Species Identity And Life History Of *Hematodinium*, The Causative Agent Of Bitter Crab Syndrome In Northeast Pacific Snow, *Chionoecetes opilio,* And Tanner, *C. bairdi,* crabs*

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PROJECT 0306 FINAL REPORT

to

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INTRODUCTION

A. Identification of Problem:

Bitter Crab Syndrome (BCS) is a fatal disease of North Pacific Tanner (*Chionoecetes bairdi)* and snow (*C. opilio*) crabs that is caused by a parasitic dinoflagellate of the genus *Hematodinium*. The type species was described in France (Chatton & Poisson 1931) and until the mid 1980's, members of the genus were only sporadically encountered in crustacean populations (Table 1). In 1986, large mortalities of Tanner crabs were reported in Southeast Alaska and subsequent research identified the causative agent as *Hematodinium* sp. (Meyers et al. 1987). At about the same time, mortalities of *Cancer pagurus* in association with *Hematodinium* infections were reported in France (Latrouite et al. 1988). Shortly afterward, BCS was reported in Bering Sea snow and Tanner crabs, but at much lower prevalences than in Southeast Alaska (Meyers et al. 1990, 1996). In 1994, a second species of the parasitic dinoflagellate, *H. australis,* was described in a crab from Moreton Bay, Australia (Hudson & Shields 1994). Since 1985, 23 *Hematodinium* related papers have been published in peer-reviewed journals which cite 21 crustacean species infected worldwide (Table 1, Fig. 1). These reports signal the recognition of *Hematodinium* associated disease as an important worldwide emerging infectious disease of commercially important crustaceans. However, the full extent to which non-commercial crustacean species are affected is unknown.

Despite this recent interest in *Hematodinium* associated disease and the potential impact of related mortalities on affected populations, much is unknown. For example, the method of infection is unknown as is the life history of the parasite outside of the host. In addition, the underlying mechanisms for the recent worldwide increase in frequency of *Hematodinium*

associated disease are not being adequately investigated.

For Bering Sea commercially important crabs, these uncertainties are critical in attempting to understand recent changes in their abundance trends (Fig. 2) and distribution patterns. In the mid 1970s, Bering Sea Tanner crab, *C. bairdi,* became a major commercial species. Shortly afterward, the Tanner crab population declined and has to this date not recovered. After the decline of the Bering Sea red king crab fishery in the early 1980s, the snow crab quickly became the major crab fishery with respect to both landings and over-all value, just by sheer size of the fishery. Reaching a peak harvest level in 1991, the snow crab fishery has generally been on the decline with the exception of peak harvest periods in the late 1990s (NMFS 1999). The cause or causes of the decline for both snow and Tanner crabs are unknown, but a key signature during this general downward trend appears to be poor recruitment (Rugulo et al. 2003).

BCS was originally thought to occur at low prevalences and to be restricted to adult animals, however, recent reports indicate the contrary. For example, infection prevalences gathered during assessment surveys and commercial activities have approached 100% in certain populations of Southeast Alaska Tanner (Meyers et al. 1987) and east coast blue (*Callinectes sapidus*) crabs (Messick 1994). Other researchers have reported *Hematodinium* infection prevalences in adult crustaceans that exceed 30% (Table 1). However, recent observations gathered from more extensive and detailed surveys indicate that *Hematodinium* infections occur in crustaceans of all sizes and age classes. Messick (1994), Wilhelm & Mialhe (1996), Hudson et al. (1993), Stentiford et al. (2001a) and Dawe (2002) indicate that sub-adult and juveniles in particular are especially susceptible to infection. In the Bering Sea, personal observations

suggests that while overall prevalences in Bering Sea snow and Tanner crabs may be relatively low (e.g., 2-4%), infection prevalences in crabs less than 45 mm carapace width may exceed 10% for both crab species (Fig. 3). Observations by Meyers et al. (1996) further indicate that infection prevalences increase in Bering Sea snow crabs with increase in latitude. These data suggest that the effects of BCS on small size classes of snow crab may be even more pronounced in northern latitudes.

Because of the growing worldwide occurrence and prevalence of *Hematodinium*-like diseases, recent research has taken on added importance. Several limitations in research techniques have been recognized and are being addressed. First, greater effort is being directed at random sampling of all host size groups to better monitor age/size class susceptibilities. Second, more powerful statistical tests such as logistic regression are being applied to biological and prevalence data to better understand risk factors and their interactions that may predict host susceptibility to disease (Corsin et al. 2001; McAllister et al. 2000; Leung et al. 2000; Morado et al. 1999). Third, *in vitro* culture is being attempted to characterize parasitic life history stages within the host (Appleton & Vickerman 1998), but little progress has been made toward identifying late internal and external life history stages. Fourth, traditional techniques of disease detection involving the examination of blood smears or pleopods under transmitted light are not accurate; therefore, disease prevalence is generally underestimated. Field & Appleton (1996) determined that traditional methods of *Hematodinium* detection underestimated prevalence by 3 to 5% when compared to immunological methods. As a result, immunological methods that offer increased sensitivity and speed of diagnosis relative to traditional methods are being utilized in disease detection and monitoring (Field & Appleton 1996; Stentiford et al. 2001b;

Stentiford et al. 2002; Sheppard et al. 2003). At the molecular level, the polymerase chain reaction (PCR, Saiki et al. 1988) has become increasingly widespread in pathogen identification and disease detection/monitoring and is particularly useful in the diagnoses of cryptic organisms such as dinoflagellates and parasites (e.g., Carnegie et al. 2000; Stokes et al. 2000; Ford et al. 2001; Gruebl et al. 2002; Lee & Frischer 2004). Ribosomal DNA has been the focus of many successful PCR-based diagnostic assays, including an assay for *Hematodinium* (Gruebl et al. 2002). Fifth, because so few morphological features exist that can be reliably used to differentiate species of *Hematodinium*, sequence differences of the first internal transcribed spacer (ITS1) have supported the notion of multiple species of *Hematodinium* (Hudson & Adlard 1996). However, the use of genetic techniques to designate species is viewed as controversial (for discussion, see Kunz 2001) and caution must be exercised, particularly in the case of regions with complex secondary structure.

On the molecular level, secondary structure of rDNA is providing unique insight on possible function and significance not provided by simple sequencing. Ribosomal DNA (rDNA) are repetitive sections in genomic DNA that encode for the type of RNA (rRNA) making up ribosomes, cell organelles in which translation from mRNA to proteins occurs. Because of their central position in gene expression and cell metabolism, transcription of rDNA and associated proteins constitutes a major component of the total cellular transcription effort in eukaryotic cells; in yeast, for example, transcription of ribosomal genes constitutes about 60% of the total transcription of the cell (Planta 1997).

Ribosomal DNA genes occur as multiple tandem repeats in the nuclear DNA and consist of three genes, two internal transcribed spacers, an external transcribed spacer and a non-

transcribed spacer (Fig. 4, Russell 2002). All components, except for the non-transcribed spacer, are transcribed into a single-stranded rRNA, which is then folded into a complex secondary structure based on complementary sequences in different parts of the rRNA. External and internal transcribed spacers provide an important role in the correct folding of the molecule, but are removed by enzymatic reactions in the course of maturation of rRNA. The secondary structure of both rDNA genes and the transcribed spacer is an important aspect of their evolution and function, and need to be routinely considered as phylogenetic or species identification markers (Hillis & Dixon 1991).

Because of their functional importance, parts of rDNA genes are highly conserved. This high level of conservation facilitates their applications as population and species specific markers, as PCR primer affinity can often be used to distinguish between species and larger taxonomic groups. In addition, the high degree of conservation has allowed the reconstruction of very deep phylogenies, and indeed, recently provided data that has revolutionized our understanding of animal evolution (Halanych 2004). In prokaryotes, where species definitions are complex, 16S rDNA differentiation is used to identify species (Kemp & Aller 2004). Similar approaches have been used in eukaryotes (Adachi et al. 1996), although discordance in differentiation from more commonly used morphological characters complicate the use of rDNA as species identifiers in eukaryotes.

In contrast to rDNA itself, the internal transcribed spacers (Fig. 4) 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 (bp insertions/deletions), often so much that sequences cannot be aligned with confidence. However, despite this variability in the DNA sequence, the

secondary structure is often conserved across surprisingly large taxonomic groups (e.g., ITS 2 in green algae and flowering plants, Mai & Coleman 1997). This high degree of conservation suggests some significance of ITS secondary structure in the folding of rDNA (Musters et al. 1990), and may also suggest some significance of ITS in the regulation of RNA transcription (van Herwerden et al. 2003).

In recent years, the 18S rDNA gene and ITS1 (Hudson & Adlard 1996) or only the 18S rDNA (Gruebl et al. 2002) regions of the *Hematodinium* genome have been sequenced, but their secondary structure has not been investigated. Such an analysis of the *Hematodinium* genome may reveal, at another level of resolution, inter and intraspecific relationships of known species/variants of the parasitic dinoflagellate that could influence efforts to manage the disease in affected crustacean populations.

B. Project Goals/Objectives:

This project was designed to address three key research issues. First, it is necessary to determine how many species of *Hematodinium* exist in the North Pacific and worldwide, because if a single species infects many hosts, scores of crustacean species should be screened for *Hematodinium* to accurately assess infection rates of the disease. In efforts to address this objective, *Hematodinium* was collected from several decapod hosts and the ITS1 and 18S rDNA regions were sequenced. Ribosomal DNA is frequently used to distinguish between species and even strains of dinoflagellates (Rowan & Powers 1991; Saldariagga et al. 2004). Second, current detection and monitoring methods are cumbersome, tedious and time consuming. It was our intent to critically evaluate the efficacy of previously published *Hematodinium* primers and develop a PCR-based protocol that is more sensitive, accurate and rapid than currently employed

methods. Such a method would permit a more thorough estimation of prevalence with larger sample sizes. Third, the PCR protocol should enhance our efforts to determine the presence or absence of free-living parasite stages that might exist in water and sediment. However, because amplification of DNA by PCR is destructive in nature, morphological characterization of external putative life history stages is not possible. To overcome this predicament, a fluorescent antibody protocol was developed to compliment the molecular diagnostic assay so that putative life history stages of *Hematodinium* in PCR positive water and sediment samples could be fluorescently tagged and examined morphologically. The importance of this work plan has gained added importance as *Hematodinium* infections are increasing with respect to both number of hosts and geographic areas affected.

APPROACH

Methods 1 - Sample Collection: The Alaska Fisheries Science Center (AFSC), Alaska Department of Fish & Game (ADF&G) and Department of Fisheries & Oceans Canada (DFO) conduct several annual stock assessment surveys that provide routine and opportunistic windows for sample collection (Table 2,3,4,5,6). Blood and tissue samples were collected from Bering Sea snow, *C. opilio*, and Tanner, *C. bairdi*, crabs in 2003 and 2004, and Gulf of Alaska Tanner crabs in 2003, Southeast Alaska Tanner crabs in 2003 and 2004 and snow crab from Newfoundland, Canada in 2004. Specific, but limited samples intended for molecular analysis and preserved in 100% ethanol from *Callinectes sapidus* (Maryland and Virginia, USA), *C. angulatus*, *C. tanneri* (British Columbia, Canada), and *Nephrops norvegicus* (Scotland, UK) were submitted by colleagues.

Sample collections were for the following intended purposes. First, to continue our time series of monitoring BCS in North Pacific snow and Tanner crab populations, but also augmenting this effort with initial testing of a PCR-based protocol. Second, to collect blood and tissue samples from other populations of crabs known to be parasitized by *Hematodinium*. Samples from these host species had a dual purpose; assisting colleagues (DFO and ADF&G) in monitoring the disease in affected populations while contributing to our efforts to determine the number of *Hematodinium* species that exist worldwide. Third, environmental samples (i.e., water, sediment) were collected with the purpose of detecting and identifying putative free-living parasite stages.

