2011

Molecular genetic studies of Hematodinium sp., a parasitic dinoflagellate of the blue crab, Callinectes sapidus

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Molecular genetic studies of *Hematodinium* sp., a parasitic dinoflagellate of the blue crab, *Callinectes sapidus*

A Dissertation

Presented to

The Faculty of the School of Marine Science

The College of William and Mary

In Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

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by

Katrina M. Pagenkopp Lohan

2011

APPROVAL SHEET

This dissertation is submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

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Approved, August 2011

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DEDICATION

This dissertation work is dedicated to the love of my life, my husband, Christopher Lohan, who taught me that anything is possible as long as we have each other, and to my parents, Paul and Margaret Pagenkopp, who have loved, encouraged, and guided me throughout my life.

I also dedicate this work in loving memory to the four family members I lost while conducting this work: my cousin, Zia Fiorella; my grandfather, Richard Gardner; my mother-in-law, Kimberly Squares; and my great uncle, J. Robert Gardner.

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ACKNOWLEDGMENTS

First and foremost, I acknowledge and sincerely thank my advisors, Drs. Kimberly Reece and Jeffrey Shields, for their guidance, patience, and enthusiasm throughout my time in their labs, which has aided in my personal and professional development.

I thank my committee members, including Dr. David Gauthier, Dr. Emmett Duffy, and Dr. Allen Place for their advice, expertise, and critical appraisal of this project as it evolved. Dr. Place also contributed samples of blue crabs from numerous locations, which greatly aided in our understanding of the range of this parasite species.

I give a special thanks to Dr. Jan McDowell, who helped immensely with many aspects of the microsatellite work conducted for this project. Dr. McDowell was an integral part of the development of the microsatellite library, the optimization of the markers, and the data analysis. I could not have completed this research without her expertise, creativity, and patience.

I thank current and former members of the Shields lab, including Dr. Caiwen Li, Dr. Tom Dolan, Dr. Hamish Small, Dr. Terrence Miller, Dr. Jessica Moss Small, Anna Coffey, Peter Coffey, Erin Jenkins, and Kelly Delaney. All these individuals assisted with collecting both alternate and blue crab hosts as well as screening blue crabs and alternate hosts for the parasite. Additionally, Drs. Small, Miller, and Moss Small provided genetic laboratory assistance and essential brainstorming sessions throughout the development of my dissertation work.

I thank current and former members of the Reece lab. Gail Scott, Alanna MacIntyre, and Georgetta Constantin all provided laboratory assistance and advice. Dr. Jan Cordes provided technical assistance and expertise for the microsatellite data. Dr. Jie Xiao assisted with the development of the *Hematodinium* sp. microsatellite library and offered her expertise on microsatellite scoring. Wendi Quidort provided laboratory assistance as well as offered her services as a running partner/counselor, which was much needed throughout this project. Annie Dershem provided technical assistance while freely sharing her enthusiasm for biology and learning.

I thank Dr. Corinne Audemard, Dr. Ryan Carnegie, and Kristy Hill for their expertise as well as the numerous scintillating conversations and brainstorming sessions. I also thank Laurie Sorenson for her technical assistance with microsatellite library construction, microsatellite scoring, and troubleshooting the microsatellite PCR assays. I thank Dr. John Graves for the use of his lab facilities for the development of the microsatellite library for *Hematodinium* sp.

I owe a very special acknowledgement to the following members of the VIMS Eastern Shore laboratory: Sean Fate, Alan Birch, Edward Smith, and PG Ross. All of these individuals were critical for obtaining specimens, especially alternate hosts, and I am extremely grateful for their assistance and flexibility.

I thank Jon Gain (Texas A&M University, Corpus Christi, Texas), as well as Drs. Marc Frischer and Richard Lee (Skidaway Institute of Oceanography, Georgia) for their contribution of infected blue crab samples, which aided in our understanding of the range of this parasite species.

I thank those individuals that assisted through my Master's program and pushed me to continue my education, including Dr. Robert Fleischer, Dr. Ted Schultz, Dr. Sean Brady, Dr. Nancy Zeller, and Faridah Dahlan. I learned an immense amount of information about troubleshooting PCR assays and stepping outside my comfort zone. Thank you for their continued support and encouragement.

