



# Molecular detection of *Hematodinium* sp. in Northeast Pacific *Chionoecetes* spp. and evidence of two species in the Northern Hemisphere

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**ABSTRACT:** *Hematodinium* is a genus of parasitic dinoflagellates that infects crustaceans worldwide including Tanner crabs *Chionoecetes bairdi* and snow crabs *C. opilio* in the Northeast Pacific Ocean. The present study describes the optimization of a PCR-based assay for the detection and monitoring of *Hematodinium* sp. in snow and Tanner crabs. Two fragments, 1682 and 187 bp, were amplified from the 18S ribosomal DNA region of the parasite. The assay performed well in 6 additional decapod species (1 lobster and 5 crabs) infected with *Hematodinium* spp., suggesting that it could be used to detect *Hematodinium* spp. in other decapods. We also report *Hematodinium* spp. infections in the majid crab, *Hyas coarctatus*, and the lithodid crab, *Lithodes couesi*. Sequencing of 18S rDNA and the adjacent internal transcribed spacer 1 (ITS1) region of *Hematodinium* spp. isolated from 7 host species in the present study revealed the presence of 2 *Hematodinium* clades, one in the blue crab *Callinectes sapidus* and a second in all other host species. The ITS1 sequences of the 2 clades could not be aligned, but showed a conserved secondary structure that may be related to a functional diversification during a host switch. Comparison of our data with 18S and ITS1 sequence data available in GenBank placed the north Pacific *Hematodinium* sp. in a clade separate from the *Hematodinium* sp. infecting the portunoids, *C. sapidus*, *Liocarcinus depurator* and *Scylla serrata*, and within a second clade that infected all other decapod hosts located in the North Pacific and North Atlantic Oceans.

**KEY WORDS:** ITS1 · Secondary structure · 18S rDNA · Parasite · PCR · Crustacean · Parasitic · Dinoflagellate · Disease

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## INTRODUCTION

Bitter crab syndrome (BCS) is a fatal disease of Tanner crabs *Chionoecetes bairdi* and snow crabs *C. opilio* in the North Pacific Ocean that is caused by a parasitic dinoflagellate in the genus *Hematodinium*. The type species of *Hematodinium perezii* was described from *Liocarcinus depurator* in France (Chatton & Poisson 1931) and until the mid-1980s, members of the genus were sporadically encountered in crustacean populations. In the late 1980s to early 1990s, large mortalities

resulting from *Hematodinium* infections were reported in Tanner crabs in southeastern Alaska (Meyers et al. 1987), snow and Tanner crabs in the Bering Sea (Meyers et al. 1996) and *Cancer pagurus* and *Necora puber* in France (Latrouite et al. 1988, Wilhelm & Mialhe 1996). In 1994, a second species, *H. australis*, was described in sand crabs *Portunus pelagicus* from Moreton Bay, Australia (Hudson & Shields 1994). Since 1975, over 40 crustacean species distributed across the northern Atlantic and Pacific Oceans have been reported as being infected with *Hematodinium* spp.,

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including *Portunus pelagicus*, Norway lobsters *Nephrops norvegicus* and blue crabs *Callinectes sapidus* (reviewed in Stentiford & Shields 2005, Small et al. 2006, 2007b). Despite the potential economic and ecological impact of *Hematodinium*-related mortality on affected populations, little is known about the method of infection, the life history of the parasite outside the host and the underlying causes for the recent worldwide increase in frequency of *Hematodinium*-associated disease.

Traditional techniques of disease detection involve macroscopic examination of pleopods that generally result in detection of only relatively advanced infections (Stentiford et al. 2001) or microscopic examination of hemolymph smears. More sensitive, immunological methods for *Hematodinium* detection (Field & Appleton 1996, Stentiford et al. 2001, Small et al. 2002) are of insufficient specificity or may not recognize life stages of *Hematodinium* absent in the original inoculum or in alternate hosts (Bushek et al. 2002, Small et al. 2006). Use of the PCR has become increasingly widespread in pathogen identification and disease detection and monitoring and is particularly useful in the diagnosis of cryptic organisms such as dinoflagellates and parasitic stages (e.g. Cunningham 2002, Gruebl et al. 2002, Lee & Frischer 2004, Small et al. 2006). Ribosomal DNA (rDNA) has been used in many successful PCR-based diagnostic assays, including assays for *Hematodinium* in blue crabs (Gruebl et al. 2002, Small et al. 2007a) and Norway lobsters (Small et al. 2006).

The internal transcribed spacers (ITS) between the 18S and 5.8S rDNA gene (ITS1) and between the 5.8S and 28S gene (ITS2) are often highly variable with a large proportion of indels (insertions and/or deletions), so much so that sequences often cannot be aligned with confidence. However, the secondary structure of the ribosome is often conserved across surprisingly large taxonomic groups (e.g. ITS2 in green algae and flowering plants, Mai & Coleman 1997). The high degree of conservation suggests some significance of ITS secondary structure in the folding of rDNA (Musters et al. 1990), and perhaps in the regulation of RNA transcription (Van Herwerden et al. 2003). Although the 18S rDNA gene and ITS1 (Hudson & Adlard 1996, Gruebl et al. 2002, Small et al. 2007a,b) regions of several *Hematodinium* isolates have been sequenced, their secondary structure has not been investigated.

PCR-based assays for the detection of *Hematodinium* spp. in *Callinectes sapidus* and *Nephrops norvegicus* were developed from parasites isolated from those hosts with limited or no testing on infected Pacific species of *Chionoecetes* (Gruebl et al. 2002, Frischer et al. 2006, Small et al. 2006, 2007a). As differ-

ences have been noted between *Hematodinium* isolates infecting different host species, including *Chionoecetes* spp., with regards to DNA sequences, morphology, virulence, seasonality of infection, length of infection and environmental salinity (e.g. Hudson & Shields 1994, Hudson & Adlard 1996, Small et al. 2007b, J. F. Morado pers. obs.), we questioned whether PCR-based assays developed for *Hematodinium* spp. ex *C. sapidus* or *N. norvegicus* would perform well on *Hematodinium* sp. infecting Pacific *Chionoecetes* spp. Therefore, we optimized and evaluated an efficient and sensitive assay for *Hematodinium* sp. in Tanner and snow crabs. Our goal was to develop such an assay and to investigate DNA sequence differences between *Hematodinium* spp. derived from *Chionoecetes* spp. and other host species. Based on 18S sequences and the secondary structure of ITS1, we present evidence of 2 clades of *Hematodinium* in the Northern Hemisphere, probably deserving of species status.