Multiple kinds of samples were collected from randomly and non-randomly selected individuals, but blood smears were always prepared from sampled individuals for microscopic examination. From a randomly selected set of crabs blood smears, and blood and/or muscle samples for molecular analysis were simultaneously collected. Samples for molecular analysis were preserved in 100% ethanol, frozen or applied to FTA® cards (Whatman Inc., New Jersey, USA) for archival purposes. Non-random samples (i.e., visibly diseased crabs) were collected and preserved in 100% ethanol for use in development of the PCR-based *Hematodinium* assay. Samples requested from colleagues were intended for molecular analysis and were submitted to us in 100% ethanol. Hemolymph for in vitro culture and long-term storage of the parasitic dinoflagellate was collected by syringe from patently infected crabs, transferred to vacutainers, placed in a refrigerator and shipped cold to the laboratory.

Southeast Alaska (Fig. 5) was identified as the target area from which both water and sediment samples could be collected from historically known areas of high and rare (or non-

existent) BCS prevalence. Water and sediment samples from high prevalence areas provided the best opportunity to encounter, detect and morphologically characterize putative free-living stages of *Hematodinium*. Alitak Bay, Kodiak Island, was identified as a secondary site for environmental sampling.

Blood smears regardless of host were prepared from each sampled individual and transported to the laboratory where they were stained by modified Wright's stain. All smears were read blind, in no particular order and independent from samples for molecular analysis. Twenty random fields were examined per each slide and the overall composition of host hemocytes, trophonts, pre-spores and apparent dinospores was rated between one (1-10%) and five (90-100%).

Methods 2 - PCR Diagnostic Protocol: Genomic DNA (crab and, if present, parasite DNA) was extracted from tissue samples with DNeasy Tissue Kit (Qiagen Inc., Valencia, CA, USA) columns according to the manufacturer's instructions for animal tissue. Two separate 30 ul elutions were collected. Because preliminary tests showed that the second elution resulted in higher yields in DNA amplifications, DNA from the second elution was routinely used in subsequent PCR runs. DNA from ethanol preserved blood samples was extracted using a modified Qiagen protocol, as follows. A sample of ethanol preserved blood was shaken to redistribute material that had settled to the bottom of the tube, 250 µl was aliquoted into a 1.5 ml tube and spun down (1600 g for 5 min), the supernatant was decanted and the pelleted material was allowed to air dry. The pellet was then processed on DNeasy columns as a tissue sample. All extractions were visualized on 2% agarose gels stained with SYBR® Green (Molecular Probes Inc., Eugene, OR, USA).

Genomic DNA extractions from nonrandom blood or tissue samples collected from patently infected crabs were used for development and practical testing of the PCR-based diagnostic protocol. Randomly collected samples were evaluated with the PCR protocol under blind conditions and compared to results obtained from microscopic examination of blood smears from the same crabs. Only after samples were analyzed according to each protocol were the results compared. If the results of the microscopic examination and the PCR assay differed, the corresponding slides were reexamined as previously described (typically by another reader) and template DNA was re-amplified.

Using published primer sequences reportedly specific for *Hematodinium* (Table 7) we optimized the amplification conditions for the small subunit (SSU or 18S) and the first internal transcribed spacer (ITS 1) region of ribosomal DNA in *Hematodinium* (Fig. 4). To reduce the number of gels run and to simplify scoring, PCR products from the two 18S PCR runs were combined before loading onto a gel. PCR products were visualized on 2% agarose gels stained with SYBR® Green.

Methods 3 - Species Identification: We adopted a comparative molecular approach for the identification of *Hematodinium* species infecting *C. bairdi* and *C. opilio*. Amplified 18S and ITS1 fragments were sequenced from PCR products in both forward and reverse directions on either an ABI 3730XL DNA Analyzer by the DNA Sequencing Facility at the University of Washington, Department of Biochemistry or a Li-Cor Biosciences model 4200 DNA Analyzer. Three to five separate PCR products per template were combined prior to sequencing. Sequences of specific isolates were combined to contigs in Sequencher version 4.2 (Gene Codes Corp, Ann Harbor, MI, USA) and sequences were aligned in BioEdit version 7.0.1 (Hall 1999).

Sequences published by Hudson and Adlard (1996) and Gruebl et al. (2002) were included in that alignment. We also investigated the number of restriction sites available in the 18S region using BioEdit.

As alignment of sequences was not possible between ITS1 sequences, an alignment of secondary structure (three dimensional folding structure of single stranded RNA) was carried out using the program DYNALIGN (Mathews 2005). Dynalign finds the lowest free energy sequence alignment and secondary structure common to two, unaligned sequences by using the mutual information of the two sequences to constrain secondary structure prediction. This can result in a large improvement in the accuracy of secondary structure prediction. RNAStructure (Mathews et al. 2004) was used to fold individual sequences and to modify secondary structure predictions produced by DYNALIGN, although the more commonly used web based m-fold (Zuker, 2003) provided identical results. RNAViz (de Rijk et al. 2003) was used to draw structures based on the output of DYNALIGN. Regulatory motifs suggestive of promoter regions (van Herwerden et al. 2003) were identified using BioEdit (Hall 1999).

Methods 4 - Hematodinium Life History Studies: Progress in this area was purposely delayed until the published primers were rigorously tested. However, samples for this part of the project were collected, processed and preserved in preparation for final analysis.

Of the regions that we were able to monitor, Southeast Alaska met our requirement for possessing both reference (i.e., Icy Strait, Glacier Bay) and high (i.e., Stephens Passage) BCS prevalence sites. Using a 2.2-liter horizontal water sampler, water was collected just off bottom and stored at 4°C for a maximum of 24 hr before filtering the sample. Salinity of water samples was measured prior to each being filtered through a graded series of 47 mm diameter filters (5.0,

2.5 and 1.5µ filters). The final number of filters of each pore size varied as the number of filters required to process a sample was dependent upon the amount of particulate matter in each sample. Regardless, each final filter was cut in half with one-half fixed in HistoChoice® (Amresco, Solon, OH, USA) for histological, microscopic, or immunological analyses and the other half preserved in 100% ethanol for molecular analysis.

Inhibitors that impede extraction of DNA and the PCR assay exist in many types of samples and are a particular problem in environmental samples. DNA isolated from filters using standard Proteinase K-SDS lysis, phenol chloroform extraction and ethanol precipitation (Sambrook et al. 1989) followed by PCR with the 18S primer sets (Table 7) resulted in inconsistent amplification of spiked positive controls (see below) and could be not improved. DNA isolation of positive controls using a methods modification of Audemard et al. (2004) utilizing Qiagen's QIAamp DNA Stool Mini Kit yielded consistent results from positively spiked controls. This method was subsequently used for DNA isolation from all water and sediment samples. As an indicator of the presence of materials interfering with DNA extraction, each water filter was split prior to extraction and one preparation was spiked with genomic DNA from a BCS positive crab. Isolation of DNA from preserved filters was as follows. Filters were cut into eight equal sections with one section placed into a 1.5 ml tube containing 800 µl QIA amp DNA Stool Mini Lit lysis solution Buffer ASL. Another section was placed in a the same buffer, but spiked with 100 ng genomic DNA previously isolated from a BCS positive crab. Positive controls were processed in parallel with unspiked samples. Both tubes were subjected to three cycles of freezing at -20°C and thawing in an effort to more thoroughly lyse cells. Extraction then followed the manufacturer's instructions except that one-half of an InhibitEX

tablet per tube was used. A final elution using 30 µl sterile water was performed, samples were dried in a vacuum concentrator and resuspended in 10 µl TE buffer (10 mM Tris, 1.0 mM EDTA). PCR was performed using both 18S primer sets (Table 7) on 1.0 µl of material isolated from spiked and unspiked water filters. Additionally, a PCR trial from each of the two extractions was spiked with 20 ng genomic DNA, resulting in a total of eight PCR runs per water filter: four for each primer pair with only one of each set of four not spiked at any point in the protocol. After the initial round of PCR runs on material extracted from a water filter, the process was repeated, but with varying volumes of template until the template material was exhausted. This was done to increase the probability of detecting low concentrations of *Hematodinium* in the samples.

Sediment samples were collected with a 205 x 255 mm center pivot benthic grab with a bite depth of 70 mm. A top centimeter of each sediment sample was transferred to a clear 120 ml glass jar and placed in a refrigerator for later processing.

Sediment samples were processed in the laboratory in a manner similar to Bolch (1997, 2001), but with some modifications. All samples and processing solutions were kept cold and all processing steps were performed on ice. Sediment samples were thoroughly mixed in the original sediment container using a non-metallic spatula. A 10cc subsample of sediment was transferred into a clean borosilicate container after which artificial filter-sterilized seawater (AFSW) was added to attain a final volume of 50 ml. The sample was then sonicated for 2 min to dislodge detritus and passed through a series of Nytex mesh sieves (i.e., 243, 53 and 20 µm sieves). The 20µm filtrate was collected and transferred to a 250 ml separation funnel which was placed in a refrigerator overnight. The settled material was collected by draining the bottom

50 ml from the funnel, sonicated for 2 min and then centrifuged to concentrate the pellet.

The supernatant was poured off and the pellet was resuspended in a minimal volume of AFSW and transferred to a density gradient. A two step density gradient using sodium polytungstate (SPT) solutions (first, 1.0 /1.3 and then 1.3/1.6 g/cm^{-3}) was applied to the resuspended pellet and then centrifuged for 10 min. at 1600 g. The procedure was the same for each fraction. The accumulation of organic materials at the interface was removed and then washed three times (1000 g for 2 min) to remove SPT. The final pellet from each fraction was resuspended in a minimal amount of AFSW and divided into two equal aliquots. Both aliquots were transferred to 1.5 ml tubes, one was filled with 100% ethanol while the other was filled with HistoChoice® MB fixative. Duplicate sediment samples were prepared from randomly selected sediment samples, spiked with trophonts at three processing steps: prior to Nytex sieving, prior to over-night separation, and prior to density gradient separation and then processed as noted previously. For sediment samples that yielded a *Hematodinium*-sized amplicon, the HistoChoice® MB fixed sediment fraction was used in the fluorescent antibody assay (see Fluorescent Antibody Assay below).

DNA from the ethanol-fixed sediment material was extracted in an identical manner to that of water samples, except that sediment material was not sub-sampled as were water filters, but processed in their entirety. Sediment material was also split and spiked with BCS positive and negative DNA prior to extraction and amplification in the same fashion as water samples.

Fluorescent Antibody Assay: Polyclonal antibodies against *Hematodinium* from both North Pacific snow and Tanner crabs were developed by a contractor (Harlan Bioproducts for Science, Madison, WI). Because cultures of *Hematodinium* are not available, hemolymph from

heavily infected snow and Tanner crabs provided the antigen. The antibody developmental protocol was as follows. Parasite densities in crab hemolymph were determined and if necessary, multiple hemolymph samples from the same host species and geographic region were pooled to increase density to 100-200 x 10^6 cell/ml. Individual or pooled hemolymph was centrifuge at 1500 g for 5 min at 9C. The pellet was then washed three times in 1ml of sterile *Nephrops* saline solution and centrifuged at 1500 g for 5 minutes. Parasite densities were again determined on the final 1ml volume to assure the appropriate density after which 10µl of 37% formaldehyde was added (final concentration of 1%), vortexed and incubated at 9°C for 1 hour to fix the cells. Cells were washed four times in 1.25 ml of *Nephrops* saline solution (centrifuged at 1500 g for 5 min). The final volume was then forwarded to Harlan for rabbit polyvalent antibody production. The contractor optimized conditions for an ELISA reaction which served as the basis for testing each respective polyvalent antibody on blood smears or wet mounts of *Hematodinium*.

Smears of *Hematodinium* from cultured and stored hemolymph were prepared on poly-L-lysine coated slides. Coated slides without *Hematodinium* and slides with only fetal bovine serum and uninfected hemolymph served as controls. Fluorescent cell staining as outlined in Harlow & Lane (1988) was followed using rabbit anti-*Hematodinium* and FITC-conjugated goat anti-rabbit IgG as primary and secondary antibodies, respectively. After initial testing, the IFAT protocol was applied to water and sediment samples that were identified by the PCR assay as containing *Hematodinium*.

RESULTS

Results 1 - Sample Collections: *Hematodinium* samples were collected from a wide range of decapod hosts and areas (Table 2,3,4,5,6). They included parasitic dinoflagellate samples from the Norway lobster, the blue crab, snow crab from Newfoundland and the Bering Sea, Tanner crab from the Bering Sea, Gulf of Alaska and Southeast Alaska. In addition, parasitic dinoflagellates were also collected from Vancouver Island triangle and grooved Tanner crabs. Despite the cooperation of international colleagues, we were unable to obtain the parasite from both France and Australia.

