial tests, the *Hematodinium*-specific primer set yielded product only when DNA from *Hematodinium* was present, although the generic 18S rRNA targeted primers produced product from each of the dinoflagellate test strains and the protozoan pathogens. These studies demonstrate that both primer sets are specific for *Hematodinium*.

Detection sensitivity

In addition to determining the specificity of the probes designed in these studies, the sensitivity of the PCR-based *Hematodinium* diagnostic method was also determined for the Univ-F-15 and Hemat-R-

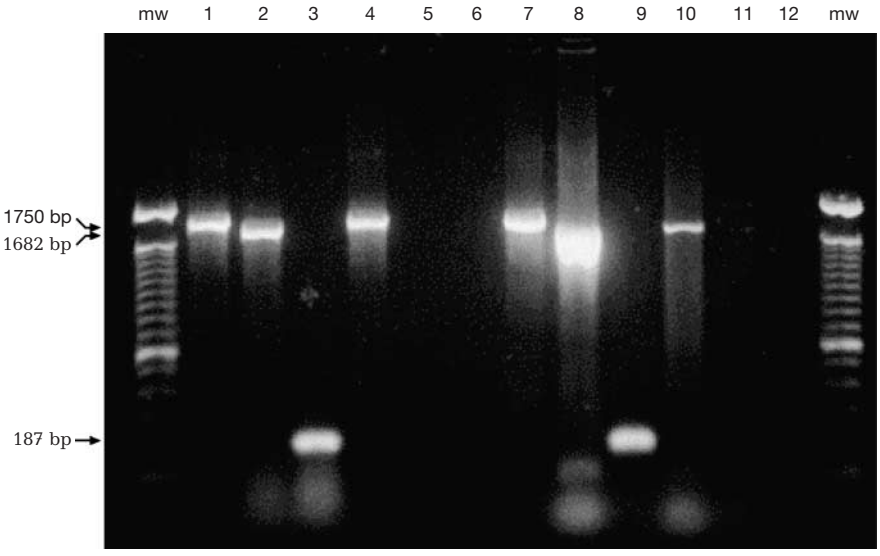

Fig. 2. Specificity of primer sets developed in this study. PCR amplification of genomic DNA purified from hemolymph from an uninfected blue crab (lanes 4–6), genomic DNA from the hemolymph of a *Hematodinium* infected blue crab (lanes 7–9), and genomic DNA from the oyster parasite, *Perkinsus marinus* (lanes 10–12). Genomic DNA purified from a nearly pure culture of *Hematodinium* cells served as a positive control (lanes 1–3). Amplification was accomplished using the generic universal primer set Univ-F-15/Univ-R-1765 (lanes 1, 4, 7, 10), the *Hematodinium*-specific primer set Univ-F-15/Hemat-R-1654 (lanes 2, 5, 8, 11), and the *Hematodinium*-specific primer set Hemat-F-1487/Hemat-R-1654 (lanes 3, 6, 9, 12). PCR products were electrophoresed and visualized on a 1% agarose gel. Molecular weights (mw) are indicated

1654 primer set and the Hemat-F-1487 and Hemat-R-1654 primer set. The sensitivity of detection using the primer set Hemat-F-1487 and Hemat-R-1654 was 2 orders of magnitude greater (0.06 parasite cells; Fig. 3A) than the sensitivity obtained with the primer set Univ-F-15 and Hemat-R-1654 (6 cells; Fig. 3B). Calculating from initial parasite concentration and volume of hemolymph initially purified for these studies, the sensitivity of the PCR diagnostic technique for the primer pair Hemat-F-1487 and Hemat-R-1654 is 1 parasite cell in 300 000 hemocyte cells or approximately 3 orders of magnitude greater than the sensitivity that can be achieved with the histological method. It was observed that in heavily infected crabs, i.e. with an infection of 90 to 95% intensity, the DNA that could be purified from the hemolymph cells was generally highly degraded and consisted largely of low molecular weight fragments. Since the Hemat-F-1487 and Hemat-R-1654 primer set amplified a small fragment (187 bp), these primers consistently amplified DNA from these degraded samples. However, it was not possible to amplify the expected 1682 bp *Hematodium*-specific product using the Univ-F-15 and Hemat-R-1654 primer set (data not shown), probably since the DNA recovered from these samples did not contain fragments larger than 1000 bp. Thus, because of greater sensitivity and consistency, the primer set Hemat-F-1487 and Hemat-R-1654 was used for all further routine diagnostic studies.