## MATERIALS AND METHODS

**Sample collection.** Paired samples of ethanol-preserved hemolymph and hemolymph smears were collected from random samples of *Chionoecetes bairdi* (n = 150) from the Bering Sea, Gulf of Alaska and southeastern Alaska, and *C. opilio* (n = 61) from the Bering Sea and Newfoundland in 2003 and 2004 during annual stock assessment surveys by the Alaska Fisheries Science Center, Alaska Department of Fish and Game, and Department of Fisheries and Oceans Canada. Additional samples were collected from 15 visibly diseased *C. bairdi*. Ten-fold serial dilutions of hemolymph from 3 visually BCS positive *C. bairdi*, using hemolymph of 3 visually BCS negative *C. bairdi* as diluents, were prepared at sea. Before the dilutions were preserved in ethanol, 3 hemolymph smears were prepared from each hemolymph dilution (n = 81), the undiluted hemolymph (n = 9) and each BCS negative crab (n = 9).

Colleagues provided ethanol-preserved hemolymph samples from *Hematodinium*-infected *Callinectes sapidus* (Maryland and Virginia, USA), *Chionoecetes angulatus*, *Chionoecetes tanneri* (British Columbia, Canada) and *Nephrops norvegicus* (Scotland, UK). A single *Hyas coarctatus* specimen collected in the Bering Sea showing clinical signs of *Hematodinium* infection was frozen at sea and later transferred to 100% ethanol. A hemolymph sample from a *Lithodes couesi* specimen captured near Vancouver Island, Canada, was preserved in 100% ethanol. Samples of *Hematodinium australis* could not be located.

Hemolymph smears were prepared and air-dried at sea, transported to the laboratory and stained using a

modified Wright's stain (Thompson 1966). Twenty random fields were examined per each slide and the composition of host hemocytes, trophonts, and pre-spores was rated between 1 (<<1 to 10%) and 5 (90 to 100%); however, the combined qualitative rating for each sample could not exceed 5. For example, a rating of 1, 3, 1 (sum = 5) indicated that hemocytes, trophonts and pre-spores were all present; trophonts were most abundant but a transition phase from trophonts to pre-spores was evident. All smears were read independently of the molecular analysis.

**PCR diagnostic assay.** For DNA isolation, the ethanol-preserved hemolymph was centrifuged, the supernatant decanted and the pelleted material air-dried. The pellet was processed on DNeasy Tissue Kit (Qiagen) columns according to the manufacturer's instructions for animal tissue. Genomic DNA extractions from 10 infected hosts (n = 2 *Callinectes sapidus*, 2 *Nephrops norvegicus*, 2 *Chionoecetes tanneri*, 1 *Chionoecetes angulatus*, 3 *Chionoecetes bairdi*) and 6 uninfected crabs (n = 2 *C. tanneri*, 2 *C. angulatus*, 2 *C. bairdi*), as identified by hemolymph smear examination, were used for optimization of the PCR-based assay. We first attempted to optimize the amplification conditions for the 18S and ITS1 regions using the published primer pairs Hsp 1f/2r (Hudson & Adlard 1994), Univ-F-15/Hemat-R-1654 and Hemat-F-1487/Hemat-R-1654 (Gruebl et al. 2002), which are specific for *Hematodinium* sp. ex *Callinectes sapidus*, by varying primer, *Taq* polymerase (in Storage Buffer A, Promega), MgCl<sub>2</sub> and bovine serum albumin concentrations, and annealing temperatures. Positive and nega-

tive controls were included for all PCR reactions. The PCR products were visualized on 2% agarose gels stained with SYBR® Green. To investigate inhibitory effects of large amounts of parasite DNA, amplification of 5 heavily infected samples, as revealed by histological examination, were evaluated using 5 times the usual volume of template in PCRs. New primers targeting the ITS1 rDNA region were designed (Table 1, Fig. 1) using Amplicon (Jarman 2004) from an alignment of *Hematodinium* sequences from the present study and dinoflagellate sequences obtained from GenBank. PCR products generated by the new primers were sequenced (see next section) to confirm ITS1 amplifications.

Samples were evaluated with the PCR assay under blind conditions and compared with blind results obtained from microscopic examination of paired hemolymph smears. If results of the microscopic examination and the PCR assay differed, the corresponding slides were re-examined and template DNA was re-amplified.

**18S and ITS1 sequencing.** Amplified 18S and ITS1 rDNA fragments were purified using a Qiaquick Purification Kit (Qiagen) and sequenced bidirectionally using BigDye Terminator® cycle sequencing kit (ABI) on an ABI 3730XL DNA Analyzer by the DNA Sequencing Facility at the University of Washington, Department of Biochemistry. To minimize *Taq*-generated sequencing errors, 3 to 5 separate PCR products per template were combined before sequencing. Sequences were assembled in Sequencher v. 4.2 (Gene Codes) and aligned using ClustalW in BioEdit v. 7.0.1

Table 1. *Hematodinium* spp. rDNA primer pairs, amplicon size, reaction conditions and primer sequence sources. Standard PCR conditions: 1 µl genomic DNA, 100 µM each dNTPs, 1× buffer (50 mM KCl, 10 mM Tris-HCl, 0.1% Triton® X-100), 0.4 mg ml<sup>-1</sup> bovine serum albumin and sterile water to 25 µl. Standard PCR temperature profile: denaturation at 95°C for 1 min, number of cycles as designated at 94°C for 15 s, annealing temperature as designated for 15 s, 72°C for 30 s, 4°C for 5 min