The total number of random and non-random hemolymph and tissue samples collected from snow and Tanner crabs for molecular analysis were as follows: preserved in 100% ethanol (N = 271; Table 2) , frozen (N= 167) or applied to FTA® cards (Whatman Inc., New Jersey, USA; N=302). Randomly collected samples for the PCR protocol were slightly fewer in number (N = 190 *C. bairdi* and 70 *C. opilio*).

Hematodinium samples were collected from 6 host species from both the North Atlantic and Pacific Oceans. To date, 207 random samples have been screened via the PCR diagnostic tool and 67 or 32.4% tested positive. In contrast, *Hematodinium* was detected in 13 of 15 (86.7%) non-random samples (two crabs were macroscopically misdiagnosed). Infection prevalences were highest in Tanner crabs from Southeast Alaska (59.4%) and followed by Eastern Bering Sea snow crabs (45.5%).

In contrast, overall prevalence as determined by traditional methods was 31.6%. Infection prevalence was highest in Bering Sea snow (32.4%) and Tanner (41.9%) and followed closely by Newfoundland snow crab (25.8%; Table 3).

In 2003, water and sediment samples were collected from Stephens Passage, Glacier Bay

and Icy Strait, Southeast Alaska (Tables 5,6; Fig. 5)). In that same year, additional water samples were collected from within and just outside Alitak Bay, Kodiak Island (Fig. 4). We revisited Stephens Passage and Icy Strait again in 2004 to collected additional water and sediment samples.

Although 191 samples were collected for culture, not all samples were adequate for this purpose (Table 4). Many were contaminated with bacteria or of insufficient density. Appropriate samples were seeded in media formulated by Appleton & Vickerman (1996) and routinely examined.

Results 2 – PCR-Based Diagnostic Protocol: Genomic DNA extractions from 10 BCS positive hosts (N = 4 *N. norvegicus*, 2 *C. tanneri*, 1 *C. angulatus*, and 3 *C. bairdi*) and BCS negative crabs (N=2 *C. tanneri* and 2 *C. angulatus* examined and provided by S. Bower, DFO Nanaimo, and two putative BCS negative *C. bairdi*, as identified by examination of blood smears) were used for development and practical testing of the PCR-based diagnostic protocol.

Under our testing protocols, PCR primers Hsp 1 and 2 (Hudson & Adlard 1994) produced numerous nonspecific bands (Fig. 5) in both BCS positive and negative crabs, some of which were similar in size (approximately 680 bp) to the expected band. The cleanest amplifications were regularly obtained from highly infected crabs. However, as the intensity of the infection dropped, nonspecific bands began to appear with uninfected crabs often having the highest number of nonspecific bands. PCR amplifications of a small number of BCS negative crab samples did not produce nonspecific bands. Attempts to improve specificity by re-optimization of PCR conditions were unsuccessful and we discontinued use of Hsp 1 and 2 in routine screening of hemolymph and tissue samples.

Once PCR conditions were optimized (Table 7), both the Univ-F-15/Hemat-R-1654 and Hemat-F-1487/Hemat-R-1654 (Greubl et al 2002) 18S amplifications yielded consistent bands of the expected size in BCS positive hosts without nonspecific fragments in either BCS positive or BCS negative samples (Fig. 6). In individual crabs, both amplifications either produced a band, or neither did. Also, as the level of infection increased, the amplicon yield also increased. Amplifying DNA from each sample twice, that is with both 18S primer pairs, provided a control for false negatives due to human error during PCR procedures. Additionally, both positive (a BCS positive extraction) and negative (sterile distilled water) controls were run during all PCR runs. From the 70 collected *C. opilio*, PCRs with both 18S primer pairs resulted in 44 positives (Table 8). Microscopic examination resulted in 39 of the same 70 samples scored as positive; all samples scored as negative in the PCR assay were also negative by microscopic examination. Reamplification of the 11 samples of uncertain status did not result in a change in the PCR results. Reexamination of the blood smears from the 11 samples resulted in their reclassification as negative for BCS. From the 190 collected *C. bairdi*, microscopic examination and PCR runs with both 18S primer pairs resulted in 49 positives in common (Table 8). An additional 20 samples were rated as infected by examination of the blood smears, but were PCR negative. Reexamination of these 20 samples resulted in reclassification of all 20 as histologically negative, while reamplification did not result in a change in their PCR status. Two of the 190 samples were scored as PCR positive and histologically negative; reexamination of the blood smears resulted in one sample being reclassified as positive, while the second remained negative. Reamplification of these two samples produced both 18s bands.

Using sequence information (see below), two enzymes were selected to restrict the 1682

bp 18S amplicon. Restriction with EcoRI (Invitrogen Corp., Carlsbad, CA, USA) produced two bands of the expected sizes, 1569 and 113 bp, and restriction with Hpa I (New England Biolabs, Beverly, MA, USA) resulted in a 1308 bp and a 374 bp fragment (Fig. 8).

Results 3 - Species Identification of *Hematodinium*: Sequencing of 18S revealed five mismatches to the Hsp1 primer sequence (Hudson & Adlard 1994); however, while the primer pair Hsp 1 and 2 proved to be insufficiently specific for use in a diagnostic assay, they did amplify ITS1 cleanly in heavily infected hosts and we were able to sequence the amplicons.

Sequence variation of ITS1 showed two very differentiated clades of *Hematodinium* sp. in the isolates of the present study: one clade (A) was isolated from blue crab (*Callinectes sapidus*), and the other clade (B) was found in all other species from both the North Atlantic and Pacific. These two clades were so different, that an alignment was not possible, with the exception of a highly conserved region at the beginning of the sequence (position 22-41 in *Chionoecetes bairdi*). Within each of the clades, the vast majority of the variation was due to indels, and here particularly the loss or gain of repeats in repetitive sequences. There was only one substitution between the clades.

Despite these differences in sequence between the two clades, the secondary structure was remarkably similar, consisting of three major and three minor helices along a linear structure (Fig. 9 A,B,C). However, in clade B a second secondary structure was possible that greatly increased the length of stem III and resulted in a lower free energy alignment (compare Fig. 9B to 9C). The GC content was, as expected, generally higher in helices than in intervening regions (Table 9). Generally, variability of both sequence and secondary structure appeared to increase from 5' to 3' end of the sequence.

Regulatory motifs were found in both clades (Table 10). Interestingly, the two clades harbored either reverse (TATAAT vs TAATAT) or complement (CCCGCC vs. GGGCGG) motifs of each other, but never the same motifs. Motifs were mostly located in the stems and tips of helices. The relative conserved position of the GC box in helix I suggested a true functional significance, while TA boxes appear more randomly distributed

Results 4 - Life History Studies: Sediment samples from five sites, two of which are historically negative (i.e., Icy Strait and Glacier Bay), and one historically positive (i.e., Stephens Passage) for *Hematodinium* were processed and DNA extracted from the material retained on the 1.3 and 1.6 density layers. None of the PCR trials of unspiked sediment samples that had been sieved produced any bands with either set of primers in either the 1.3 or 1.6 fractions. A sediment sample from a historically positive site in Stephens Passage which was processed only through the 243 μm Nytex meshed sieve (sediment texture did not permit further sieving) and then applied directly to an SPT gradient produced a *Hematodinium*-sized band of roughly 1700 bp in the 1.3 fraction only (Fig. 10), and with only the Univ-F-15/Hemat-R-1654 primers; the Hemat-F-1487/Hemat-R-1654 primers did not produce a band. All PCRs of the DNA from the sediment samples that had been spiked with trophonts during the sieving procedure were negative, unless spiked with *Hematodinium* DNA immediately before DNA extraction or PCR. All aliquots spiked with *Hematodinium* DNA prior to extraction or PCR produced a single band of the expected size.

A total of 17 water samples from 5 sites (Table 5) (in 2003, 14 samples; in 2004, 3 samples) were processed and the DNA extracted from material retained on the filters. At least 1/4 (excluding the filter extraction that is spiked with *Hematodinium* DNA) of a 0.65 um filter

was processed for each water sample and at least 1/4 filter of a 11.0 um and 20.0 um filter for each historically positive site. None of the PCR runs using material recovered from unspiked water samples produced any bands with either set of 18S primers. PCR runs with DNA isolated from material that had settled to the bottom of the tubes containing the 0.65 filters did not produce amplicons with either set of primers. All PCR trials of aliquots spiked with *Hematodinium* DNA prior to extraction or PCR produced a single band of the expected size.

Generally, *Hematodinium* life history forms were not detected by PCR in either water or sediment samples and, as a result, there was little cause to implement the planned indirect fluorescent antibody technique (IFAT). However, in preparation for use, the IFAT technique was blindly applied to negative and positive BCS blood smears. BCS positive blood smears were stained positive (Fig. 12). The IFAT technique was not applied to the only potentially positive sediment sample because it was exhausted during the PCR assay.

DISCUSSION

Discussion 1 - Sample Collections: A number of regions throughout Alaska were targeted as collection sites. The intent was to expand coverage of our BCS monitoring program in snow and Tanner crab populations while also testing the null hypothesis that only one species of *Hematodinium* exists in the North Pacific. Despite our best efforts, our sampling efforts fell short of optimal because; 1, assessment surveys did not develop for those areas outside of the standard NMFS surveys that we identified as regions of interest, 2, continued population declines of Tanner crabs made target sample numbers difficult or impossible to attain and 3, Bering Sea warming trends and snow crab populations trends apparently caused shifts in snow

crab populations that made target sample numbers difficult to meet. As a result, snow crab sample numbers from the Chukchi Sea and Norton Sound, and Tanner crab sample numbers from Bristol Bay, Alitak Bay (Kodiak) and Prince William Sound were not possible or fell short of expectation. In addition, despite the valiant effort of international colleagues, samples of *Hematodinium* from France and Australia could not be found. However, our approach toward investigating the effects of *Hematodinium* on affected populations generated considerable national and international interest and cooperation.

Discussion 2 - PCR based Diagnostics: We wanted to rigorously test the published *Hematodinium* primers (Hudson and Adlard 1994; Gruebl et al. 2002) on C. bairdi and *C. opilio* in hopes that they would prove adequate for development of a PCR-based diagnostic tool. While the Hsp 1 and 2 primers (Hudson and Adlard 1994) were not robust and could not be used for screening of *C. bairdi* and *C. opilio* for *Hematodinium*, both 18S primer pairs (Gruebl et al. 2002) were reliable and satisfactory for use in a diagnostic assay. Gruebl et al. (2002) indicated difficulty in obtaining high molecular weight DNA and amplifying the 1682 bp18S region from intensely infected *Ca. sapidus*. To overcome this difficulty they designed a new primer, Hemat-F-1487, for use with Hemat-R-1654 that produced a shorter amplicon of 187 bp. *Hematodinium* 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 blood smears, and allowed use of either primer set with all samples. Both primer sets performed well with samples of *Hematodinium* from *C. tanneri, C. angulatus, Ca. sapidus* and *N. norvegicus*; however, because we do not have infection intensity information for these samples we can not comment on their performance relative to infection intensity. Gruebl et al. (2002) isolated DNA

with phenol-chloroform while we used Qiagen columns. Phenol-chloroform extraction provides a more representative sampling of DNA fragments while Qiagen columns tend to retain relatively less small molecular weight DNA (personal observation); the *C. bairdi* and *C. opilio* extractions may have contained relatively more copies of high molecular weight DNA leading to better performance of Univ-F-15/Hemat-R-1654 in PCR.

The PCR assay was both more sensitive and more specific than blood smear examination in identifying crabs parasitized by *Hematodinium*. The PCR assay detected *Hematodinium* at levels below that which slide readers detected. Collection of *C. bairdi* and *C opilio* were late in the year so that relatively few low intensity infections were encountered; therefore, we do not have an estimate of how much more effective the PCR assay was at detecting low level infections. Slide readers tended to error on the side of caution and rated smears BCS positive when abnormal cells, i.e., cells that could not be identified as crab hemocytes that were consistent with *Hematodinium* in some features, were encountered. Use of the PCR assay eliminates the need to visually identify cells with ambiguous features. While the results indicate that the PCR-based assay is more sensitive for detection of BCS than traditional light microscopy methods, further research is necessary to determine the sensitivity of the protocol.