I thank all of my friends, especially Aura Paige, Courtney Elkins, Danielle Palmer, and Kelli Reddy. I would not have gotten through this project without their love and support.

I extend a special and heartfelt thank you to my family, especially my parents and siblings, for loving and supporting me throughout my academic career, even when I had to completely ignore everyone to write grants, papers, and this dissertation. I would not be the person I am today without them.

Lastly, I extend warm and loving thank you to my husband, Christopher; our loveable Labrador, Jade; and our clamorous cat, Gina. Thank you for your love, support, laughter, and for never letting me take myself too seriously.

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ABSTRACT

Economically viable fisheries for the American blue crab, *Callinectes sapidus*, occur in the USA from Delaware Bay to the Gulf of Mexico. Unfortunately, a number of factors, including overexploitation, degraded habitats, and disease, have contributed to the decline of blue crab populations in the USA. In April 2008, the Secretary of Commerce declared the blue crab fishery in the Chesapeake Bay a "commercial fishery disaster." One factor potentially contributing to the decline of blue crab populations in high salinity waters is infection with *Hematodinium* sp., a parasitic dinoflagellate, which can cause significant mortalities in blue crab populations.

Since the first report of this parasite infecting blue crabs in 1975, few studies have used molecular techniques to examine life history characteristics, population structure, geographic range, and host specificity of the species of *Hematodinium* that infects this host. To examine host specificity, a variety of crustaceans were collected from the Delmarva Peninsula, a region that has high prevalence of *Hematodinium* sp. in blue crabs. Sequence data obtained from the first internal transcribed spacer (ITS1) region of the ribosomal RNA (rRNA) gene complex from *Hematodinium* sp. in blue crabs and potential alternate hosts was used to determine that this species of *Hematodinium* is a host generalist, capable of infecting many decapods and possibly amphipods along the Delmarva Peninsula. Additional sequence data obtained from *Hematodinium* sp. from infected blue crabs collected from Georgia, the Gulf Coast of Florida, and Texas showed that one species of *Hematodinium*, the same species found in Virginia, infected blue crabs in all of these locations.

Due to the lack of variation observed in the ITS1 region sequences, an enriched microsatellite library was developed using a non-clonal primary culture of *Hematodinium* sp. from an infected blue crab. From this library, eleven microsatellite markers were identified and optimized for *Hematodinium* sp. to assess intra-specific variation. Eight microsatellite markers were polymorphic and these were used to genotype *Hematodinium* sp. from infected blue crabs from six locations along the Delmarva Peninsula. Allele frequencies from these microsatellite markers, along with analyses of the multi-locus genotypes recovered from single genotype infections, indicated that the Delmarva Peninsula contains a single population of this parasite. Microsatellite data also indicate that this parasite is haploid in the hemolymph of the blue crab and that multiple infections are common. The observed genotypic variation was extremely high, indicating that sexual reproduction occurs at some point in the life cycle of this parasite, though the exact life history stage that undergoes sexual reproduction is unknown. This is the first report for evidence of ploidy, multiple infections, population structure, and sexual reproduction for this species of *Hematodinium*.

Molecular genetic studies of *Hematodinium* sp., a parasitic dinoflagellate of the blue crab, *Callinectes sapidus*

INTRODUCTION

The Host: *Callinectes sapidus*

Life cycle and ecology

In Chesapeake Bay, female blue crabs hatch their eggs at the mouth of the estuary and the zoeal larvae develop off the coast in the high salinity waters of the mid-Atlantic Bight (Epifanio 2007). The zoeae molt at least four times to become megalopae, which re-enter the estuary via tidal selective transport (Epifanio 2007). The megalopae metamorphose into juveniles, and colonize seagrass beds or other protective habitats (Lipcius et al. 2007, Hines 2007). The juveniles continue to grow and molt into adults, moving to various habitats, such as shallow mud and sand bottom habitats, deep channels, and saltmarshes, as they increase in size. Blue crabs can undergo extensive migrations during this period (>200km), reaching the low salinity waters of the upper Chesapeake Bay (Van Engel 1958, Hines et al. 1987, Hines 2007). As adults, males will mate multiple times while females generally mate only once (Jivoff et al. 2007). Inseminated females then migrate to the high salinity waters near the mouth of Chesapeake Bay where they oviposit and release their eggs, while males tend to remain in the less saline waters.