Primer pair	Sequence (5'-3')	Region amplified	Expected amplicon size (bp)	Annealing temperature (°C)	MgCl <sub>2</sub> conc. (mM)	<i>Taq</i> conc. (U µl <sup>-1</sup> )	Primer conc. (µM)	No. ampl. cycles	Source
Hsp1f Hsp2r	gtt ccc ctt gaa cga gga att c cgc att tcg ctg cgt tct tc	Partial 18S and ITS1	650	54	2.5	0.04	0.5 0.5	33	Hudson & Adlard (1996)
Univ-F-15 Hemat-R-1654	ctc cca gta gtc ata tgc ggc tgc cgt ccg aat tat tca c	Partial 18S	1682	53	2.5	0.04	0.5 0.5	34	Gruebl et al. (2002)
Hemat-F-1487 Hemat-R-1654	cct ggc tcg ata gag ttg ggc tgc cgt ccg aat tat tca c	Partial 18S	187	56	3	0.05	0.216 0.18	30	Gruebl et al. (2002)
Hsp 6f Hsp 9r	gct gat tac gtc cct gc ttc acg gaa ttc tgc aat tcg	Partial 18S and ITS1	637	53	2.5	0.04	0.125 0.125	34	Present study
Hsp 7f Hsp 9r	agt cat cag ctg gtc ctg a	Partial 18S and ITS1	650	53	2.5	0.04	0.125 0.125	34	Present study
Hsp 8f Hsp 9r	tgc cct tag atg ttc tg	Partial 18S and ITS1	806	53	2.5	0.04	0.125 0.125	34	Present study

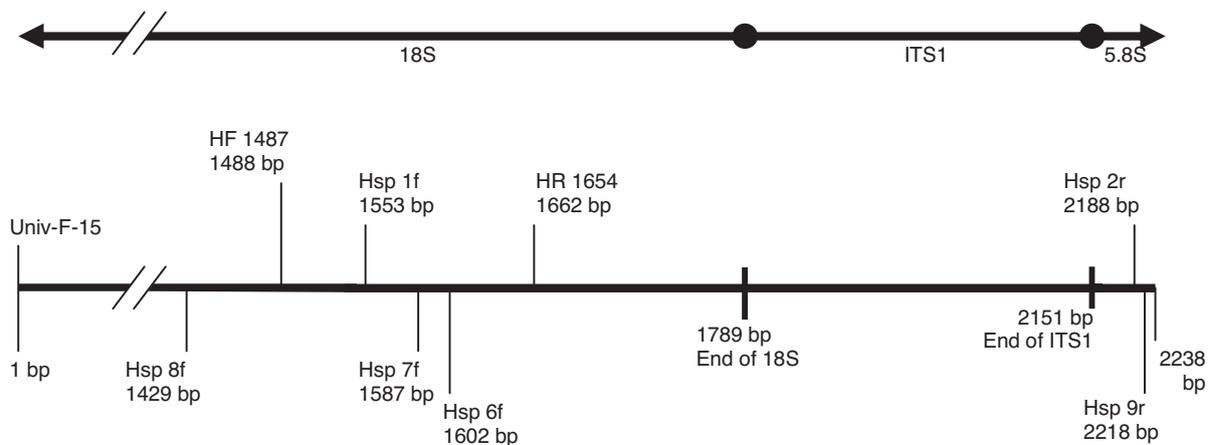


Fig. 1. *Hematodinium* spp. Schematic showing primer locations in the 18S, ITS1 and 5.8S rDNA regions of *Hematodinium*

(Hall 1999); sequences available from GenBank and sequences published by Hudson & Adlard (1996) and Gruebl et al. (2002) were included in the alignment. Based on restriction enzyme analysis in BioEdit, *EcoRI* (Invitrogen) and *HpaI* (New England Biolabs) were used to restrict the 18S amplicon.

The neighbor-joining (NJ) method (Saitou & Nei 1987) and bootstrap test (500 replicates; Felsenstein 1985) in MEGA4 (Tamura et al. 2007) were used to construct a phylogram of 25 (21 from this study, 4 from GenBank: accession # EU856716, EF065717, AF286023, DQ925237) 18S sequences; *Syndinium* sp. ex *Corycaeus* sp. (GenBank accession # DQ146406) was used as an outgroup. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option); there were a total of 1541 positions in the final dataset.

As sequence alignment was not possible between ITS1 sequences obtained from *Hematodinium* sp. ex *Callinectes sapidus* and *Hematodinium* from the other hosts, similarities of predicted secondary structure confirmations were investigated for one sequence from each clade (Clade A: *Hematodinium* ex *Callinectes sapidus*, GenBank accession # FJ844431; Clade B: *Hematodinium* ex *Chionoecetes bairdi*, GenBank accession # FJ844416) using Dynalign (Mathews 2005), part of the software package RNAstructure (Mathews et al. 2004). Default values were used for the alignment, with an M value (maximum separation of aligned nucleotides) of 15 and a gap penalty of 0.4. Twenty structures were produced but only the one with the least free energy is predicted here. In addition, mfold (Zuker 2003) was used to assess folding in individual sequences and confirm secondary structure predictions produced by Dynalign. Default values were used for all variables, including 37°C folding temperature, a linear RNA molecule and 1 M NaCl

concentration. RnaViz (De Rijk et al. 2003) was used to draw predicted structures based on the output of Dynalign. Regulatory motifs suggestive of promoter regions (Van Herwerden et al. 2003) were identified using BioEdit (Hall 1999). The sliding window feature in DnaSP 4.20.2 (Rozas et al. 2003) was used to assess nucleotide diversity,  $\pi$ , along the RNA molecule.

## RESULTS

### PCR-based diagnostic assay

Amplifications using ITS1 primers Hsp 1f/2r (Hudson & Adlard 1994) routinely produced numerous non-specific bands from infected and uninfected samples of *Chionoecetes* spp., some of which were similar in size to the expected band of 680 bp. Attempts to improve specificity by re-optimization of PCR conditions were unsuccessful and Hsp 1f/2r were not used in subsequent sample assessments. Hsp1f/2r did amplify ITS1 cleanly in some heavily infected hosts and we were able to sequence those amplicons for use in designing new primers. The newly designed primers, Hsp 6f/9r, Hsp 7f/9r and Hsp 8f/9r, produced fragments of the expected sizes with 1 or 2 non-specific bands also produced in some samples; these primers were satisfactory for sequencing ITS1, but were not used in diagnostic assays.