For detection of *Hematodinium* in *C. bairdi* and *C. opilio* tissue samples, we recommend a PCR-based diagnostic test using Qiagen DNeasy Tissue Kit columns for extractions and two PCR trials with 18S primers Univ-F-15/Hemat-R-1654 and Hemat-F-1487/Hemat-R-1654 (Greubl et al. 2002); the completed PCRs can be combined prior to visualization on a gel. Presence of both amplicons of the expected size would be evidence of a *Hematodinium* infection. Additionally, there are numerous restriction sites in the 18S region that can be assayed for

confirmation that resultant amplicons were derived from *Hematodinium*. These primers amplified *Hematodinium* 18S derived from a variety of hosts and may be suitable for routine assays of other host species.

Discussion 3 - Species Identification: Amplification and comparison of 18S rDNA indicate that sequences of all contrasted parasitic dinoflagellates regardless of host are remarkably similar. The sequences were so similar that over a 1700bp length of amplified DNA, only one of two bp differences existed. The results suggest that all tested isolates are the same species. In contrast, the primers developed by Hudson & Adlard (1994,1996) amplified a region of parasitic DNA (ITS1) that can provide some information on species identity.

While our data showed very limited variability at the 18S rDNA, extensive variability at the internal transcribed spacer region (ITS1) was indicated to the extent that two clades were revealed. The sequences from the two clades could not be aligned with confidence. Sequences of the ITS1 region revealed no differences between *Hematodinium* isolated from *C. bairdi, C. opilio, C. angulatus, C. tanneri* and *N. norvegicus* except in allele length at microsatellite loci. Within clade sequences only differed by indels, primarily resulting from loss or gain of tandem repeat units. The ITS1 region of *C. sapidus* was sufficiently unique that it formed its own clade.

Although it is well known that 18S rDNA is more conserved and thus generally less variable than ITS (Hillis & Dixon 1991, Hudson & Adlard 1996, LaJeunesse 2001), the difference in variability between the two 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.* 2001). Possible explanations include amplification of

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

Suggested amplification of host DNA appears unlikely, as the ITS1 primers of Hudson & Adlard (1994) also allowed sequencing of 220-240 bp of the adjacent 18S region, which aligned perfectly with 18S amplified using the primers by Gruebl (2002). Furthermore, sequences of both clades showed high similarity with the sequences of Hudson & Adlard (1996). We are reasonably confident that both 18Sr DNA and ITS1 sequences originate from *Hematodinium* and not the host. The observed nonspecific bands are due to mismatches between the primer site and Hsp1 rendering it less specific for *Hematodinium* than desired and resulting in strong specific bands only when relatively large amounts of parasite DNA are present.

Pseudogenes are genes that have lost their function and thus accumulate mutations much more rapidly than their functional counterparts (Alvarez & Wendel 2003). 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 two clades with completely different sequences can be explained by pseudogenes, especially as the secondary structure of the ITS1 region was maintained (Fig. 9). Furthermore, the GC content of helices and intervening regions was remarkably constant between the two clades (Table 9).

Intra-individual variation is a potential problem in a marker that is repeated multiple times in each genome, such as rDNA and ITS. However, past research has shown that intra-individual variation, although occasionally present, is usually small compared to variation between individuals and populations (Alavarez & Wendel 2003). In parasitic species like *Hematodinium*, intra- and inter-individual variation may be confounded if hosts are infected by several, genetically differentiated genotypes. Because the amplified PCR products were not cloned, it is uncertain whether one or several clones were present in each crab. However, sequences were generally clean and showed no evidence of contamination by other sequences. Furthermore, there is no reported case of intra- or inter-individual variability that would prevent alignment between sequences. Finally, if intra-individual variation was the cause of the variation observed, it would appear unlikely to find one sequence in blue crab and the other in all other species. It would appear even less likely that Hudson & Adlard (1996) found exactly the same patterns. Yet, the world-wide distribution of the two clades does pose a riddle; why is *Hematodinium* from the Norway lobster not more similar to the blue crab variant? It has been proposed that *Hematodinium* from the blue crab is most similar to the type species that was likely introduced into the United States with one of its hosts, the green crab, *Carcinus maenus*, which has a European distribution from Northern Norway, and southern Iceland to Mauritania (http://www.tmu.uit.no/crustikon/Decapoda/Decapoda2/Species_index/Carcinus_maenas.htm).

A specific case of intra-individual polymorphism at the rDNA region was found in the malaria-causing parasite *Plasmodium falciparum*, which has two functionally distinct rRNA in the vertebrate and the mosquito host (Mercereau-Puijalon *et al.* 2002). *Plasmodium* rDNA differs in several respects from other eukaryotic rDNA in that: (i) there are very few copies of

rDNA, varying from five to eight, whereas other species have 100s to 1000s of copies; (ii) there are no tandem arrays of rDNAs as in other eukaryotes and (iii) paralogues of rDNA appear to accumulate mutations independently (Rogers et al. 1995). Expression studies show that different rDNA is expressed in the vertebrate host and the mosquito, and knock-out experiments suggest that both rDNAs are required (Mercereau-Puijalon *et al.* 2002). *Plasmodium* has therefore a very specific structure of the rDNA multigene family, which has evolved in adaptation to its life cycle in two different hosts. As the life cycle of *Hematodinium* is currently unknown, a similar genome structure may be responsible for the observed variation at ITS. However, the fact that different ITS1 sequences were discovered in different species contradicts this hypothesis. Furthermore, different copies of *Plasmodium* ITS1 still have a sequence similarity of 63% - 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 two different species of *Hematodinium*, one in blue crab and the other in widely distributed decapod species. The extreme difference between almost invariable 18S rDNA and not alignable ITS1 sequences could either be explained by conservation of 18S rDNA or by 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). We therefore hypothesize that the ITS1 region may have evolved particularly rapidly, possibly in connection with functional diversification during a host switch.

A functional role of internal transcribed spacers?

The secondary structure of the two divergent ITS clades found in the present study were remarkably similar to each other, and to previously published secondary structures of other dinoflagellates species (Gottschling & Plötner 2004). This remarkable conservation of the secondary structure of ITS among dinoflagellates (Gottschling & Plötner 2004) as well as larger phylogenetic groups (Mai & Coleman 1997) suggests some functional significance, despite the general high frequency of insertions and deletions observed in the ITS regions and in this study. 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 2004). In particular, stem regions in the ITS regions may bind to proteins to form ribonucleoprotein complexes that protect nascent RNA from nuclease digestion, help the assembly of rRNAs into ribosomal subunits or act as a 'work bench' for other RNA – protein complexes (Lalev et al. 2000). The secondary structure of ITS may therefore have a significant role in the efficiency of the production of ribosomes.

Despite the similarity in the ITS secondary structure, there were also some differences, most notably in the length and the GC content of helix III (Fig 9, Table 9). Similarly, the ITS2 region of hard ticks (Ixodidae, Arthropoda) mainly evolved by changes in the length of stem regions of conserved helices (Hlinka *et al.* 2002). The functional significance of such changes is currently unknown and needs further investigation.

In addition to similarities in the secondary structure, we also found regulatory motifs in both ITS1 sequences of *Hematodinium*. Such regulatory motifs are important in the initiation and regulation of transcription, and often occur in tandemly repeated sequences in the external transcribed spacers (ETS), where transcriptional rates are positively correlated to the number of

repeats present (Busby & Reeder 1993). Similar regulatory motifs have been found in the ITS1 region of some flatworm species (van Herwerden et al. 2003). Interestingly, the two clades had either the reverse (TATAAT vs TAATAT) or complement (CCCGCC vs. GGGCGG) 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,* however, which expresses different sets of rDNA in different host species (Mercereau-Puijalon et al. 2002), make such a possibility conceivable, although in *Plasmodium,* rDNA regions adjacent to the ITS1 region show some differentiation as well. On the other hand, evidence for host specificity of ITS1 genotypes is equivocal in *Symbiodinium*, symbiotic dinoflagellates in coral reef dwelling cnidarians, mollusks, platyhelminthes and protests. 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 ITS1 strains of the commensal (Rodriguez-Lanetty et al. 2004). Although this clearly 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*, and may warrant further investigation.

In summary, we found clear evidence for two distinct ITS1 clades among the *Hematodinium* clades analyzed here, which correspond well to previous data by Hudson &

Adlard (1996) and probably deserve species status. The secondary structure between these two 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, although this notion needs further clarification.

Despite the remarkable advances of molecular technology in systematics, the question as to what defines the species has still not been adequately answered. A review of the literature indicates that there is no consensus as to which loci are most useful, nor what degree of divergence between loci in organisms of interest identify separate species. Other researchers suggest that sequencing and contrasting multiple loci greatly enhances resolution of phylogenetic relations. For example, Saldarriaga et al. (2004) developed a phylogenetic tree for dinoflagellates based on combined small and partial large subunit rRNA sequences.

Discussion 4 - Life History Studies: Extracting amplifiable DNA from environmental samples, e. g., water and sediment, is notoriously difficult due to abundant PCR inhibitors that isolate with DNA. Our positive controls demonstrated that the extraction procedure we used did allow recovery of amplifiable *Hematodinium* DNA when using the Univ-F-15/Hemat-R-1654 and Hemat-F-1487/Hemat-R-1654 primers. The lack of amplicons from PCRs of DNA recovered from the sediment samples that had been spiked with trophonts during the sieving procedure probably indicates that trophonts can not survive the abrasive sieving. While it remains to be seen if the sequence of the amplicon produced from the apparent BCS positive from Stephens Passage is consistent with *Hematodinium* 18S, it is interesting to note that this is the only sample to produce an amplicon and the only sample that was not sieved. It is possible that life history stages found outside the host do not remain intact throughout our procedure to

recover cells from water and sediment samples for DNA isolation. Even though our positive controls indicated that we could amplify *Hematodinium* DNA from the water and sediment samples, our methods may not have been sensitive enough to detect *Hematodinium* at the levels present in the environmental samples.

From the beginning, we understood that identification of external lie history stages was possibly our most difficult goal to address. Over the last twenty years, the increased worldwide occurrence of *Hematodinium* related disease has been met with increased effort to more adequately identify the pathogen and understand disease pathogenesis and parasite ecology. However, the recent combined effort directed at *Hematodinium* related disease is small when compared to historical effort toward the oyster disease caused by *Perkinsus marinum* (23 publications versus 181, ASFA literature search). As with *Hematodinium*, little was previously known of the life history of *P. marinum* outside of the oyster host, but only recently was success reported in detecting life history stages of the parasite in water and sediment samples via development of a PCR based protocol (Corinaldesi et al. 2005; Audemard et al. 2004). Directed effort in this area should continue as significant information could be revealed on disease dissemination and seasonal infection cycles, mode of infection, the possible existence of reservoirs and complete elucidation of the life history of *Hematodinium*. The last point is particularly important because an understanding of its life history could reveal vulnerable stages where disease transmission could be interrupted or managed.

PROJECT SUMMARY/EVALUATION

This research project identified three main objectives; first, to determine the number of

Hematodinium species that exist world-wide, second, develop a PCR-based assay for detecting and monitoring BCS in affected crustacean populations and, third, detect and describe *Hematodinium* life history stages that may exist in the environment. Considerable progress was made in two of the three objectives, but deficiencies are also acknowledged. With respect to species identification, the type species of *Hematodinium* (France) could not be obtained. Collections of *Hematodinium* from *Portunus pelagicus* (Australia) were also not possible. Regardless, our data indicate that two clades/species exist, one infecting the blue crab, while the other appears to be cosmopolitan in distribution and infects all other examined decapod hosts. Contributions from France and Australia are necessary to develop a complete phylogeny of the taxon. Significant progress in development of the PCR assay was made. It is our opinion that the technique was critically tested and deployment into the field is warranted. The PCR-based diagnostic protocol will form the basis of future BCS monitoring efforts. Little or no success is noted in addressing the third objective. DNA extraction from environmental samples is difficult and only recently has significant progress been made in overcoming natural inhibitors in soil, sediment, water and feces. Even though areas of known high BCS prevalences were targeted, it is uncertain whether putative free-living life history stages exist, were present during the time of sampling or are ephemeral in nature.