The blue crab diet varies with ontogenetic stage and prey availability. In general, the preferred food item for both juvenile and adult blue crabs is bivalves (Laughlin 1982, Alexander 1986, Mansour 1992); however, the number and kind of bivalve consumed depends on the age and location of the blue crab as well as on prey abundance and diversity (Hines et al. 1990, Eggleston 1990, Mansour 1992, Birch 2000). Blue crabs are opportunistic feeders and will expand their diet to include other prey items if their preferred food source is scarce (Laughlin 1982, Mansour 1992, Birch 2000). Other prey include crabs (e. g. blue crabs and various mud crab species), amphipods, worms, fish,

shrimp, snails, plants, and detritus (Laughlin 1982, Mansour 1992). In general, larger adult blue crabs have a less varied diet and engage in cannibalism more frequently than smaller juvenile blue crabs (Mansour 1992). In some higher salinity areas of Chesapeake Bay, conspecifics can account for ~ 25% of the adult blue crab diet (Mansour 1992).

The blue crab fishery

The blue crab fishery is historically important in the Chesapeake Bay. From 1989 to 1993, average annual blue crab landings from the Atlantic and Gulf coasts exceeded 213 million pounds with a dockside value of US$137 million in 1994 (Johnson et al. 1998). In the Chesapeake Bay alone, the blue crab fishery had an average dockside value of US$53.1 million per annum between 1990-1994 (Rugolo et al. 1998). During the 1990s, the blue crab fishery in the Gulf of Mexico accounted for 29% of the total U.S. catch (Guillory et al. 1998). More recently, there have been major declines in many blue crab fisheries. In 2007, the fishery in the Chesapeake Bay had a total harvest of 43.5 million pounds, which was the lowest on record since 1945 (Chesapeake Bay Advisory Report 2008). Along coastal Georgia, blue crab landings have dropped to 1.8 million pounds, well below the 45-year average of 3.9 million pounds (Lee and Frischer 2004). Overexploitation, pollution, summer hypoxia, degraded nursery habitat, and disease are all problems the blue crab faces and, as such, are all possible factors affecting the decline of the blue crab fisheries (Kennedy and Cronin 2007).

In April 2008, the Virginia Marine Resources Commission instituted a winter dredge moratorium for Chesapeake Bay waters and reduced the duration of the fall crab pot season (http://www.mrc.virginia.gov/). On September 23, 2008, the Department of Commerce Secretary Carlos M. Gutierrez declared the Chesapeake Bay blue crab fishery a "commercial fishery disaster." In 2009, the Chesapeake Bay Blue Crab Advisory Committee reported a 70% increase in the adult blue crab population when compared to 2008 (Chesapeake Bay Advisory Report 2009). Most of these adult crabs were likely those that were not fished with the implementation of the moratorium on winter dredging. Unfortunately, the same document showed that blue crab recruitment, the number of young blue crabs entering the Chesapeake Bay, had only slightly increased and still remained well below the target numbers. The total harvest in the Chesapeake Bay in 2008

was estimated at 48.6 million pounds. The Chesapeake Bay Blue Crab Advisory Committee again reported an increase (41%) in the number of adult blue crabs in 2010 compared to 2009 (Chesapeake Bay Advisory Report 2010). The increase in adult blue crabs was again attributed to the retention of crabs not fished due to the continued moratorium on winter dredging. They also reported that blue crab recruitment had doubled since 2009. The estimated 2009 harvest for the Chesapeake Bay was 53.9 million pounds. Though these results are encouraging, the Chesapeake Bay advisory committee elected to maintain the conservation efforts over the next few years.

The parasite: *Hematodinium* sp.