Once PCR conditions for the primers targeting the 18S rDNA region were optimized for the *Hematodinium* species infecting *Chionoecetes bairdi* and *C. opilio* (Table 1), both the Univ-F-15/Hemat-R-1654 and Hemat-F-1487/Hemat-R-1654 (Gruebl et al. 2002) 18S amplifications yielded consistent bands of the expected sizes, ~1680 and 190 bp, respectively, in BCS positive hosts without non-specific amplification in either BCS positive or negative samples (Fig. 2). From

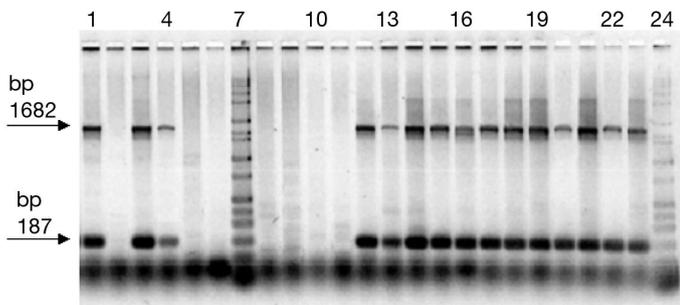


Fig. 2. *Hematodinium* sp. PCR screening of *Chionoecetes bairdi* samples with primer pairs Univ-F-15/ Hemat-R-1654 (1682 bp) and Hemat-F-1487/ Hemat-R-1654 (187 bp). All sample lanes contain combined products of both PCRs. Lanes 2, 5, 6, 8, 9, 10 and 11 containing no bands are negative for *Hematodinium*; Lane 4 is a microscopically *Hematodinium* negative sample; Lanes 1, 3, 4 and 12 to 23 are positive for *Hematodinium*; Lanes 7 and 24 are size standards

individual hemolymph samples, either both or neither primer set produced visible amplifications, providing a control for false negatives due to mutations in the priming site or human error during PCR procedures. The PCR reactions with 5 times the standard volume of template DNA showed no visible reduction in amplicon quality or quantity in either 18S PCR product. Restriction of 18S with *EcoRI* and *HpaI* yielded fragments of the expected size.

Of the 211 randomly collected samples of *Chionoecetes opilio* and *C. bairdi*, there were 35 cases of disagreement (16.5%) in BCS diagnoses between the PCR assay and hemolymph smear examination (Table 2). For those 35 crabs, re-amplification of DNA with both primer pairs did not change the PCR result, while re-examination of 33 of the 35 hemolymph smears by a different reader resulted in a change in diagnoses to that obtained from the PCR assay. Re-examination of 2 of the 35 hemolymph smears, origi-

nally diagnosed as negative did not result in a change in status; DNA re-extraction and subsequent amplification with both 18S primer pairs produced faint PCR products, i.e. the crabs had extremely light infections.

Hemolymph from the 3 crabs used for diluents in serial dilutions was negative for *Hematodinium* as determined by PCR and examination of hemolymph smears; the 3 visually positive crabs were confirmed positive by PCR and microscopic examination. Smears from the serial dilutions were consistently scored as positive by both readers through the fourth dilution in the first series and the third dilution in the second and third series. PCR results were positive for both 18S amplifications through the fourth, sixth and fifth dilutions in the first, second and third series, respectively.

### Species identification of *Hematodinium*

Hsp 1f/2r and Univ-F-15/Hemat-R-1654 primer pairs amplified the 5' end of 18S resulting in ~130 bp of overlapping sequence, allowing assembly of the 18S and ITS1 amplicons. 18S and ITS1 sequences for *Hematodinium* spp. isolated from 4 *Chionoecetes bairdi*, 3 Pacific *C. opilio*, 1 *C. angulatus*, 3 *C. tanneri*, 1 *Hyas coarctatus*, 1 *Lithodes couesi*, 2 Newfoundland *C. opilio*, 3 *Nephrops norvegicus* and 2 *Callinectes sapidus*, have been deposited in GenBank (accession numbers FJ844412 to FJ844431).

Sequence variation in 18S and the NJ tree (Fig. 3) showed 2 different clades of *Hematodinium* spp. in the isolates in the present study: one clade (A) was isolated from *Callinectes sapidus* and *Liocarcinus depurator*, and is henceforth referred to as the portunoid clade, and the other clade (B) was found in all other host species from both the North Atlantic and Pacific Oceans and is henceforth referred to as *Chionoecetes* clade according to the predominant host genus. Comparison

Table 2. *Hematodinium* sp. Results and re-examinations of samples from *Chionoecetes opilio* and *C. bairdi* evaluated both microscopically and by PCR assay

Species	n	Initial results				No. of discrepancies	Adjusted results <sup>a</sup>				No. of remaining discrepancies
		PCR +	PCR -	Hemo-lymph smear +	Hemo-lymph smear -		PCR +	PCR -	Hemo-lymph smear +	Hemo-lymph smear -	
<i>C. opilio</i>	61	50	11	39	22	11 <sup>b</sup>	50	11	50	11	0
<i>C. bairdi</i>	150	39	111	55	95	24 <sup>c</sup>	39	111	37	113	2 <sup>d</sup>

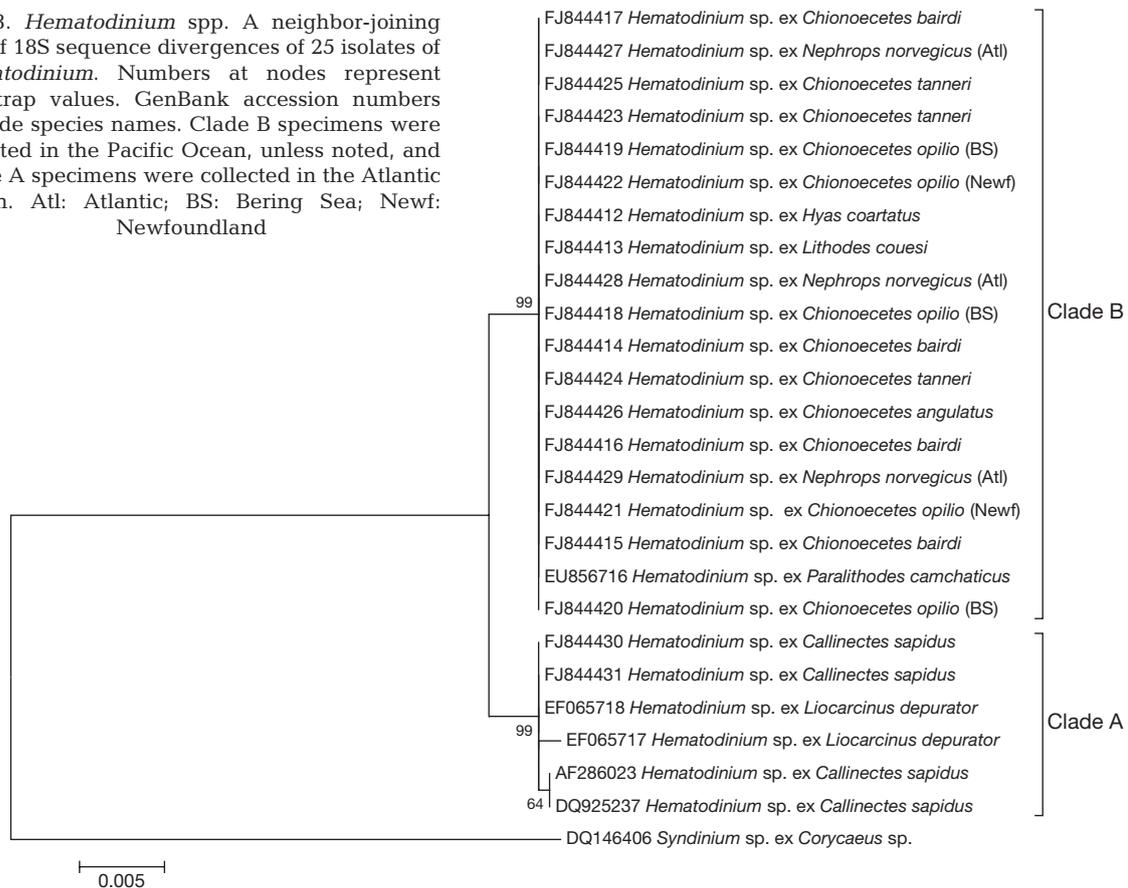
<sup>a</sup>When microscopic and molecular results were in disagreement, each sample was re-amplified and the corresponding hemolymph smear re-examined