In general, the results of this study will advance efforts to understand the effects of BCS on affected populations and effort should continue at addressing acknowledged short-comings of this project.

Future directions: Methodology does not exist to completely separate host and parasitic genomic DNA from each other during or after DNA extractions. We plan to focus future effort

on developing a method to separate host and parasite cells so that DNA can be extracted from each separately; due to the sensitive nature of many molecular techniques, we are limited in investigations of *Hematodinium* by 'contamination' with host DNA. Primers available for amplifying DNA regions of interest in dinoflagellates have been developed from either free-living or commensal dinoflagellates that have been separated from host cells prior to DNA extraction. Available primers appear to occasionally amplify host DNA, as was the case for Hsp 1 and 2 (this study) and universal dinoflagellate large subunit, or 24S, rDNA primers (unreported data), complicating efforts to amplify parasite DNA. Due to the difficulty of morphological studies of *Hematodinium*, it would be very helpful to sequence other regions of DNA typically used to distinguish closely related species of dinoflagellate, e. g., large subunit rDNA (Saldarriagga et al. 2004). Acquisition of *H. australis* for comparative studies is also critical.

Presence of a possible *Hematodinium* amplicon in the sediment sample that was partially sieved is intriguing; we plan to collect more sediment samples and vary our processing techniques. Identification of life history stages in environmental samples will contribute to our understanding of the transmission of the parasite.

We also expect to determine the level of sensitivity of the PCR protocol so that disease onset and progression can be more closely followed. Based on previous studies, it is believed that BCS develops over many (12-18) months and that disease onset is associated with molting. Both hypotheses require testing and the PCR protocol, especially quantitative PCR, would enhance our efforts in addressing these issues.

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Table 1. Summary of *Hematodinium* related disease as reported by host species, location and prevalence.

Citation	Parasite Species	Host Species	Size (mm)	Location	Prevalence
Chatton & Poisson 1931	*Hematodinium perezi*	*Carcinus maenas*		Roscoff, France	0/3000
	H. perezi	*Polybius depurator*		Luc-ser-mer, France	3/470
	H. perezi	*Carcinus maenas*		Arcachon, France	1/1000
Gallien 1938	*H. perezi*	*Platyonichus latipes*		France	18/80
Newman & Johnson 1975	*Hematodinium* sp.	*Callinectes sapidus*	70-170	NC, GA, FL	0-30%
McLean & Ruddell 1978	*Hematodinium* sp.	*Cancer irroratus*		Mid-Atlantic Bight	3/698
	Hematodinium sp.	*Cancer borealis*		Mid-Atlantic Bight	5/125
	Hematodinium sp.	*Ovalipes ocellatus*		Mid-Atlantic Bight	1/155
Couch 1983	*Hematodinium* sp.	*Callinectes sapidus*		Gulf of Mexico	Positive
Johnson 1986	*Hematodinium* sp.	13 amphipod species		Northeast USA	0.8-67%
Meyers et al. 1987	*Hematodinium* sp.	*Chionoecetes bairdi*	>70	Southeast Alaska	95%
Latrouite et al. 1988	*Hematodinium* sp.	*Cancer pagurus*		France	21%
Wilhelm & Boulo 1988	*Hematodinium* sp.	*Liocarcinus puber*	>15	Brittany, France	33.16%
Meyers et al. 1990	*Hematodinium* sp.	*C. bairdi*	>70	Southeast Alaska	26%
Eaton et al. 1991	*Hematodinium* sp.	*C. bairdi*		Southeast Alaska	68.30%
Shields 1992	*Hematodinium* sp.	*Scylla serrata*		Australia	1.50%
Field et al. 1992	*Hematodinium* sp.	*Nephrops norvegicus*	>20	Scotland	21%
Hudson et al. 1993	*Hematodinium* sp.	*Trapezia aerolata*	11	Australia	20%
	Hematodinium sp.	*Trapezia coerulea*		Australia	Positive
Love et al. 1993	*Hematodinium* sp.	*C. bairdi*		Auke Bay, AK	0-97%
Hudson & Shields 1994	*H. australis*	*Portunus pelagicus*		Queensland, Australia	0.9-4.0%
Hudson & Lester 1994	*Hematodinium* sp.	*Scylla serrata*		Australia	1.50%
Messick 1994	*H. perezi*	*C. sapidus*	5-180	Maryland, Virginia	0-100%
Taylor & Khan 1995	*Hematodinium* sp.	*Chionoecetes opilio*	92-120	Newfoundland	0.05%
Meyers et al. 1996	*Hematodinium* sp.	*C. bairdi*		Prince William Sound	0-7.2%
	Hematodinium sp.	*C. bairdi*		Cook Inlet	0.00%
	Hematodinium sp.	*C. bairdi*		Kodiak Island	3.60%
	Hematodinium sp.	*C. bairdi*		Alaska Peninsula	1.30%
	Hematodinium sp.	*C. bairdi*		Eastern Aleutian Islands	0.00%
	Hematodinium sp.	*C. bairdi*		Eastern/NE Bering Sea	0-2.4%
	Hematodinium sp.	*C. bairdi*		Western Bering Sea	0.90%
	Hematodinium sp.	*C. opilio*		Norton Sound	14.6-29.1%
	Hematodinium sp.	*C. opilio*		Chukchi Sea	13.3-15.5%
	Hematodinium sp.	*C. opilio*		Western Bering Sea	1.10%
Field et al. 1998	*Hematodinium* sp.	*N. novegicus*		Scotland	0.9-59.7%
Stentiford et al. 2001	*Hematodinium* sp.	*N. novegicus*	20-52	Scotland	0-35%
Dawe 2002	*Hematodinium* sp.	*C. opilio*	6-141	Newfoundland	1.40%
Stentiford 2002	*Hematodinium* sp.	*C. pagurus*		UK Channel Islands	No data
Urban & Byersdorfer 2002	*Hematodinium* sp.	*C. bairdi*		Alitak Bay, Kodiak	10.10%

Table 2. Total number of hemolymph and tissue samples collected by region and host species for molecular analysis.

Region	Sub-Region	Species	Samples Analyzed	No. BCS +	No. BCS -
SE Alaska	Douglas Island	*Chionoecetes bairdi*	32	19	13
	Glacier Bay		1	0	1
	Icy Strait		0	0	0
Prince William Sound			-	-	-
Kodiak Island	Alitak Bay	*Chionoecetes bairdi*	4	4	0
	Not Alitak Bay		13	0	13
Bering Sea		*Chionoecetes bairdi*	140	26	114
		Chionoecetes opilio	19	5	14
Newfoundland*		*Chionoecetes opilio*	51	49	2
Cheasepeake Bay*		*Callinectes sapidus*	2	2	0
Scotland*		*Nephrops norvegicus*	4	4	0
Vancouver Island*		*Chionoecetes angulatus*	1	1	0
		Chionoecetes tanneri	4	3	1
TOTAL			271	113	158

Table 3. Total number of blood smear collections by region and host species for determination of BCS prevalence.

Region	**Sub-Region**	**Species**	**Samples Analyzed**	**No. BCS +**	**Prevalence**
SE Alaska	Douglas Island	*Chionoecetes bairdi*			
	Glacier Bay				
	Icy Strait				
Prince William Sound					
Kodiak Island	Alitak Bay	*Chionoecetes bairdi*	7	3	42.9
	Not Alitak Bay		53	1	1.9
Bering Sea		*Chionoecetes bairdi*	418	175	41.9
		Chionoecetes opilio	262	85	32.4
Newfoundland		*Chionoecetes opilio*	519	134	25.8
Cheasepeake Bay		*Callinectes sapidus*			
Scotland		*Nephrops norvegicus*			
Vancouver Island		*Chionoecetes angulatus*	0		
		Chionoecetes tanneri	0		
TOTAL			1259	398	31.6

Table 4. Hemolymph collections for *in vitro* culture by location and host species.

Region	**Sub-Region**	**Species**	**No. Samples**
SE Alaska	Douglas Island	*Chionoecetes bairdi*	102
	Glacier Bay		13
	Icy Strait		8
Prince William Sound			0
Kodiak Island	Alitak Bay	*Chionoecetes bairdi*	3
	Not Alitak Bay		0
Bering Sea		*Chionoecetes bairdi*	17
		Chionoecetes opilio	9
Newfoundland		*Chionoecetes opilio*	25
Chesapeake Bay		*Callinectes sapidus*	1
Scotland		*Nephrops norvegicus*	0
Vancouver Island		*Chionoecetes angulatus*	0
		Chionoecetes tanneri	13

Table 5. Water samples collected for immunological analysis by region.

Year	Region	Sub-Region	Species	No. Samples	No. BCS + by PCR	IFAT Results
2003	SE Alaska	Stephens Passage	*C. bairdi*	4	0	-
		Glacier Bay	*C. bairdi*	3	0	-
		Icy Strait	*C. bairdi*	4	0	-
	Kodiak Island	Alitak Bay	*C. bairdi*	3	0	-
		Not Alitak Bay	*C. bairdi*	-	0	-
2004	SE Alaska	Stephens Passage	*C. bairdi*	2	0	-
		Glacier Bay	*C. bairdi*	-	-	
		Icy Strait	*C. bairdi*	1	0	-
	Kodiak Island	Alitak Bay	*C. bairdi*	-	-	
		Not Alitak Bay	*C. bairdi*	-	-	

Table 6. Sediment samples collected for immunological analysis by region.

Year	Region	Sub-Region	Species	No. Samples	No. BCS + by PCR	IFAT Results
2003	SE Alaska	Stephens Passage	*C. bairdi*	6	0	-
		Glacier Bay	*C. bairdi*	6	0	-
		Icy Strait	*C. bairdi*	4	0	-
2004	SE Alaska	Stephens Passage	*C. bairdi*	4	0	-
		Glacier Bay	*C. bairdi*	-	-	
		Icy Strait	*C. bairdi*	1	0	-

Table 7. rDNA targeted primer pairs, amplicon size, reaction conditions, and primer sequence sources.

Primer pair [a,b]	Sequence (5' – 3')	Region amplified	Expected amplicon size (bp)	Annealing temperature (˚C)	MgCl concentration [mM]	*Taq* concentration [c] [U/ul]	Primer concentration [μM]	No. amplification cycles	Reference
Hsp1 Hsp2	gtt ccc ctt gaa cga gga att c cgc att tcg ctg cgt tct tc	ITS1 & partial 18S	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	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	18S	187	56	3	0.05	0.216 0.18	30	Gruebl et al. 2002

[a] Standard PCR conditions: 1μl genomic DNA, 100μM each dNTPs, 1X buffer (50mM KCl, 10mM Tris-HCl {pH 9.0 at 25□C} and 0.1% Triton® X-100), 0.4 mg/ml Bovine Serum Albumin (BSA), sterile water to 25μl. [b] Standard PCR temperature profile: initial template denaturation at 95˚C for 1 min; number of cycles designated above at 94˚C for 15s; annealing temperature as above for 15s; 72˚C for 30s; followed by a final incubation at 4˚C for 5 min. [c] DNA Polymerase in Storage Buffer A (50mM Tris-HCl (pH 8.0), 100mM NaCl, 0.1mM EDTA, 1mM DTT, 50% glycerol and 1% Triton® X-100), (Promega Corp., Madison, WI, USA).

Table 8. Results and re-examinations of samples evaluated both microscopically and by PCR assay.

		Initial results					Adjusted results [a]				
Species	n	PCR +	PCR -	Blood smear +	Blood smear -	No. discrepancies	PCR +	PCR -	Blood smear +	Blood smear -	No. remaining discrepancies
C. opilio	61	50	11	39	22	11	50	11	50	11	0
C. bairdi	150	39	111	57	93	22	39	111	40	110	1 [b]

[a] When microscopic and molecular results were in disagreement, each sample was re-amplified and the corresponding blood smear reexamined by an independent slide reader. [b] One sample was scored, and re-scored, as negative by microscopy, and successfully amplified twice with both 18S primer pairs.