Table 3. Specificity testing of *Hematodinium*-specific and generic 18S rRNA targeted primer against reference dinoflagellate cultures and protozoan parasites. nd: not determined; see 'Empiric testing of primer specificity' for source abbreviations

Species	Source	Amplification with Hemat-F-1487/ Hemat-R-1654	Amplification with Dino-F-1108/ Dino-R-1275	Amplification with Univ-F-15/ Univ-R-1765
Heterocapsa pygmaea	CCMP 1322	No	Yes	nd
Gymnodinium simplex	CCMP 420	No	Yes	nd
Symbiodinium microadriaticum	CCMP 827	No	Yes	nd
Katodinium rotundatum	CCMP 1542	No	Yes	nd
Gyrodinium galatheanum	CCMP 2064	No	Yes	nd
Pfiesteria piscicida	CCMP 1928	No	Yes	nd
Unid. sp. (Pfiesteria-like)	CCMP 1973			
Gymnodinium nelsoni	G. Paffenhöffer (SkIO)	No	Yes	nd
Haplosporidium infected	S. Ford (Rutgers)			
Oysters	L. Ragone (VIMS)	No	Yes	nd
Perkinsus marinus	F.-L. Shu (VIMS)	No	nd	Yes
Hematodinium sp.	Wassaw Sound, Georgia (USA)	Yes	Yes	No