<sup>b</sup>All originally classified as bitter crab syndrome (BCS) negative

<sup>c</sup>Twenty samples originally scored as BCS positive and 4 as BCS negative

<sup>d</sup>Two samples were scored and re-scored as BCS negative by microscopy, while extracted and successfully amplified twice with both 18S primer pairs

Fig. 3. *Hematodinium* spp. A neighbor-joining tree of 18S sequence divergences of 25 isolates of *Hematodinium*. Numbers at nodes represent bootstrap values. GenBank accession numbers precede species names. Clade B specimens were collected in the Pacific Ocean, unless noted, and Clade A specimens were collected in the Atlantic Ocean. Atl: Atlantic; BS: Bering Sea; Newf: Newfoundland



of 1771 bp of 18S sequence generated in this study, Hudson & Adlard's (1996) and Gruebl et al.'s (2002) sequences yielded 13 variable sites, or 0.73% divergence. Three sites are variable only within *Hematodinium* sp. ex *Callinectes sapidus* sequences while 9 sites show fixed differences between *Hematodinium* sp. ex *C. sapidus* and all other *Hematodinium* sequences generated in this study. One site is variable only within Clade B, but differs only in 2 of the Hudson & Adlard (1996) sequences. Inclusion of an additional 110 partial 18S sequences from GenBank revealed 4 and 7 additional variable sites within the (A) portunoid and (B) *Chionoecetes* clades, respectively, or 1.4% divergence; however, each of these substitutions is unique to only one sequence. The 18S sequences amplified with the Univ-F-15/Hemat-R-1654 primers from the *Hyas coarctatus* and *Lithodes couesi* samples were in 100% agreement with the sequences obtained from *Hematodinium* sp. ex *Chionoecetes* spp. The NJ tree produced a clear division between the 2 clades with bootstrap support of 0.99 at each clade's ancestral node (Fig. 3).

Sequencing of 18S and 5.8S revealed 5 and 4 mismatches to the Hsp 1f and Hsp 2r primer sequences (Hudson & Adlard 1994), respectively. The ITS1 se-

quences obtained with Hsp 1f/2r were 328 to 352 bp and aligned with the sequences for *Hematodinium* sp. ex *Callinectes sapidus* or *Hematodinium* sp. ex *Chionoecetes* spp. reported by Hudson & Adlard (1996); length variation was due to indels of repetitive sequences. As in 18S, sequence variation of ITS1 showed 2 different clades of *Hematodinium* spp.; Clade A was isolated from blue crab, while Clade B was found in hosts from North Atlantic and North Pacific Oceans. Alignment of the 2 clades was not possible, with the exception of a highly conserved region at the beginning of the sequence (positions 1 to 9). Alignment with ITS1 sequences available in GenBank (n = 112, data not shown), representing *Hematodinium* spp. isolated from at least 9 host species, show sequence agreement with either Clade B (83 isolates from hosts: *Pagurus bernhardus*, *P. prideaux*, *Munida rugosa*, *Nephrops norvegicus*, *Chionoecetes opilio*, *Cancer pagurus* and *Carcinus maenas*) or Clade A (1 unknown source and 28 from *Callinectes sapidus*, *Liocarcinus depurator* and *Scylla serrata*). Within each of the clades, virtually all of the variation was due to indels in the repetitive regions. There were only 1 and 2 substitutions within Clades A and B, respectively, in this study; both Clade B substitutions were within repetitive elements.