Table 9: G+C content of helices and intervening regions in the ITS1 region of the two Hematodinium clades. See Fig 9 for a diagram of helices. IVR – intervening regions between respective helices.

	C.b.I		C.b. II		C. sap.	
	# bases	G+C	# bases	G+C	# bases	G+C
Total	328	46%	328	46%	350	46.00
helices	239	49%	270	51%	247	53%
IVRs	89	38%	58	26%	78	37%
IVR	31	35%	31	35%	35	14%
helix a	18	56%	18	56%	21	52%
IVR	7	14%	7	14%	7	29%
helix I	83	67%	83	67%	90	51%
IVR	6	17%	6	17%	3	33%
helix II	47	55%	47	55%	52	54%
IVR	6	33%	6	33%	1	0%
helix b	50	24%	50	24%	35	57%
IVR	15	53%	2	0%	12	67%
helix III	19	47%	50	56%	21	38%
IVR	18	61%			25	28%
helix c	22	23%	22	23%	28	68%
IVR	6	0%	6	0%	20	30%

Table 10. Regulatory motifs and their positions in clades A (*Callinectes sapidus*) and B (*Chionoecetes bairdi*).

Motif	**Clade A**	**Clade B**	**Location**
TATAAT	208-213		Stem of helix II
	314-319		Tip of helix c
TAATAT		17-22	Linear
CCCGCC	78-83		Stem of helix I
GGGCGG		102-108	Tip of helix I

Figure 1. World-wide reports of *Hematodinium* related disease. Red dots indicate general regions where *Hematodinium* has been reported. Blue polygon indicates recent report.

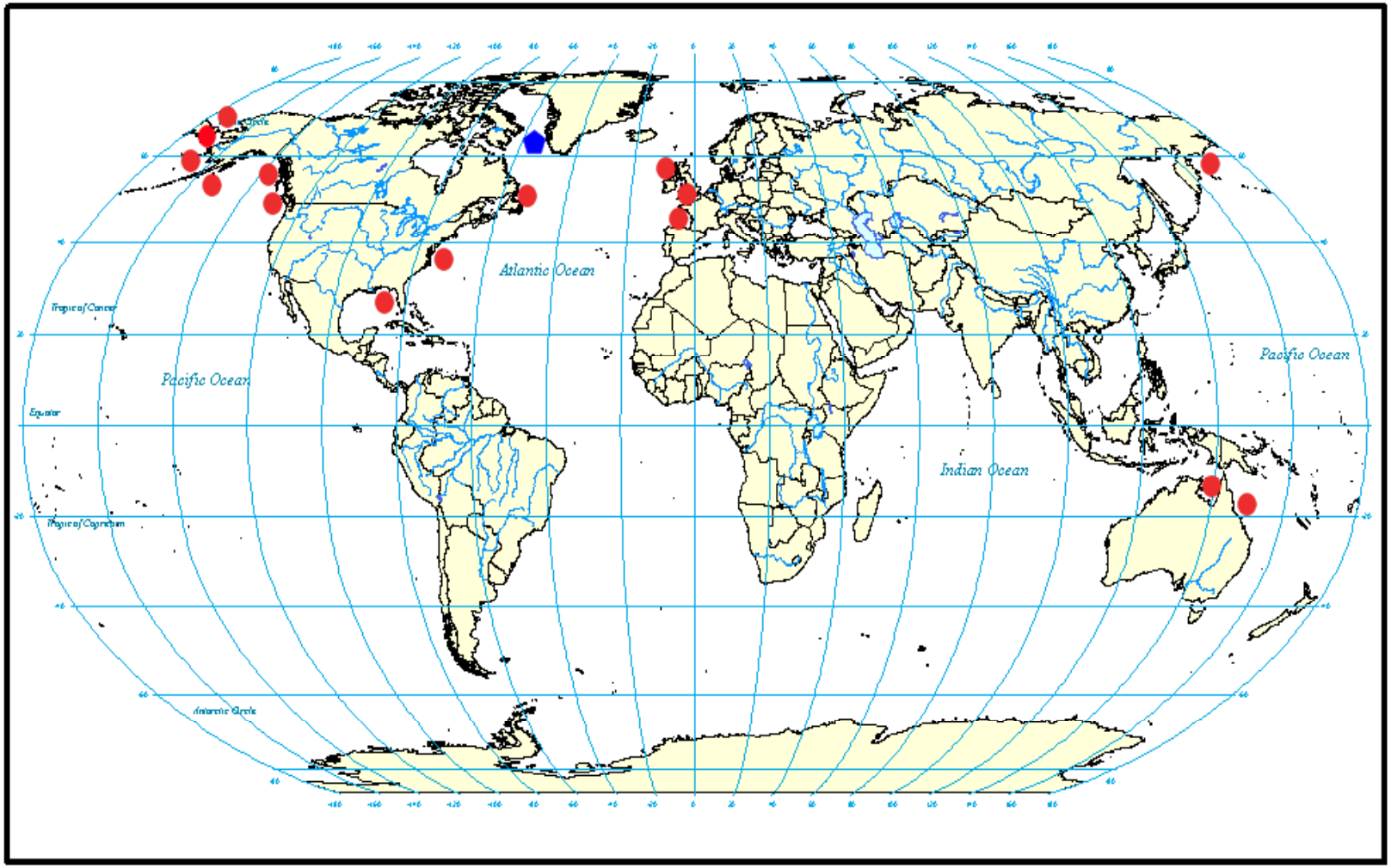


Figure 2. Historical landing trends of North Pacific commercial crabs.

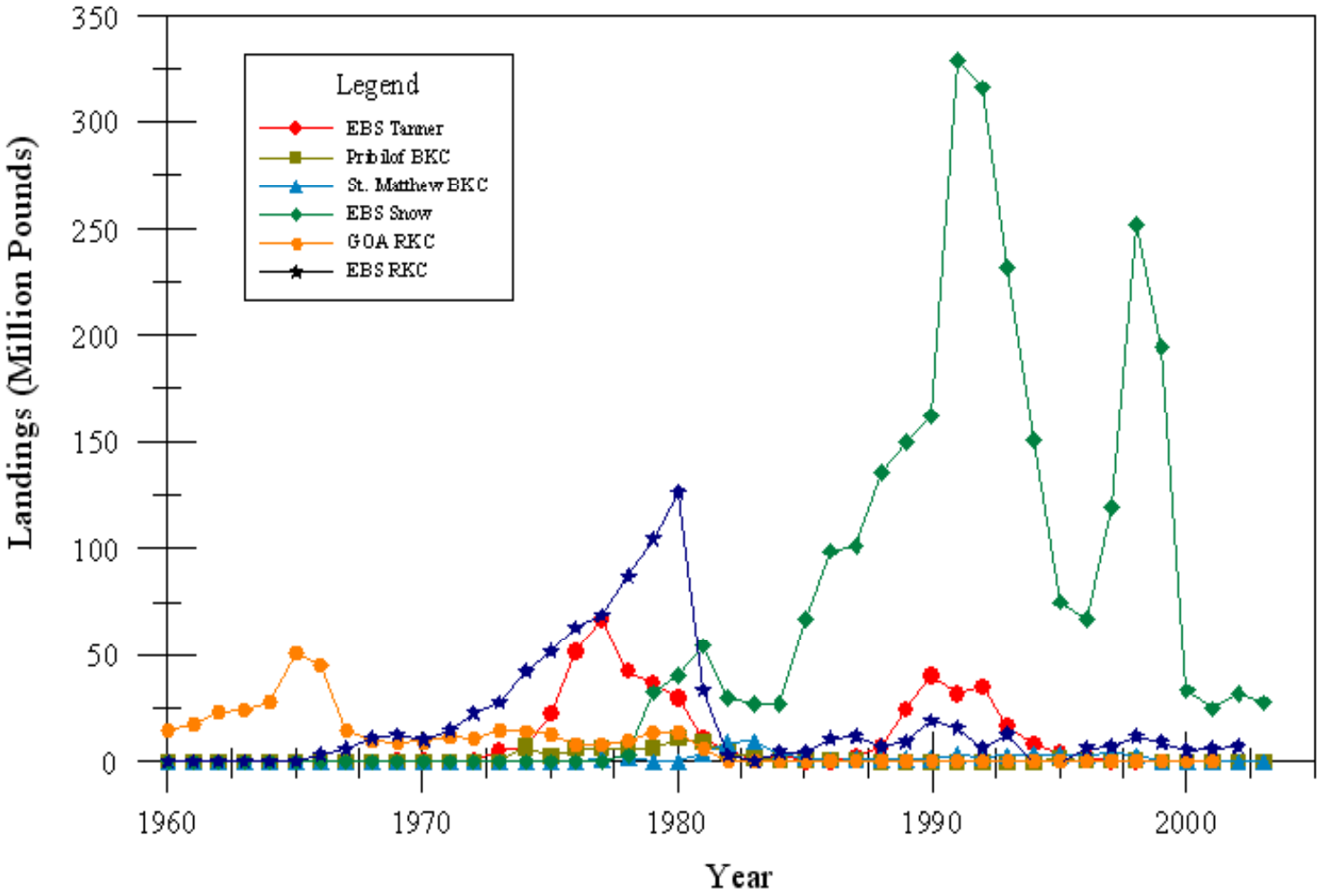
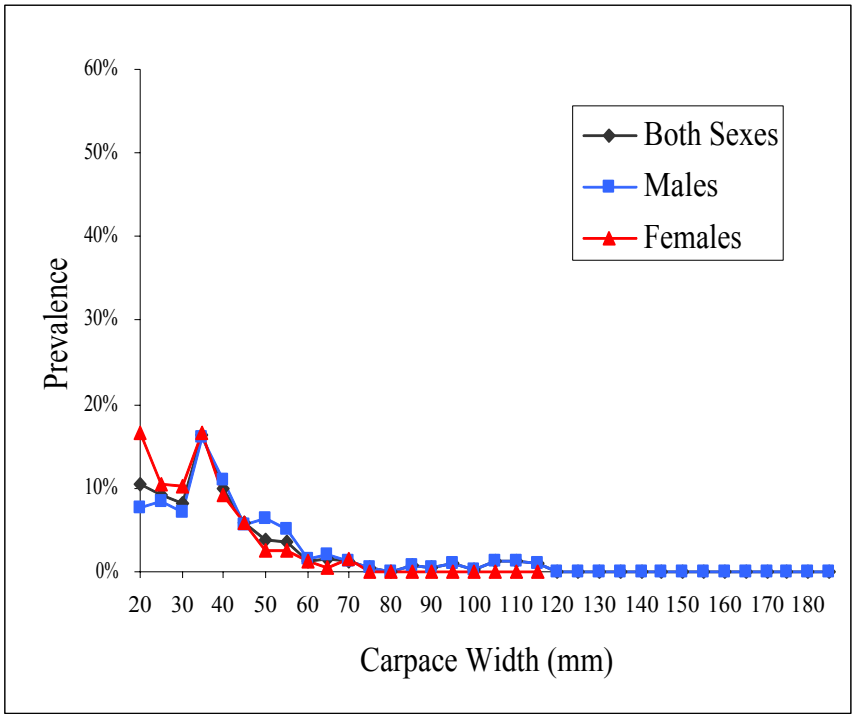


Figure 3. Prevalence of BCS in Eastern Bering Sea snow and Tanner crabs, 1988-1998.