Approximately 7% of dinoflagellates are parasitic (Drebes 1984) and these species belong to four orders: Phytodiniales, Gymnodiniales, Blastodiniales, and Syndiniales (Coats 1999). The parasitic dinoflagellates of marine crustaceans are within the Blastodiniales and Syndiniales (for complete review see Shields 1994). *Hematodinium* spp. are in the Syndinidae (Chatton and Poisson 1931), a family of parasitic dinoflagellates that reside in the host hemocoelom. Currently, there are only two described species of *Hematodinium*: the type species, *Hematodinium perezi*, described from *Carcinus maenus* and *Liocarcinus depurator* from France (Chatton and Poisson 1931) and *Hematodinium australis* described from *Portunus pelagicus* from Australia (Hudson and Shields 1994). Newman and Johnson (1975) were the first to report *Hematodinium* sp. infections in blue crabs from the USA. Based on histological data, they stated that the parasite found in the blue crab matched many of the characteristics of *H. perezi*, the type species described by Chatton and Poisson (1931); however, recent molecular evidence indicates that *Hematodinium* sp. from blue crabs is distinct from the type species (Small et al., in review). Unfortunately, the ability to distinguish between different species of *Hematodinium* has been hindered by a lack of distinguishing morphological characteristics as well as the development of only a few molecular markers for these parasites (Stentiford and Shields 2005, Small et al., in review).

Hematodinium sp. has distinct physiological effects on its blue crab host. The main signs of infection are lethargy, milky white hemolymph that will not clot, and low hemocyte numbers (Newman and Johnson 1975, Shields and Squyars 2000). Blue crabs

with *Hematodinium* sp. infections die from physiological disruption due to nutrient depletion caused by decrease in glucose levels, loss of clotting ability due to decline in hyalinocytes, respiratory dysfunction due to declines in hemocyanin levels, starvation due to increased lethargy, and lethal secondary infections (Shields et al. 2003).

The vast majority of blue crabs infected with *Hematodinium* sp. only survive for a short period of time. In one study, all naturally-infected crabs died within 35 days of being caught and all experimentally infected crabs died 55 days post-inoculation (Messick and Shields 2000). Shields and Squyars (2000) observed an 86% mortality rate in experimentally infected blue crabs held for 40 days, with infected crabs having a 7-8 fold increased chance of dying compared to uninfected crabs. They also reported that four blue crabs appeared to be resistant to infection. Based on their data, Shields and Squyars (2000) predicted three possible outcomes for blue crabs infected with *Hematodinium* sp.: (1) acute infection leading to death within 40 days, (2) chronic infection leading to survival for up to 90 days with infections leading to dinospore formation, or (3) resistant to infection.

Environmental and host factors contribute to *Hematodinium* sp. infection in blue crabs. The parasite is most prevalent during fall months (August-November; Newman and Johnson 1975, Messick 1994, Messick and Shields 2000). In blue crabs, *Hematodinium* sp. has only been detected in salinities greater than 10ppt (Newman and Johnson 1975, Messick et al. 1999, Messick and Shields 2000) and temperatures higher than 3°C (Messick et al. 1999, Messick and Shields 2000). All studies showed increased prevalence with higher salinities and temperatures. Infection is more common in juveniles (5-89 mm CW) and postmolt crabs (Messick 1994). In a more recent study, juveniles (3-30 mm CW) were shown to have higher prevalence compared to other age classes and prevalence was significantly higher in immature compared to mature crabs during the fall months in the coastal bays of Maryland (Messick and Shields 2000).

Hematodinium sp. may be capable of overwintering in blue crabs, potentially explaining how the parasite is maintained in the blue crab population. Messick et al. (1999) found seven crabs that appeared to be uninfected at first examination, but when the water temperature was increased, the crabs developed overt infections. The researchers suggested that latent infections could have been activated upon the increase in

temperature. Similarly, Frischer et al. (2006) used PCR data to show that latent or low-level infections can survive in blue crabs in low salinity waters. However, no published studies have directly examined the crab tissues for overt infections in winter months.