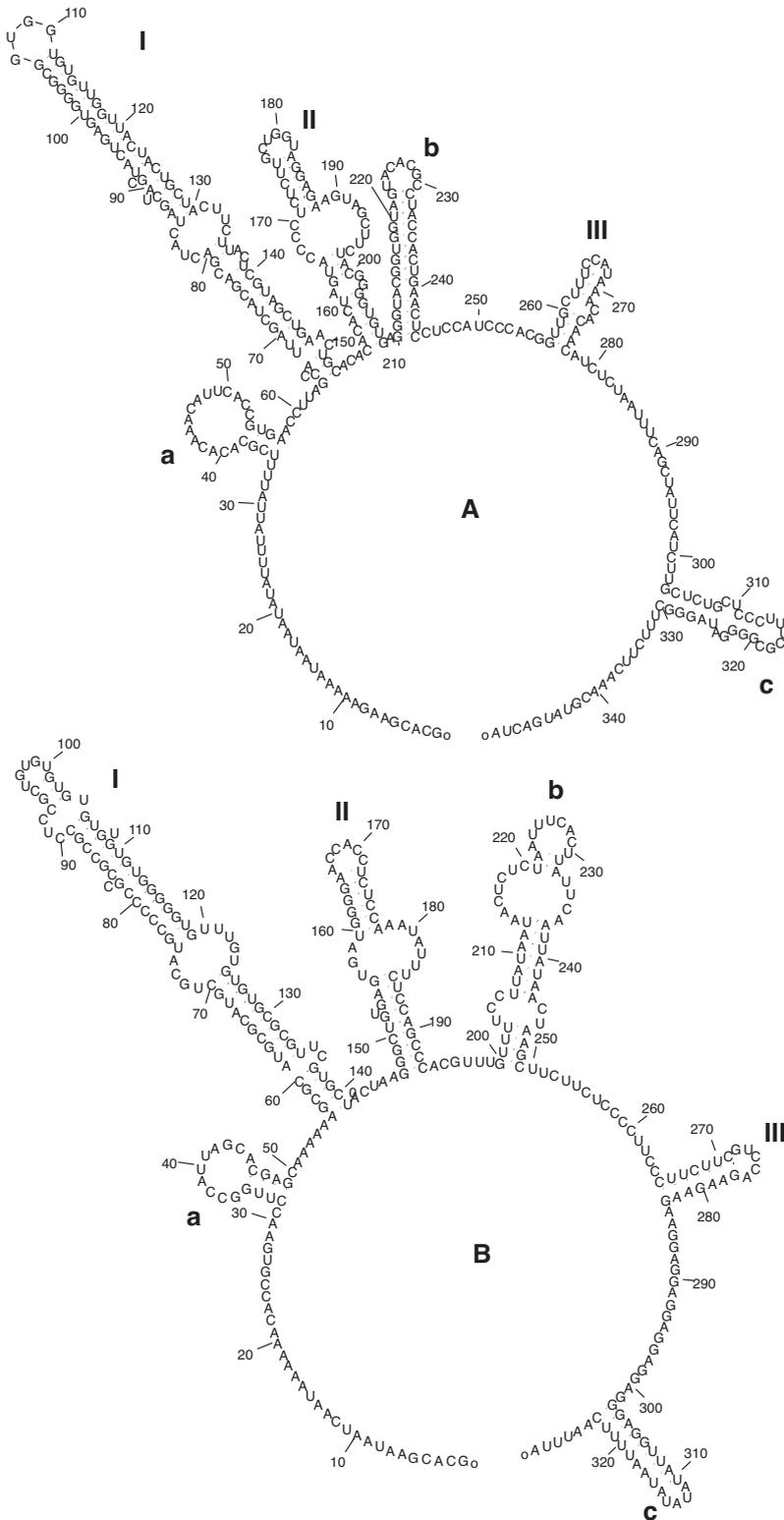


Fig. 4. *Hematodinium* spp. Internal transcribed spacer 1 (ITS1) transcript predicted secondary structure for Clades A (*Callinectes sapidus*, GenBank accession # FJ844431) and B (*Chionoecetes bairdi*, GenBank accession # FJ844416). Free energy for both structures was  $\Delta G = -121.1$  kcal mol<sup>-1</sup>. Major helices are labeled I to III while minor helices are labeled a to c

Despite these differences in sequence between the 2 clades, the predicted secondary structure was remarkably similar, consisting of 3 major and 3 minor helices along a linear structure (Fig. 4). Both structures had the same free energy ( $\Delta G = -121.1$  kcal mol<sup>-1</sup>), which was similar to the free energy of structures obtained for individual RNA molecules in mfold (*Callinectes sapidus*:  $\Delta G = -118.9$  kcal mol<sup>-1</sup>, *Chionoecetes bairdi*:  $\Delta G = -119.8$  kcal mol<sup>-1</sup>). The GC content was generally higher in helices than in intervening regions (Table 3). Variability of sequence ( $\pi = 0.0$  from 1 to 300 bp;  $\pi$  increased from 0.012 to 0.015 from 300 to 362 bp) appeared to increase from 5' to 3' end of the sequence. Regulatory motifs, mostly located in the stems and tips of helices, were found in both clades (Table 4), either reverse (TATAAT versus TAATAT) or complement (CCCGCC versus GGGCGG) motifs of each other, but never the same motif in both clades. The relative conserved position of the GC box in Helix I suggested a true functional significance, while TA boxes appeared more randomly distributed.

## DISCUSSION

### *Hematodinium* spp. clades

We found clear evidence for 2 distinct ITS1 clades among the *Hematodinium* spp. analyzed here, with North Pacific *Hematodinium* spp. falling into the clade composed of all isolates except those from the portunoids, *Callinectes sapidus*, *Liocarcinus depurator* and *Scylla serrata*, which corresponds well to recent hypotheses regarding *Hematodinium* systematics. The secondary structure between these 2 clades is highly conserved and contains regulatory motifs, though these motifs are different between clades. We suggest that these patterns of differentiation and similarity may represent a functional adaptation to a new host species.

Amplification and comparison of 18S rDNA indicated that sequences of *Hematodinium* regardless of host were remarkably similar with only 13 variable sites in over 1700 bp. While the differences clearly separated the sequences into 2 groups, Clade A (*Hematodinium* sp. ex portunoids) and Clade B (*Hematodinium* sp. from all other hosts), the

Table 3. *Hematodinium* spp. G+C content of helices and intervening regions in the ITS1 region of *Callinectes sapidus* (GenBank accession # FJ844431; portunoid Clade A) and *Chionoecetes bairdi* (GenBank accession # FJ844416; *Chionoecetes* Clade B). See Fig. 4 for a diagram of helices. IVR: intervening regions between respective helices in order of appearance from start

	Clade A		Clade B	
	No. bases	G+C	No. bases	G+C
Total	350	46%	328	46%
Helices	247	53%	239	49%
IVRs	78	37%	89	38%
IVR	35	14%	31	35%
Helix a	21	52%	18	56%
IVR	7	29%	7	14%
Helix I	90	51%	83	67%
IVR	3	33%	6	17%
Helix II	52	54%	47	55%
IVR	1	0%	6	33%
Helix b	35	57%	50	24%
IVR	12	67%	15	53%
Helix III	21	38%	19	47%
IVR	25	28%	18	61%
Helix c	28	68%	22	23%
IVR	20	30%	6	0%

Table 4. *Hematodinium* spp. Regulatory motifs and their positions (bases) in *Callinectes sapidus* (GenBank accession # FJ844431, Clade A) and *Chionoecetes bairdi* (GenBank accession # FJ844416, Clade B)

Motif	Clade A	Clade B	Location
TATAAT		208–213 314–319	Stem of Helix b Tip of Helix c
TAATAT	17–22		Linear
CCCGCC		78–83	Stem of Helix I
GGGCGG	102–108		Tip of Helix I

divergence at 18S between the 2 groups was low. In contrast, sequence divergence at ITS1 was so extensive that sequences from the 2 clades could not be aligned. Within Clade B, sequences from *Hematodinium* were similar and only differed by indels of tandem repeat units.