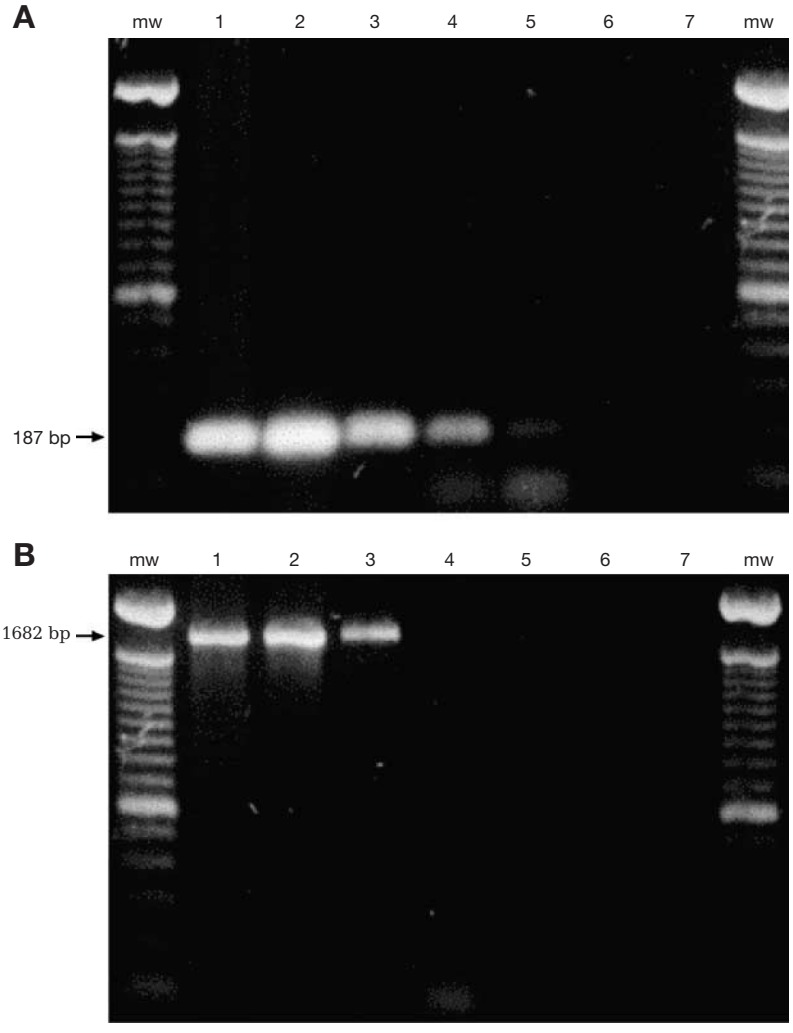

Fig. 3. *Hematodinium* detection sensitivity using *Hematodinium*-specific primer sets. Serial dilution of *Hematodinium* cells were amplified using the (A) Hemat-1487 and Hemat-1654 primer set and (B) Univ F-15 and Hemat-1654 primer set. Specific amplification of the *Hematodinium* 18S rRNA gene from DNA extracted from 600 (lane 1), 60 (lane 2), 6 (lane 3), 0.6 (lane 4), 0.06 (lane 5), 006 (lane 6) and 0.0006 (lane 7) *Hematodinium* cells are shown. PCR products were electrophoresed and visualized on a 1% agarose gel. Molecular weights (mw) are indicated

Detection of *Hematodinium* infection in native blue crabs

Initial studies were conducted to utilize the PCR *Hematodinium* diagnostic technique in field samples. Ten blue crabs were collected from a high-salinity estuary (Skidaway River) and low-salinity estuary (Ogeechee River) located in southeastern Georgia, USA. Crabs were collected in July and August 2000, respectively, and genomic DNA was purified from hemolymph. DNA yields were generally 100 to 300 µg DNA per ml of hemolymph. During 1999/2000 *Hematodinium* disease in blue crabs was reported in the Skidaway River estuary but not in the Ogeechee River estuary (Lee 2000). Of the 20 crabs examined, only 2 from the Skidaway River estuary showed a mild *Hematodinium* infection as indicated by histological procedures (SkIO 3 and 4), while all of the others appeared to be uninfected (Table 4). PCR examination of these samples confirmed that Crabs 3 and 4 from the Skidaway River estuary were infected. In addition, a weak amplification signal of the correct-sized product was also detected in the other 8 crabs collected from the Skidaway River (Table 4). Four of these amplification products were cloned and sequenced and their identity confirmed to be *Hematodinium* (99 to 100% sequence similarity; data not shown). Of the 10 crabs collected from the Ogeechee River estuary, none appeared to be infected with *Hematodinium* by histological observation or by the primer PCR assay (Table 4). These results demonstrate the reliability of the PCR diagnostic *Hematodinium* assay developed in this study, and demonstrate the use of this procedure in routine field studies.

DISCUSSION

A significant decline in the Georgia (USA) blue crab harvest during 1999 and 2000 has been partially attributed to the disease caused by the parasitic dinoflagellate *Hematodinium* (Lee 2000). However, the identification and study of this parasite has been hampered by the requirement of a histological-based diagnostic technique. Routine histological diagnosis of *Hematodinium* infection in blue crabs typically involves the examination of 100 to 300 hemocytes per sample. Thus, the sensitivity of this diagnostic technique is approximately 1 cell per 100 to 300 cells, is limited to the detection of morphologically recognized forms of the parasite, and requires the expertise of a trained parasitologist (Chatton & Poisson 1931, Messick 1994). In this study we amplified, cloned, and sequenced the 18S rRNA gene from the parasite associated with diseased crab to identify the crab parasite and to develop a novel molecular PCR-based diagnostic tool that could provide a simpler, more sensitive, and reliable new diagnostic tool for *Hematodinium* in blue crabs.