The transmission modes of all *Hematodinium* species are currently unknown, but there are several hypotheses. One hypothesis is that molting is important in transmission. Higher prevalence was found in medium and small-sized *Nephrops norvegicus* and the timing of molting was correlated with higher prevalence, indicating that molting may be involved in transmission in this system (Field et al. 1998, Stentiford et al. 2001). Similarly, there is a positive association between prevalence of a species of *Hematodinium* and molting in *Chionoecetes opilio* (Shields et al. 2005, Shields et al. 2007). Higher prevalence of *Hematodinium* sp. was found in juvenile blue crabs (Messick 1994), suggesting that molting may play a role in the transmission of *Hematodinium* sp. in blue crabs. There is PCR evidence of a free-living stage in the water column, presumably dinospores (Meyer et al. 1987, Frischer et al. 2006, Li et al. 2010). It is possible that dinospores can penetrate the crab exoskeleton, either after molting or through injury, or enter the crab during imbibition and cause infection.

Another possible mode of transmission for *Hematodinium* sp. is ingestion of the parasite, either through cannibalism or consumption of other infected prey. Sheppard et al. (2003) reported anecdotal data that blue crabs became infected with *Hematodinium* sp. after consuming infected crab tissue. Walker et al. (2009) reported that 73% (n=11 of 15) of the blue crabs in their study showed evidence of *Hematodinium* sp. infection only 48 hours after consuming infected blue crab tissue, providing further evidence for this mode of transmission in blue crabs. However, a more recent study found no development of *Hematodinium* sp. infections in either blue crabs or gammarid amphipods after ingestion of infected blue crab tissue (Li et al., in review). The consumption or ingestion of the transmissive stage of the parasite has been proposed as a mode of infection for snow crabs feeding on bivalves that have concentrated the free-living stage of the parasite, but this remains conjecture (Shields et al. 2005). This scenario is also possible in blue crabs given that a large portion of the blue crab diet is composed of bivalves (Mansour 1992, Laughlin 1982). Additionally, *Hematodinium* sp. infections have been reported in a variety of other blue crab prey including amphipods (Johnson 1986) and xanthid crabs

(Messick and Shields 2000, Sheppard et al. 2003), so consumption of these infected prey could be a source of infection for blue crabs. However, the variety of potential hosts is also suggestive of an infectious dinospore as opposed to trophic transfer of infections.

Sexual transmission is a third possible mode of transmission. A *Hematodinium* species was found in the seminal fluid surrounding the spermatophores in the vas deferens of *Chionoecetes bairdi* (Meyers et al. 1996). However, sexual transmission seems unlikely as a major mode of transmission given the higher prevalence in juveniles.

Sequence data for understanding phytoplankton ecology and evolution

As there are very few morphological markers to distinguish different species/strains of *Hematodinium*, molecular markers should be useful for uncovering information about the ecology and evolution of this parasite. Molecular techniques have been increasingly used to address questions regarding the ecology and evolution of various phytoplankton species, including various species of dinoflagellates. Molecular studies have compared toxicity and genotypes of harmful algal species (Touzet et al. 2007), examined geographic origin of a species (Masseret et al. 2009), assessed population structure (Nagai et al. 2007a, 2009), assessed clonal versus sexual reproduction (Nagai et al. 2007a, Masseret et al. 2009), and determined whether or not hybridization events between species have occurred (Kooistra et al. 2001). Many studies on geographic variation, population structure, and identification of different species of phytoplankton have used highly variable DNA sequence data, such as the first internal transcribed spacer region (ITS1) of the ribosomal RNA (rRNA) gene complex.

The ITS1 region is a commonly used molecular tool because it has multiple copies in the genomes of most organisms and is highly variable. The ITS1-5.8S-ITS2 (ITS) region is frequently used for determining "species" designations in dinoflagellates (Litaker et al. 2007). The amount of genetic distance in the ITS region between free-living dinoflagellate species is typically greater than 4%, which has been used as the standard of genetic distance to differentiate between species in this group (Litaker et al. 2007). While the majority of studies have used ITS region sequence data, this region is problematic because it has intra-cellular variation (Brown et al. 2004), with the potential to have many different variants of the same sequence in a single cell. This means that, in

order to properly assess the variation in the ITS region of a particular organism, many clones from a sample have to be sequenced. Unfortunately, this process greatly increases the cost of processing samples. Prior to this dissertation, sequence data of the rRNA gene complex was the only molecular marker available for distinguishing among and between species of *Hematodinium*.