Although it is well known that 18S rDNA is more conserved and, thus, generally less variable than ITS1 (Hillis & Dixon 1991, Hudson & Adlard 1996, La-Jeunesse 2001), the difference in levels of variability between the ITS1 and 18S regions found in the present study is highly unusual. In fact, the only other instance of such an observation of which we are aware is in the prokaryote, *Cyanobacterium prochlorococcus*, where different strains exhibited almost identical 16S rDNA, but very different ITS regions (Rocap et al. 2002). Possible explanations include amplification of host DNA,

existence of a pseudogene in one or both clades, intra-individual variation or rapid evolution of ITS1 in response to a host switch.

Amplification of host DNA appears unlikely as the overlapping ~130 bp of the adjacent 18S region amplified with ITS1 aligned perfectly with 18S amplified using 18S primers. Furthermore, sequences of both clades showed high similarity with the *Hematodinium* 18S and ITS1 sequences in GenBank. Small et al. (2006) hybridized probes based on *Hematodinium* 18S/ITS1 boundary sequences to fixed parasite cells prepared from *Hematodinium*-infected *Nephrops norvegicus* tissue samples, demonstrating that the 18S/ITS1 sequences were not derived from host DNA.

18S rDNA pseudogenes have been described in symbiotic dinoflagellates of the genus *Symbiodinium* (Santos et al. 2003) as well as some free living dinoflagellates (Rehnstam-Holm et al. 2002). Pseudogenes are often characterized by extensive deletions in their sequences (often more than 50% of the total sequence length, Santos et al. 2003), although the sequence of the remaining segment appears to remain remarkably conserved in dinoflagellates (Rehnstam-Holm et al. 2002, Santos et al. 2003). Although the mechanism of this sequence conservation is unknown (Santos et al. 2003), it appears unlikely that the existence of 2 clades with completely different sequences can be explained by pseudogenes, especially as the secondary structure of the ITS1 region was maintained (Fig. 4) and the GC content of helices and intervening regions was remarkably constant between the 2 clades (Table 3).

Intra-individual variation is a potential problem in a marker that is repeated multiple times in each genome, such as rDNA and ITS. However, intra-individual variation, when occasionally present, is usually small compared with variation between individuals and populations (Alvarez & Wendel 2003). In parasitic species like *Hematodinium*, intra- and inter-individual variation may be confounded if hosts are infected by several genetically differentiated genotypes. Small et al. (2007b) found variation only within repetitive regions in ITS1 within *Hematodinium* isolates from several host species. Because the amplified PCR products in this study were not cloned, it is uncertain whether one or several clones were present in each crab. However, ITS1 sequences showed no evidence of contamination by other sequences as would be expected if a mixture of *Hematodinium* clones were sequenced. A specific case of intra-individual polymorphism at the rDNA region was found in the malaria-causing parasite *Plasmodium falciparum*, which has 2 functionally distinct rRNAs in the vertebrate and the mosquito host (Mercereau-Puijalon et al. 2002) reflecting 2 distinct copies of ITS1. As the life cycle of *Hematodinium* is currently unknown, a similar genome structure may be

responsible for the observed variation at ITS1. However, the fact that different ITS1 sequences were discovered in different host species contradicts this hypothesis. Furthermore, different copies of *P. falciparum* ITS1 have a sequence similarity of 63 to 94% (Mercereau-Puijalon et al. 2002), much more than the unalignable sequences of *Hematodinium*.

The most likely explanation for the large variation between clades is the existence of 2 different species of *Hematodinium*, one in the portunoids, *Callinectes sapidus*, *Liocarcinus depurator* (GenBank EF153724 to EF153728 and EF065708 to EF065718) and *Scylla serrata* (GenBank EF173451 to EF173459), and the other in widely distributed decapod species, including Pacific *Chionoecetes bairdi* and *Chionoecetes opilio*. The only other portunoid from which *Hematodinium* has been isolated and sequenced, *Carcinus maenas*, harbors the Clade B type *Hematodinium* sp. Interestingly, while formally classified as a portunoid, in systematics studies *C. maenas* occasionally places outside the Portunoidea as sister to the genus *Cancer* (Cancroidea: Cancridae) (e.g. Mahon & Neigel 2008). Comparative phylogenetic studies of the parasite isolated from *C. maenas* and other Clade B and A hosts may be especially useful in understanding *Hematodinium* systematics. Our findings support the suggestions of other authors that the *Hematodinium* in blue crabs and that in other decapods (Hudson & Adlard 1996, Hamilton et al. 2007, Small et al. 2007a,b) are different species based on 18S and ITS1 sequence differences. Based on their ITS1 sequences, which showed only a 66.5% similarity between *Hematodinium* sp. ex *Nephrops norvegicus* and *Hematodinium* sp. ex *Chionoecetes* spp., Hudson & Adlard (1996) concluded that the *Hematodinium* species infecting *Nephrops norvegicus* comprised a third species. Our sequencing results are in agreement with Small et al. (2006) and the other *Hematodinium* sp. ex *N. norvegicus* sequences deposited in GenBank and do not support Hudson & Adlard's (1996) hypothesis; we confirm that other than in number of repetitive elements, the ITS1 sequences of *Hematodinium* sp. ex *N. norvegicus* is virtually identical to that of *Hematodinium* derived from all but *Callinectes sapidus* and *L. depurator*. Furthermore, variation within *Hematodinium* sp. ex *N. norvegicus* is low, with only 9 differences in ITS1, each confined to single sequences, within 34 sequences (3 in the present study, and 31 in GenBank). As noted by Small et al. (2007b), the sequence reported by Hudson & Adlard (1996) should be regarded with caution.

The extreme difference in level of variability between similar 18S rDNA and non-alignable ITS1 sequences could be explained by either conservation of 18S rDNA or fast evolution of ITS1. There is little evidence to suggest that 18S is more conserved in

dinoflagellates than in other groups; in fact, 18S is often used to define morphologically similar species of parasitic, symbiotic and free-living dinoflagellates (e.g. Salomon et al. 2003) and phylogenies of 18S rDNA correspond well to hypotheses based on morphology and other molecular markers (Taylor 2004). Indeed, the ITS1/5.8S/ITS2 sequences have been useful in distinguishing species of free-living dinoflagellates (Litaker et al. 2007). We therefore hypothesize that the ITS1 region may have evolved particularly rapidly, possibly in connection with functional diversification during a host switch. While the existence of 2 clades may be related solely to environmental factors such as temperature, salinity or latitude, we find the corresponding taxonomic separation within the known hosts, i.e. Clade A infecting portunoids versus Clade B infecting all other hosts, intriguing and suggestive of a host switch as an important factor in the divergence of the 2 clades.