Chonoecetes opilio



Chionoecetes baidi

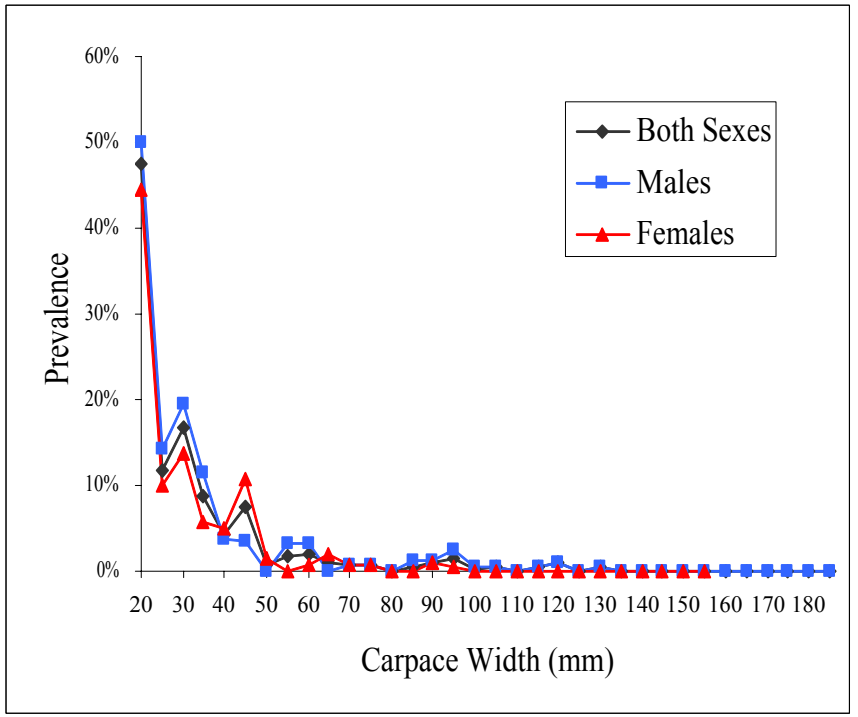


Figure 4. Graphical representation of an rDNA repeat unit consisting of a non-transcribed spacer (NTS), external transcribed spacer (ETS), small subunit 18S rDNA (18S), internal transcribed spacer 1 (ITS1), 5.8S rDNA (5.8S), internal transcribed spacer 2 (ITS2), large subunit 28S rDNA (28S) and another external transcribed spacer. For the present study, the 18S rDNA and ITS1 were sequenced.

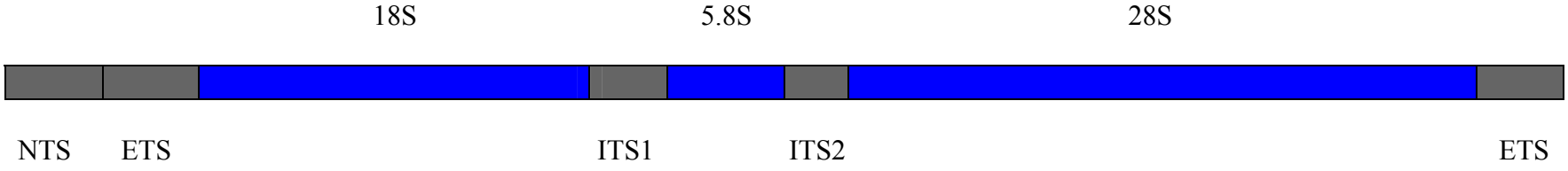


Figure 5. Focal sampling regions for Bitter Crab Syndrome. Stephens Passage in Southeast Alaska and Alitak Bay, Kodiak are known regions of high and moderate areas of disease prevalence. *Hematodinium* infections are rare or non-existent in Icy Strait, Southeast Alaska.

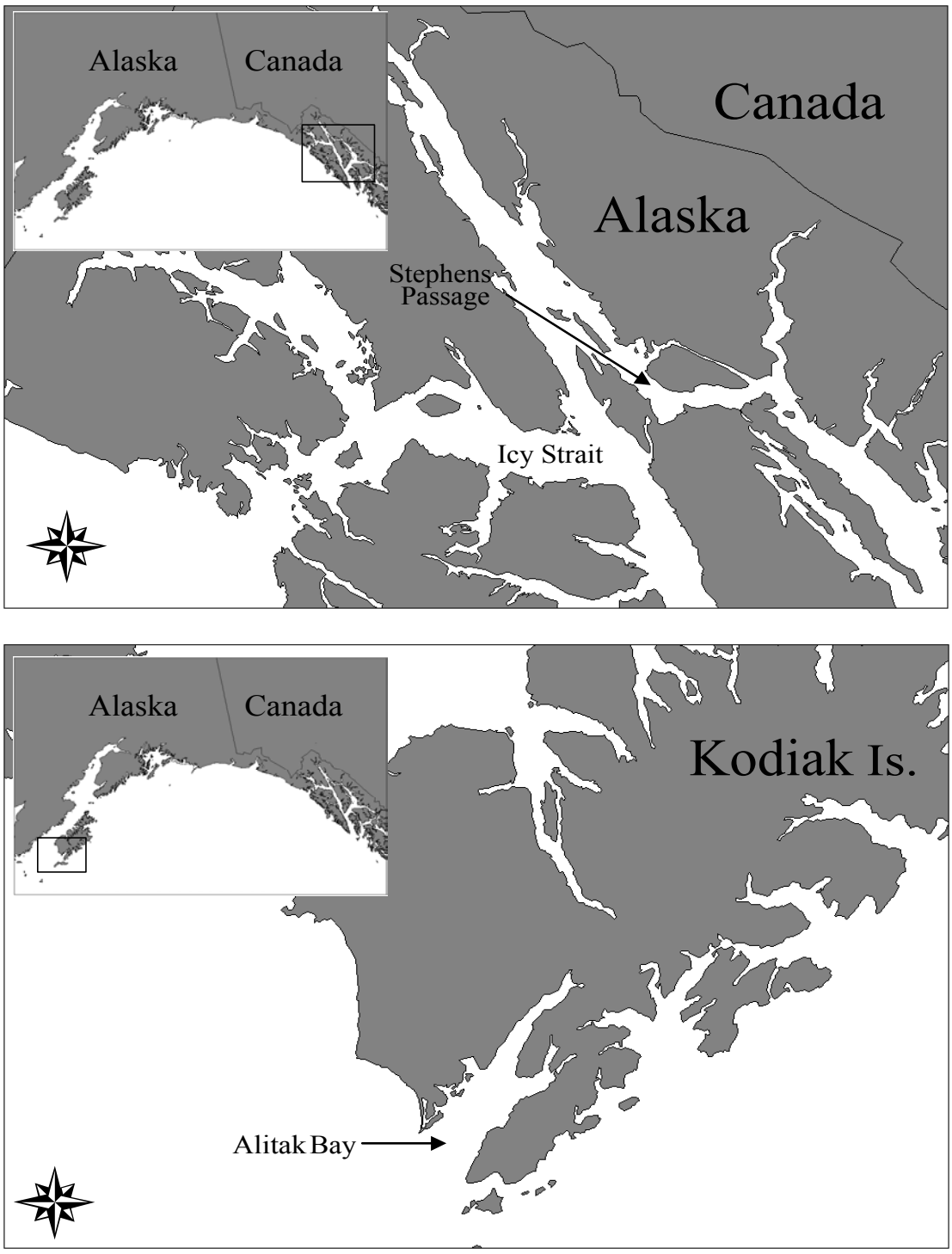


Figure 6. PCR amplifications illustrating the inconsistent banding patterns encountered in HSP 1 and 2 products. Lanes 1-5, microscopically-rated heavy infections of *Hematodinium*; Lane 6, microscopically-rated light infection of *Hematodinium*; Lanes 8, 9 microscopically negative for *Hematodinium*; Lane 10, microscopically-rated moderate infection of *Hematodinium*; Lanes 11, 12 BCS positive PCR controls; Lane 7, size standard. bp, base pairs.

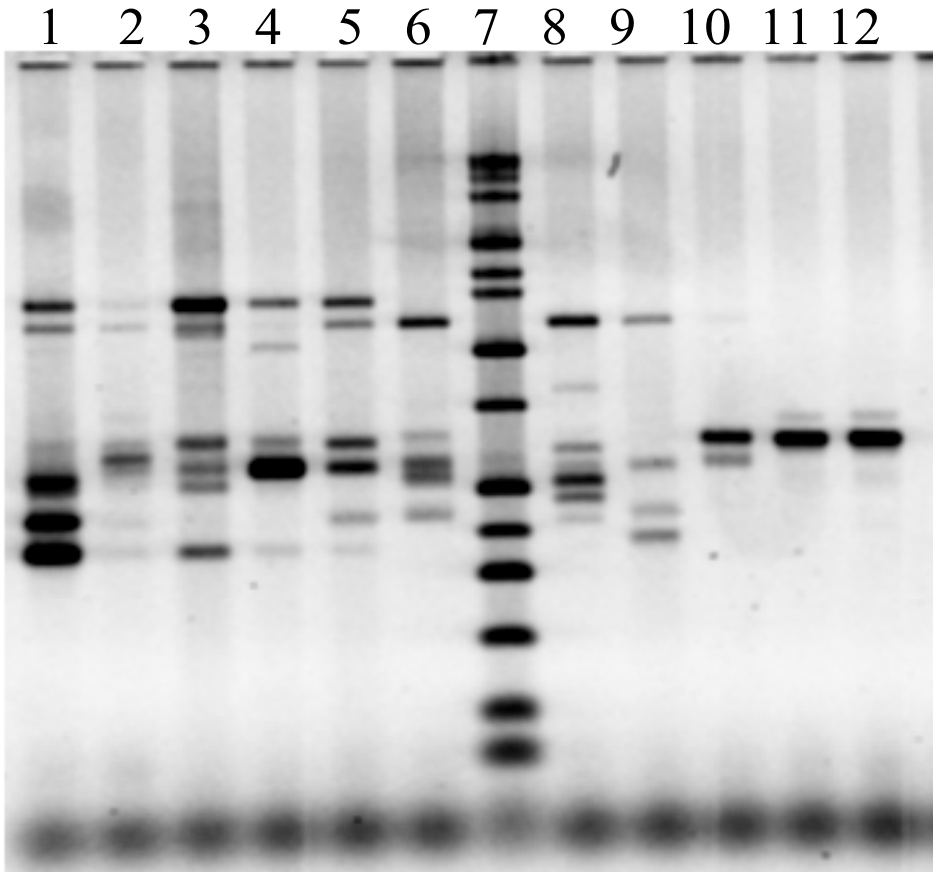


Figure 7. PCR screening of *C. bairdi* samples with UF15/HR1654 (1682 bp) and HF1487/HR1654 (187 bp). All lanes contain combined products of both PCRs. Lanes 2, 5, 6, 8, 9, 10, 11 containing no bands are negative for *Hematodinium*; Lane 4, a sample that was microscopically BSC-; Lanes 1, 3, 4, 12 – 23, positive for *Hematodinium;* Lanes 7, 24, size standard. bp, base pairs.

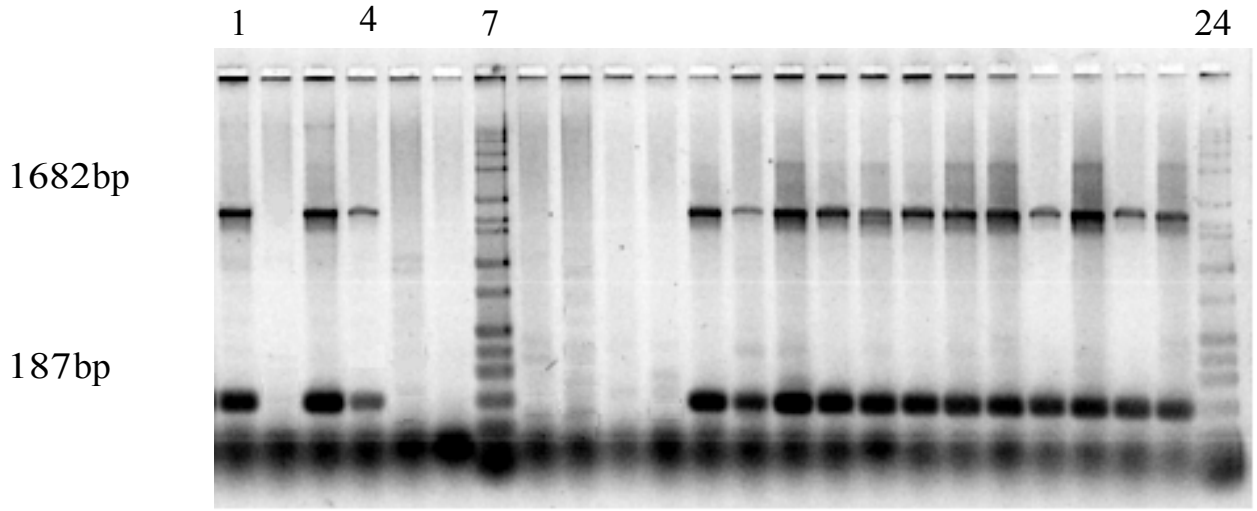


Figure 8. RFLP products of the UF15/HR1654 18S amplicon restricted with Hpa I and Eco RI. Lane 1, unrestricted UF15/ PCR product; Lanes 2 - 4 product restricted with Eco RI; Lanes 6-8, product restricted with Hpa I. Lane 5, size standard. bp, base pairs.