Currently, the relationships among the *Hematodinium* species from different hosts and from the same host from different locations are still under investigation. Molecular data show a >98% similarity with minor length variation in the ITS1 region of a species of *Hematodinium* from four hosts (*N. norvegicus*, *Cancer pagurus*, *Pagurus bernhardus*, and *C. opilio*), suggesting that these hosts are all infected with the same species of *Hematodinium* (Small et al. 2007a). A high sequence similarity (97-100%) was found between the ITS1 region sequence of a species of *Hematodinium* from *Portunus trituberculatus* and previously published ITS1 region sequences of a species of *Hematodinium* from both *P. trituberculatus* and *L. depurator* (Small et al. 2008), indicating that these hosts are probably infected with the same or a closely related species of *Hematodinium.* Using small subunit (SSU) rRNA gene sequence data, Jensen et al. (2010) found that one species of *Hematodinium* appears to infect multiple crustaceans in the boreal region including *C. bairdi, N. norvegicus, C. opilio, Chionoecetes angulatus, Hyas coarctatus, Lithodes couesi,* and *Paralithodes camchaticus.* A similar study by Hamilton et al. (2010) used sequence data from a portion of the ITS1 region to determine that *Hematodinium* species infecting crustaceans in the North Pacific and North Atlantic consisted of three distinct clades, with one clade consisting of all host species, one clade consisting of only langoustines, and one clade consisting of crab species. These studies indicate that all *Hematodinium* species examined to date appear to be capable of infecting multiple hosts and seem to occur over large geographic regions.

Current molecular evidence indicates that the *Hematodinium* sp. that infects blue crabs is distinct from the *Hematodinium* species that infects crustaceans in the boreal regions. Hudson and Adlard (1996) stated the *Hematodinium* species collected from four crustacean hosts (*C. sapidus*, *C. bairdi*, *C. opilio*, and *N. norvegicus*) were all in the genus *Hematodinium* based on the similarity within 237 bp from the SSU rRNA gene. Based on the sequence differences observed in the ITS1 region among the *Hematodinium*

species from these hosts, they proposed this genus was comprised of three distinct species of *Hematodinium*. First, they proposed that *H. cf. perezi* from the blue crab was actually a distinct species from *H. perezi*, which, as mentioned above, was described from *C. maenus* and *L. depurator* in France (Chatton and Poisson 1931). Their results also suggested that the *Hematodinium* sp. found in *N. norvegicus* was a second distinct species and that the *Hematodinium* sp. found in both *C. bairdi* and *C. opilio* was a third distinct species. Unfortunately, when other researchers attempted to confirm the findings of Hudson and Adlard (1996), they found that the sequence from *Hematodinium*-infected blue crabs from Hudson and Adlard (1996) was only 82% similar to additional sequences also obtained from *Hematodinium*-infected blue crabs, bringing the original data and its conclusions into question (Small et al. 2007b). Using SSU rRNA gene sequence data, Jensen et al. (2010) found that the *Hematodinium* sp. from *C. sapidus* and the *Hematodinium* sp. from *L. depurator* formed a single clade, while the *Hematodinium* sp. that infects boreal crustaceans formed a separate clade. Small et al. (in review) used ITS1 region sequence data to determine that the species of *Hematodinium* infecting the blue crab and the species of *Hematodinium* found in southern China are closely related to the type species, *H. perezi*, but that all three appear to be distinct genotypes. Based on this information, Small et al. (in review) has suggested that the species of *Hematodinium* that infects the blue crab be named, *Hematodinium perezi* genotype III.

Multiple studies from the United Kingdom (UK) have shown that a single species of *Hematodinium* occurs over a wide geographic area. Small (2004) found that ITS1 region sequences from a species of *Hematodinium* found in *N. norvegicus* individuals from various locations were 93-99% similar. Hamilton et al. (2007) used sequences of a 1,100 bp fragment containing of the SSU rRNA gene and the complete ITS region from the species of *Hematodinium* that infects *N. norvegicus* and *C. maenus* to confirm that the same parasite species was found in two different locations in Scotland. In comparison to the work done in the United Kingdom, less molecular work has been done to characterize the *Hematodinium* species found throughout the blue crab range. When a 1,682 bp portion of the SSU rRNA gene of *Hematodinium* sp. collected from four infected blue crabs from Virginia and Georgia were compared, the sequences were identical (Small et al. 2007b); however, the SSU rRNA gene is highly conserved, and a more variable