Table 4. Detection of *Hematodinium* in blue crabs from the Skidaway River and Ogeechee River estuaries by histological and PCR techniques

Crab	Histology		PCR[b]
	Presence	Intensity[a]	
Skidaway River, July 2000			
1	No	0	+
2	No	0	+
3	Yes	13%	++
4	Yes	16%	++
5	No	0	+
6	No	0	+
7	No	0	+
8	No	0	+
9	No	0	+
10	No	0	+
Ogeechee River, August 2000			
1	No	0	–
2	No	0	–
3	No	0	–
4	No	0	–
5	No	0	–
6	No	0	–
7	No	0	–
8	No	0	–
9	No	0	–
10	No	0	–

[a]Intensity determined by counting number of *Hematodinium* sp. cells among 300 hemolymph cells and dividing by 300

[b]Detection of the 187 bp *Hematodinium*-specific 18S rRNA gene fragment by PCR using primers Hemat-1487 and Hemat-1654. Relative strength of detection (++ strong detection; + weak detection; – no product detected)

Based on comparisons between the 18S rRNA sequence of the blue crab parasite and available sequences in the public sequence databases, we confirmed the identity of the Georgia blue crab parasite as *Hematodinium* sp. Previously *Hematodinium* had been identified in blue crabs from Georgia by histological staining (Messick & Shields 2000). The 18S rRNA gene from the blue crab parasite showed the highest similarity (95% at the nucleotide level) to the 18S rRNA gene sequence from other dinoflagellate species (including free-living and symbiotic species). Interestingly, *Hematodinium* sp. showed 91.6% nucleotide similarity with the 18S rRNA gene from *Pfiesteria piscicida* (GenBank accession number AF149793). The 18S rRNA gene sequences from *Hematodinium* sp. was significantly less similar to the blue crab (64.3% nucleotide similarity) and other protozoan pathogens associated with other marine invertebrates including *Haplosporidium nelsoni* and *Perkinsus marinus*. Both *Haplosporidum* and *Perkinsus* are protozoan parasites of the oyster and exhibit an analogous life history to *Hematodinium* in oysters. For example, both *Hematodinium* and *Perkinsus* disease outbreaks appear to be associated with high salinity and temperature conditions (Ragone & Burreson 1993, Brousseau & Baglivo 2000). *P. marinus* is currently classified in the phylum Apicomplexa (Perkins 1976, Levine 1988), but recent molecular studies based on SSU rRNA and actin gene sequences have suggested that *P. marinus* may be more closely related to the Dinophyceaea (Flores et al. 1996, Fong et al. 1993, Reese et al. 1997, Siddall et al. 1997). The *Hematodinium* sequence determined in this study was 87.7% similar at the nucleotide level to the SSU rRNA sequence of *P. marinus* strain P1 (GenBank accession number AF126013).

Molecular techniques based on PCR have become widespread as diagnostic tools to assess the presence of cryptic parasitic and non-parasitic organisms in diverse settings, including protozoan pathogens of marine invertebrates. For example, PCR diagnostic approaches have been used to detect the protozoan parasites *Haplosporidium nelsoni* and *Perkinsus marinus* in the eastern oyster *Crassostrea virginica* (Ford et al. 2000). PCR primers have also been designed to detect the parasite *Bonamia ostreae* in the flat oyster *Ostrea edulis* and the agent of QPX in the hard clam *Mercenaria mercenaria* (Carnegie et al. 2000, Stokes et al. 2000). In some cases the PCR technique was also demonstrated as being useful for differentiating the level of infection (Carnegie et al. 2000). An advantage of genetic-based diagnostic tools over histological methods for studying parasitic organisms with complex life histories is that it is not necessary for the infection to be symptomatic in the host. Additionally, molecular detection obviates the need to recognize the parasite morphologically which, especially in the case of parasitic dinoflagellates with complex life histories, can be a difficult challenge requiring highly trained personnel and complete life history information (Burkholder & Glasgow 1997). In the case of *Hematodinium* sp., it is not clear that the entire life history of the organism is known since it has not been reliably cultured. Stages of *Hematodinium* sp. identified in blue crabs include trophont, dinospores and multi-nucleated plasmodia stages (Shields & Squyars 2000). However, there may be other stages in other host organisms that can serve as reservoirs of *Hematodinium*, or resting stages may persist outside of any host in the water or sediment. The PCR primers designed in this study will therefore be useful for elucidating the life history of *Hematodinium* and will be crucial to understanding and perhaps mitigating the effects of *Hematodinium* disease on blue crab populations.