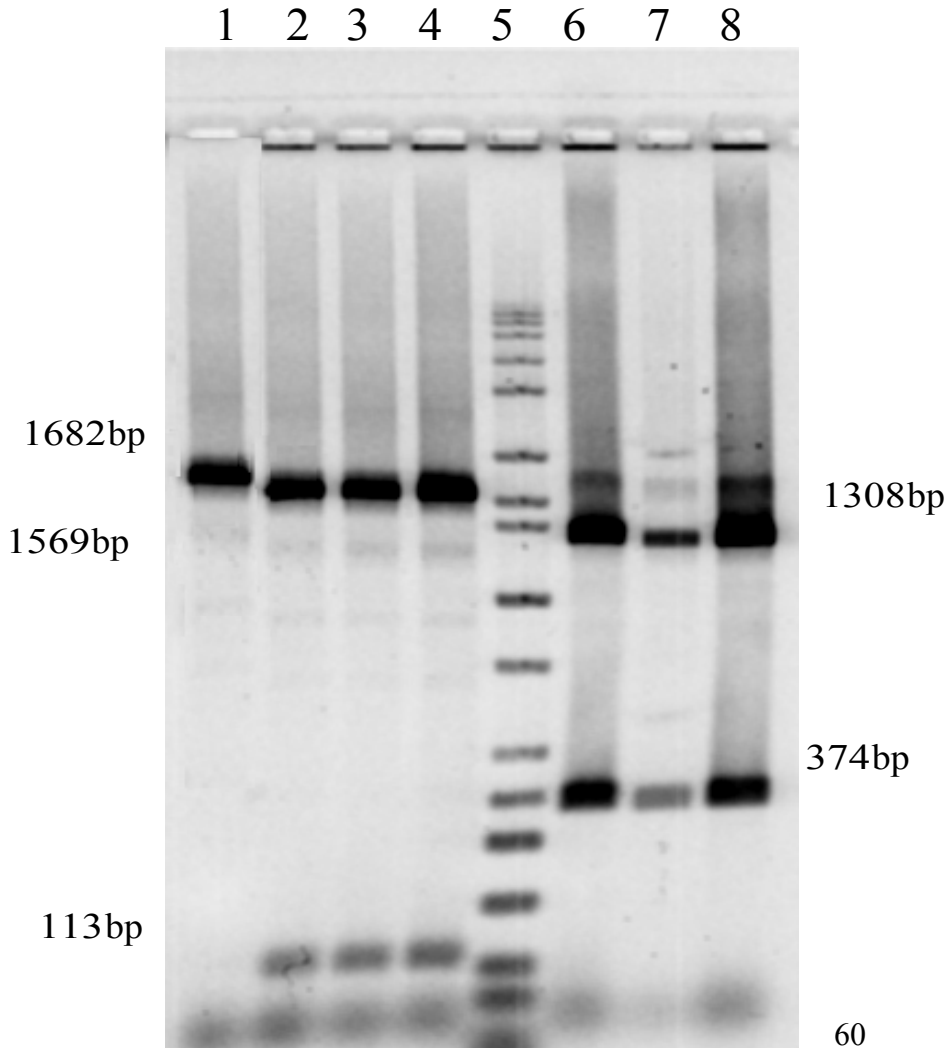


Figure 9. ITS1 transcript secondary structure for clades A (*Callinectes sapidus*) and B and C (*Chionoecetes bairdi*). C represents a lower free energy form of major helix III. D is a representative transcript for *Pfiesteria piscida* from Gottschling& Plötner (2004). Major helices are labeled I-III while minor helices are labeled a-c.

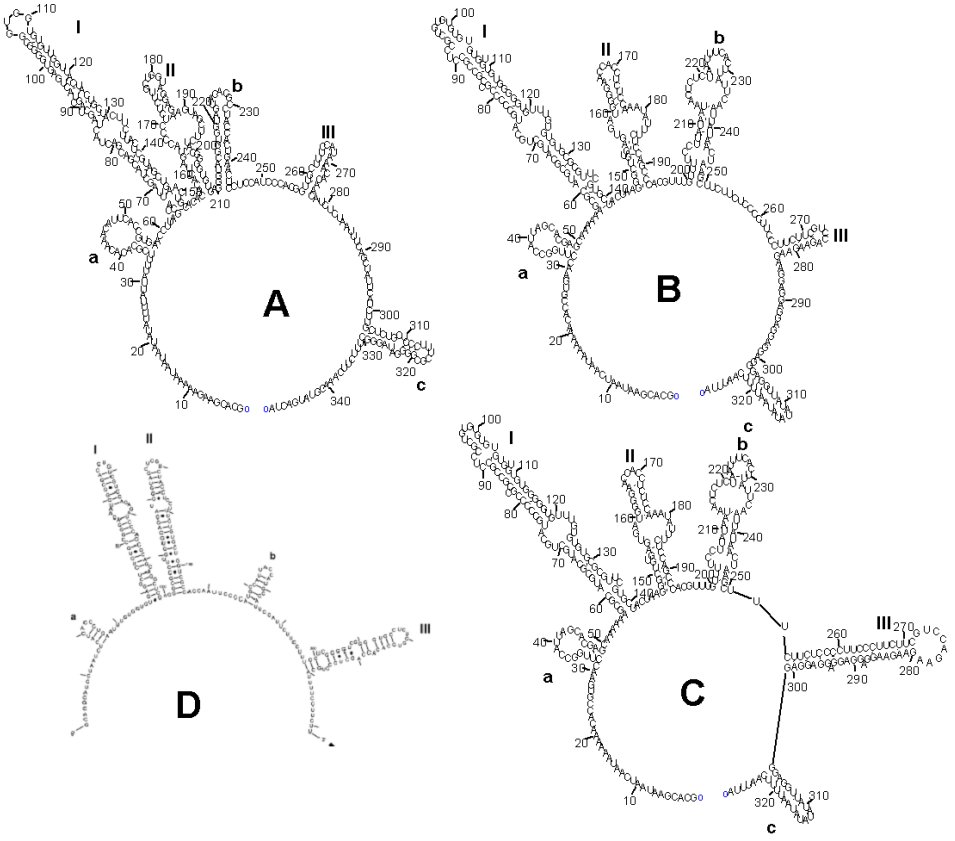


Figure 10. PCR screening of environmental water samples with positive controls. Upper half of gel contains UF15/HR1654 PCR product and lower half contains HF1487/HR1654 PCR product. Lane1, subsample of water filter spiked with *Hematodinium* DNA immediately before extraction and again before PCR, Lane 2, subsample of same water filter spiked with *Hematodinium* DNA before extraction only, Lane 3, subsample of same water filter spiked with *Hematodinium* DNA before PCR only, Lane 4, same sample unspiked. Lanes 5, 6, 7, 8, same order of controls and unspiked filter for a second water sample. Lane 9, PCR positive control, Lane 10, PCR negative control, Lane 11 unused, Lane 12, size standard.

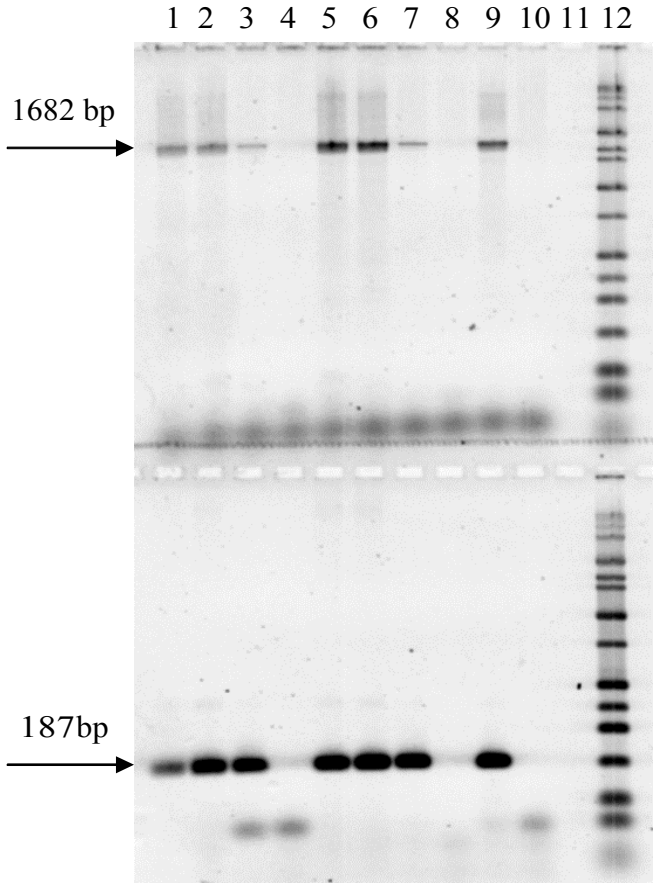


Figure 11. PCR with UF15/HR1654 of DNA isolated from a sediment sample with a *Hematodinium*-sized amplicon. Lanes 1-3, sediment positive controls. Lane 4, amplicon of a BCS positive sediment sample from Stephens Passage; Lane 5, PCR positive control; Lane 6, PCR negative control; Lane 7, unused; Lane 8, size standard. Lane 4 shows a band apparently identical in size to the expected band (1682 bp),

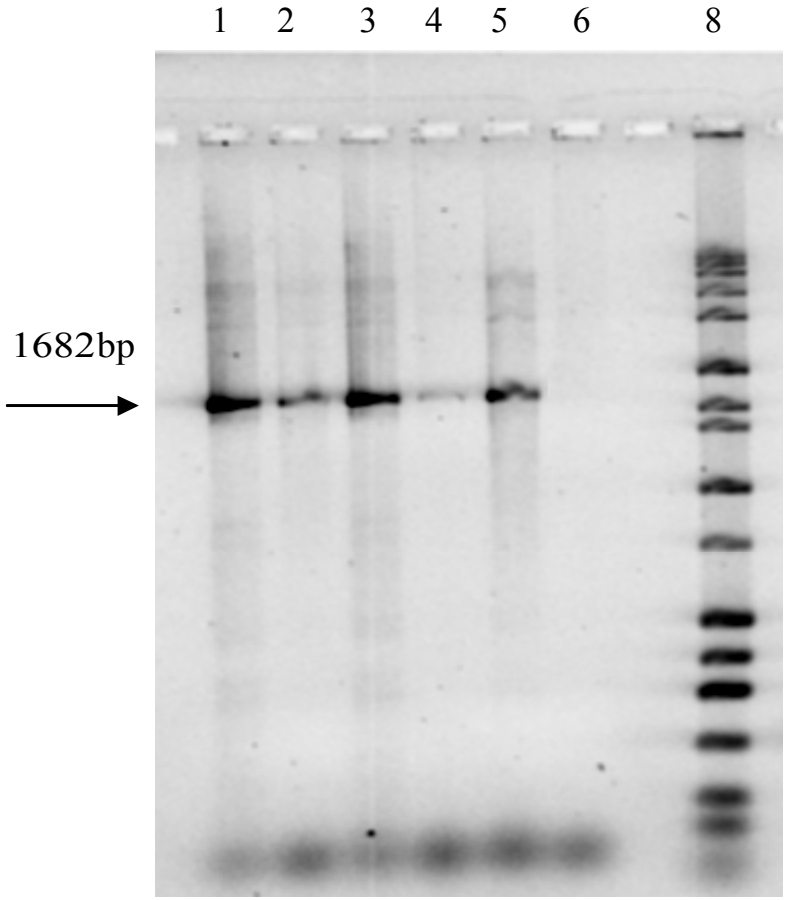


Figure 12. IFAT image of *Hematodinium* trophonts from *C. bairdi*.