region, such as the ITS1 region, could be more useful for differentiating populations of this parasite. A restriction fragment length polymorphism assay was also used to show the *Hematodinium* sp. found in blue crabs from Virginia and Georgia had identical profiles and could be distinguished from the species of *Hematodinium* found in both *L. depurator* and *P. trituberculatus* (Small et al. 2007b). Thus, prior to the current study, the only data used to determine the number of *Hematodinium* species in blue crab populations consisted of a few sequences of a highly conserved gene from a few infected blue crabs from only two locations and a few ITS1 region sequences from a handful of infected blue crabs from Virginia.

Host Specificity of *Hematodinium* spp.

The majority of the research on *Hematodinium* spp. has been conducted on infected commercially important host species. Species of *Hematodinium* have been found to infect >30 different crustaceans (for review, see Stentiford and Shields 2005). Most studies reported finding the parasite in the host via microscopy or histology, with a greater use of molecular tools utilized in the last few years for identifying and detecting infections in various host species (Small et al. 2007a, 2007b, Hamilton et al. 2009, 2010, Eigemann et al. 2010, Chualáin and Robinson 2011).

Little molecular work has been done to determine the host specificity of the *Hematodinium* sp. that infects blue crabs. By comparing partial sequences of the SSU rRNA gene, Sheppard et al. (2003) reported that the *Hematodinium* sp. in the spider crab, *Libinia emarginata*, and the stone crab, *Menippe mercenaria*, is the same or very similar to that found in blue crabs. However, as stated above, the SSU rRNA gene is highly conserved, and a more variable region, such as the ITS1 region, would have provided more confidence in the conclusion that they were the same species of *Hematodinium*. Messick and Shields (2000) reported *Hematodinium* sp. infections in *C. maenus* (8% from MD coastal bays), *C. similis* (0.7% from Georgia), and three species of mud crabs (8%). Infections of a species of *Hematodinium* also have been reported from gammarid amphipods (Johnson 1986, Messick and Shields 2000). However, it is not clear if any of these crustaceans were infected with the same species of *Hematodinium* found in *C. sapidus* as infections were determined through histological examination of infected

tissues or microscopic examination of hemolymph smears. Molecular investigations, specifically comparison of ITS1 region sequence data, should help to resolve this question.

Population genetics of microparasites, marine parasites, and dinoflagellates

Population genetic tools can be used to determine the origin of infection, compare the virulence of various isolates, and determine the amount/rate of sexual reproduction (Cacciò et al. 2001). Most population genetic studies have been conducted on microparasites that infect humans (*Plasmodium falciparum*, Razakandrainibe et al. 2005; *Cryptosporidium parvum*, Feng et al. 2000; Cacciò et al. 2001; *Toxoplasma gondii,* Ajzenberg et al. 2002). Microsatellites, a commonly used tool for population genetics, are simple sequence repeats that are usually found in noncoding regions and have high mutation rates resulting in numerous alleles within a population (Avise 2004). Due to their high mutation rates, microsatellites can show a much higher variation between populations of the same species than most other molecular markers. Within the last decade, microsatellites have been developed for several dinoflagellates within the genus *Alexandrium*, including *Alexandrium tamarense* (Nagai et al. 2004; Alpermann et al. 2006), *Alexandrium minutum* (Nagai et al. 2006a), *Alexandrium catenella* (Nagai et al. 2006c), and *Alexandrium tamiyavanichii* (Nishitani et al. 2009). Microsatellites have also been developed for other free-living dinoflagellates including *Karenia brevia* (Renshaw et al. 2006), *Cochlodinium polykrikoides* (Nishitani et al. 2007), *Heterosigma akashiwo* (Nagai et al. 2006), and *Heterocapsa circularisquama* (Nagai et al. 2007b) as well as the symbiotic dinoflagellates, *Symbiodinium* spp. (Santos and Coffroth 2003, Andras et al. 2009, Bay et al. 2009, Pettay and Lajeunesse 2009, Pinzón et al. 2011).