#### A functional role of internal transcribed spacers?

The secondary structures of the 2 divergent *Hematodinium* ITS1 sequences were remarkably similar to each other, and to secondary structures of other dinoflagellates species (Gottschling & Plötner 2004). This noteworthy conservation of the secondary structure of ITS1 among dinoflagellates (Gottschling & Plötner 2004) as well as larger phylogenetic groups (Mai & Coleman 1997) suggests a functional significance in ribosomal structure. Indeed, there is evidence that the secondary structure of ITS regions is important for transcript processing of rDNA and production of mature rRNA (Alvarez & Wendel 2003). In particular, stem regions in the ITS regions may bind to proteins to form ribonucleoprotein complexes that protect nascent RNA from nuclease digestion, aid the assembly of rRNAs into ribosomal subunits or act as a 'work bench' for other RNA-protein complexes (Lalev et al. 2000).

In addition to similarities in the secondary structure, regulatory motifs occurred in *Hematodinium* ITS1 sequences in both clades. Regulatory motifs are important in the initiation and regulation of transcription, and often occur in tandemly repeated sequences in the external transcribed spacers, where transcriptional rates are positively correlated to repeat number (Busby & Reeder 1983). Similar regulatory motifs have been found in the ITS1 region of some flatworm species (Van Herwerden et al. 2003). Interestingly, the clades had either the reverse (CCCGCC versus GGGCGG, in Clade A) or complement (TATAAT versus TAATAT, in Clade B) motif of each other. Such a change in motifs may suggest a significant change in the function of ITS1, potentially in response to a host switch.

Although the significance of ITS1 in the efficiency of rDNA transcription and maturation of rRNA is well established, its importance in adaptation to different host environments is not. Examples such as *Plasmodium falciparum*, however, which expresses different sets of rDNA in different host species (Mercereau-Puijalon et al. 2002), make such a possibility conceivable. On the other hand, evidence for host specificity of ITS1 genotypes is equivocal in the symbiotic genus *Symbiodinium*. Although ITS1 lineages exhibit some degree of host specificity, others occur in a wide range of host species and have large geographic distributions (Lajeunesse 2001, Rodriguez-Lanetty 2003). However, there are indications that *Symbiodinium*–host associations during the onset of symbiosis are highly correlated with specific *Symbiodinium* ITS1 strains (Rodriguez-Lanetty et al. 2004). Although this does not demonstrate functional significance of ITS1, it does suggest that spacers such as ITS1 cannot be discounted when examining symbiotic relations. In any case, the possibility of host-specific functional adaptation of ITS1 sequences cannot be excluded for *Hematodinium* spp., and may warrant further investigation.

With the exception of *Hematodinium* sp. ex *Chionoecetes tanneri*, the ITS1 sequences reported here in North Pacific hosts were shorter and less variable in number of repetitive elements than those reported in Atlantic hosts by Small et al. (2007b). Given the rapid divergence of ITS1 evidenced by the 2 clades, one might expect a divergence in ITS1 within the Pacific hosts if *Hematodinium* had been a long-term resident of the North Pacific Ocean. The reduced variability may indicate that the North Pacific *Hematodinium* species either passed through a bottleneck or is a recent introduction to the North Pacific (founder event), or that there is selection on ITS1 (see above).

### PCR-based diagnostics

The published *Hematodinium* primers developed by Hudson & Adlard (1994) and Gruebl et al. (2002) were rigorously tested on *Chionoecetes bairdi* and *C. opilio*. While the ITS1 primers (Hudson & Adlard 1994) proved unreliable for screening *Chionoecetes* spp. for *Hematodinium*, both 18S primer pairs (Gruebl et al. 2002) were reliable for use in a diagnostic assay. *Hematodinium* sp. recovered from *C. bairdi* and *C. opilio* amplified strongly with Univ-F-15/Hemat-R-1654 and Hemat-F-1487/Hemat-R-1654 regardless of infection intensity, as estimated from hemolymph smears. Both primer sets performed well with samples of *Hematodinium* spp. from *C. tanneri*, *C. angulatus*, *Callinectes sapidus* and *Nephrops norvegicus*. The number of infected *C. angulatus* and *C. tanneri* assayed was insufficient for a rigorous

test of the performance of the assay, but given the identical sequences of *Hematodinium* sp. infecting the 4 *Chionoecetes* species, it is likely that the PCR-based assay will perform well in these hosts. Recently published primer sequences (Frischer et al. 2006, Small et al. 2006, 2007a) for *Hematodinium* spp. from blue crabs and other hosts species were not available at the time our assay was under development, and we have not evaluated them for detecting *Hematodinium* sp. ex *C. bairdi* and *Hematodinium* sp. ex Pacific *C. opilio*.

The PCR assay was both more sensitive and more specific than hemolymph smear examination in identifying crabs parasitized by *Hematodinium* sp. The PCR assay detected *Hematodinium* at levels below that which slide readers detected in the assayed crabs and serial dilutions. Slide readers generally had difficulty detecting trace infections and, when present in low numbers on a slide, distinguishing *Hematodinium* from other cells. Use of the PCR assay resulted in a 16.5% improvement in correct BCS diagnosis over microscopic examination of slides.

To investigate *Hematodinium* spp. life histories and modes of infection, the ability to detect low intensity infections, and to recognize and distinguish *Hematodinium* species is critical. We are currently developing a quantitative PCR protocol for infected *Chionoecetes* spp. that will be useful in quantifying parasite levels within hosts and investigating disease progression. To further our understanding of *Hematodinium*, it would be helpful to sequence other regions of DNA typically used to distinguish related species of dinoflagellates, e.g. large subunit rDNA or heat shock proteins (Saldarriaga et al. 2004). There are published universal dinoflagellate primer sequences available for amplifying other dinoflagellate DNA regions; however, in North Pacific *Chionoecetes*-derived *Hematodinium* sp., these primers appear to amplify non-specific DNA; new primers are needed. Rediscovery and acquisition of *Hematodinium australis* for comparative studies are also critical.

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