Although it has been difficult to derive quantitative information based on standard end-point PCR protocols such as those utilized in this study (Raeymaekers 1993, Innis et al. 1995, Chandler 1998), our results suggest that under the described conditions, the amount of PCR product produced is at least qualitatively related to the amount of parasite present in a given sample.

For example, in the dilution series experiments designed to determine detection sensitivity, the amount of PCR product produced after 30 amplification cycles was a function of the amount of template present (Fig. 3). Only a small amount of *Hematodinium*-specific product was detected in the sample where DNA from 0.06 parasite cells was present, while samples with less DNA produced no product, and samples with more DNA produced significantly more product. However, potential PCR inhibitory effects of hemolymph were not investigated specifically in these studies, so it might not be possible to extrapolate these results to direct analyses from undiluted hemolymph samples. Nonetheless, *Hematodinium*-specific amplification from 8 of the 10 crabs collected from the Skidaway River estuary (a high-salinity estuary) yielded small amounts of products, while the other 2 crabs which tested positive for *Hematodinium* by the histological method were strongly positive. These observations provide preliminary evidence suggesting that *Hematodinium* was present in low levels, or perhaps in resting stages, in apparently asymptomatic animals. All of the crabs collected from Ogeechee River and its associated Ossabaw Sound (a low-salinity estuary) were negative for *Hematodinium*, regardless of whether the histological or PCR technique were employed. Additional quantitative PCR or *in situ* hybridization studies will be needed to confirm this hypothesis.

The *Hematodinium*-specific primer set and diagnostic method developed in his study provides a new and sensitive tool for the detection and diagnosis of *Hematodinium* sp. in blue crabs and in other host organisms. The availability of the PCR diagnostic technique should facilitate future studies of the *Hematodinium* distribution, life history, host range, and transmission pathways.

Acknowledgements. This work was supported by New York Sea Grant (NA 76RG9499) to M.E.F. and Georgia Sea Grant (NA06R60029) to R.F.L. The donation of *Perkinsus marinus* by F.-L. Chu (Virginia Institute of Marine Science), *Haplosporidium*-infected oysters by L. Rigone (Virginia Institute of Marine Science) and S. Ford (Rutgers University), and a culture of *G. nelsoni* by G. Paffenhöffer (SkIO) is gratefully acknowledged. We also acknowledge the advice and help of G. Messick (Center for Coastal Environmental Health and Biomolecular Research, Cooperative Oxford Lab, Oxford, MD) and technical assistance from J. M. Danforth, R. Juraske, and A. Trutzel. A. Boyette helped to prepare the figures and V. Patrick prepared the final manuscript.

LITERATURE CITED

Abouheif E, Zardoya R, Meyer A (1998) Limitations of metazoan 18S rRNA sequence data: implications for reconstructing a phylogeny of the animal kingdom and inferring the reality of the Cambrian explosion. J Mol Evol 47:394–405

Brousseau DJ, Baglivo JA (2000) Modeling seasonal proliferation of the parasite *Perkinsus marinus* (dermo) in field populations of the oyster *Crassostrea virginica*. J Shellfish Res 19:133–138

Burkholder JM, Glasgow HB (1997) *Pfiesteria piscicida* and other *Pfiesteria*-like dinoflagellates; behavior, impacts, and environmental controls. Limnol Oceanogr 42:1053–1075

Carlos AA, Baillie BK, Kawachi M, Maruyama T(1999) Phylogenetic position of Symbiodinium (Dinophyceae) isolates from triacnids (Bivalvia), cardiids (Bivalvia), a sponge (Porifera), a soft coral (Anthozoa), and a free-living strain. J Phycol 35:1054–1062

Carnegie RB, Barber BJ, Distel DL, Culloty SC (2000) Development of a PCR assay for detection of *Bonamia ostreae* in flat oysters, *Ostrea edulis*. J Shellfish Res 19: 643

Chandler DP (1998) Redefining relativity: quantitative PCR at low template concentrations for industrial and environmental microbiology. J Ind Microbiol Biotechnol 21:128–140

Chatton E, Poisson R (1931) Sur l'existence, dans le sang des crabes, de peridiniens parasites: *Hematodinium perezi* n.g., n.sp. (Syndinidae). CR Seances Soc Biol Paris 105: 553–557

Field RH, Chapman CJ, Taylor AC, Neil DM, Vickerman K (1992) Infection of the Norway lobster *Nephropos norvegicus* on the west coast of Scotland. Dis Aquat Org 13:1–15

Flores BS, Sidall ME, Burreson EM (1996) Phylogeny of the Haplosporidia (Eukaryota: Alveolata) based on small subunit ribosomal RNA sequence. J Parasitol 82:616–623

Fong D, Rodrigues R, Koo K, Sun J (1993) Small subunit ribosomal RNA gene sequence of the oyster parasite *Perkinsus marinus*. Mol Mar Biol Biotechnol 6:346–350

Ford S, Xu Z, DeBrosse G (2000) Acquisition and prevention of MSX and Dermo in a hatchery and land-based nursery: a DNA assay investigation. J Shellfish Res 19:571

Frischer ME, Danforth JM, Tyner LC, Leverone JR, Marelli DC, Arnold WS, Blake NJ (2000) Development of an *Argopecten*-specific 18S rRNA targeted genetic probe. Mar Biotechnol 2:11–20

Hudson DA, Adlard RD (1994) PCR techniques applied to *Hematodinium* spp. and *Hematodinium*-like dinoflagellates in decapod crustaceans. Dis Aquat Org 20:203–206

Hudson DA, Adlard RD (1996) Nucleotide sequence determination of the partial SSU rDNA gene and ITS1 region of *Hematodinium* cf. *Perezi* and *Hematodinium*-like dinoflagellates. Dis Aquat Org 24:55–60

Hudson D, Shields JD (1994) *Hematodinium* australis n.sp., a parasitic dinoflagellate of the sand crab *Portunus pelagicus* and mud crab *Sylla serrata* from Moreton Bay, Australia. Dis Aquat Org 19:109–119

Innis MA, Gelfland DH, Sninsky JJ (1995) PCR strategies. Academic Press, San Diego

Lee RF (2000) Disappearance of blue crabs from a Georgia salt marsh estuary due to parasitic dinoflagellate: linkage to high salinities caused by drought. Scenes (Newletter of the Skidaway Marine Science Foundation) 16(2,3):6–8

Levine ND (1988) The protozoan phylum Apicomplexa. CRC Press, Boca Raton, FL

Luna LG (1968) Manual of histologic staining methods of the Armed Forces Institute of Pathology, 3rd edn. McGraw-Hill, New York

Messick GA (1994) *Hematodinium perezi* infections in adult and juvenile blue crabs *Callinectes sapidus* from coastal bays of Maryland and Virginia, USA. Dis Aquat Org 19: 77–82

Messick GA (1995) Laboratory techniques to detect parasites and diseases of blue crab, *Callinectes sapidus*. In: Stolen JS, Fletcher TC, Smith A, Zelikoff JT, Kaatari SL, Ander-

son RS, Söderhäll K, Weeks-Perkins BA (eds) Techniques in fish immunology-4. SOS Publications, Fair Haven, NJ, p187–198

Messick GA, Shields JD (2000) The epizootiology of the parasitic dinoflagellate *Hematodinium* sp. in the American blue crab *Callinectes sapidus*. Dis Aquat Org 43:139–152

Messick GA, Sindermann CJ (1992) Synopsis of principal diseases of the blue crab, *Callinectes sapidus*. US Dept Commerce, NOAA Technical Memorandum NMFS–F/NEC–88

Messick GA, Jordan SJ, Van Heukelem WF (1999) Salinity and temperature effects of *Hematodinium* sp. in the blue crab *Callinectes sapidus*. J Shellfish Res 18:657–662

Meyers TR, Koeneman TM, Botelho C, Short S (1987) Bitter crab disease: a fatal dinoflagellate infection and marketing problem for Alaskan Tanner crabs *Chionoecetes bairdi*. Dis Aquat Org 3:195–216

Meyers TR, Botelho C, Koeneman TM, Short S, Imamura K (1990) Distribution of bitter crab dinoflagellate syndrome in southeast Alaskan Tanner crabs *Chionoecetes bairdi*. Dis Aquat Org 9:37–43

Newman MW, Johnson CA (1975) A disease of blue crabs (*Callinectes sapidus*) caused by a parasitic dinoflagellate, *Hematodinium* sp. J Parasitol 63:554–557

Perkins FO (1976) Zoospores of the oyster pathogen, *Dermocystidium marinum*. I. Fine structure of the conoid and other sporozoan-like organelles. J Parasitol 62:959–974

Raeymaekers L (1993) Quantitative PCR: theoretical considerations with practical implications. Anal Biochem 214: 582–585

Ragone LM, Burreson (1993) Effect of salinity of infection progression and pathogenicity of *Perkinsus marinus* in the eastern osyter, *Crassostrea virginia* (Gmelin). J Shellfish Res 12:1–7

Reese K , Siddall ME, Burreson EM, Graves JE (1997) Phylogenetic analysis of *Perkinsus* based on actin gene sequences. J Parasitol 83:417–423

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sanders GW, Hill DRA, Sexton JP, Anderson RA (1997) Small subunit ribosomal RNA sequences from selected dinoflagellates: testing classical evolutionary hypotheses with molecular systematic methods. Plant Syst Evol, Suppl 11: 237–259

Shields JD (1992) The parasites and symbionts of the blue sand crab, *Portunus pelagicus*, from Moreton Bay, Australia. J Crustac Biol 12:94–100

Shields JD (1994) The parasitic dinoflagellates of marine crustaceans. Ann Rev Fish Dis 4:241–271

Shields JD, Squyars CM (2000) Mortality and hematology of blue crabs, *Callinectes sapidus*, experimentally infected with the parasitic dinoflagellate *Hematodinium perezi*. Fish Bull 98:139–152

Siddall ME, Reece KS, Graves JE, Burreson EM (1997) 'Total evidence' refutes the inclusion of *Perkinsus* species in the phylum Apicomplexa. Parasitol 115:165–176

Smith SW, Overbeek R , Olsen G, Woese C, Gillevet PM, Gilbert W (1992) Genetic data environment and the Harvard genome database, genome mapping and sequencing. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Stokes NA, Calvo LMR, Burreson EM (2000) DNA-based molecular diagnostics for the hard clam parasite QPX (quahog parasite unknown). J Shellfish Res 19: 649

Taylor DM, Khan RA (1995) Observations on the occurrence of *Hematodinium* sp. (Dinoflagellata: Syndinidae), the causative agent of bitter crab disease in Newfoundland snow crab (*Chionoecetes opilio*). J Invert Pathol 65: 283–288

Thompson JD, Higgins DG, Gibson TJ (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680.

Woese CR (2000) Interpreting the universal phylogenetic tree. Proc Natl Acad Sci USA 97:8392–8396

Editorial responsibility: Carey Cunningham, Aberdeen, Scotland, UK

Submitted: January 15, 2001; Accepted: September 27, 2001
Proofs received from author(s): April 4, 2002