Within the last five years, several studies have used microsatellites to assess population structure for free-living dinoflagellates. Nagai et al. (2007a) used nine microsatellite markers to assess population structure of *A. tamarense* collected from ten sites along the Japanese and Korean coasts. They reported that *A. tamarense* samples taken from locations that were geographically distant were genetically similar, which they hypothesized was attributable to the transport of cells via ballast water. Masseret et al. (2009) used 12 microsatellite markers to detect high levels of intra-specific diversity

and differentiation between populations of *A. catenella* from the Mediterranean and Japan that were not detected using sequences from a portion of the ITS region and the large subunit (LSU) rRNA gene. Nagai et al. (2009) used 10 microsatellite markers to determine the presence of three distinct populations of *C. polykrikoides* from Korean and Japanese waters with only low levels of gene flow among the populations. McCauley et al. (2009) reported that LSU rRNA gene and ITS1 region sequences were able to separate *A. minutum* into a "Global" clade and a "Pacific" clade. With the use of 12 microsatellite markers that amplified *A. minutum* from the "Global" clade, the researchers were able to differentiate most of these populations by geographic origin. Alpermann et al. (2009) used 18 microsatellite markers to uncover high levels of intra-specific variation in *A. tamarense,* stating that no microsatellite multi-locus genotype was observed more than once. Lowe et al. (2010) used nine microsatellite markers to determine that *Oxyrrhis marina* had high genotypic diversity with no geographic differentiation across six geographic regions around Great Britain and Ireland.

Microsatellites have also been used to ascertain various aspects of the ecology and evolution of *Symbiodinium* spp., a group of dinoflagellates commonly referred to as zooxanthellae that inhabit the tissues of various coral species. Microsatellites were used to determine that *Symbiodinium* spp., both in culture and from coral tissue, are haploid (Santos and Coffroth 2003). Microsatellites were used to demonstrate that multiple genotypes of *Symbiodinium* sp. are capable of infecting the same coral host (Santos and Coffroth 2003, Howells et al. 2009). Data from six polymorphic microsatellite markers was used to demonstrate low levels of gene flow among *Symbiodinium* type B1/B184 inhabiting *Gorgonia ventalina* from different geographic locations within the Florida Keys (Kirk et al. 2009). They also showed population differentiation within *Symbiodinium* type B1/B184 between deep and shallow sites, indicating strains may have a preference or be selected for under certain light regimes. Thornhill et al. (2009) used three microsatellite loci for *Symbiodinium* type B inhabiting scleractinian corals in the Florida Keys and Bahamian reefs to show that genotypes were restricted to individual reefs and little to no gene flow occurred between reefs. Howells et al. (2009) used four microsatellite loci to demonstrate that *Symbiodinium* type C inhabiting *Sinularia flexibilis* were genetically differentiated with restricted gene flow on the Great Barrier Reef. They

hypothesized that the observed population structure was most likely due to passive movement of *Symbiodinium* type C by currents. Only one study thus far has shown no population structuring of a species of *Symbiodinium* on a large geographic scale. Using two microsatellite loci, Magalon et al. (2006) detected genetic differentiation in the zooxanthellae inhabiting *Pocillopora meandrina* between the Tonga and Society archipelagos in the South Pacific (2000 km); however, there was little to no differentiation observed among populations on a finer scale, such as those separated by10s of kilometers, as was observed in other studies of *Symbiodinium* spp. population structure. The findings of Magalon et al. (2006) indicate *P. meandrina* may have a different mechanism for acquiring symbionts as compared to other hosts listed above, such as maternal inheritance and dispersal with coral larvae.

Significance of Project

The main goals of this study were to determine the following: 1) whether or not the same species of *Hematodinium* that infects blue crabs was capable of infecting other crustaceans that occur with blue crabs; 2) to determine if the same species of *Hematodinium* was infecting blue crabs throughout a large geographic range; 3) to develop and utilize additional molecular markers for the species of *Hematodinium* that infects blue crabs in order to reveal aspects of the ecology and evolution of the parasite that had previously eluded researchers such as ploidy, multiple infections, genotypic variation, and population structure.

This project gives insight into the host specificity of *Hematodinium* sp. from the western mid-Atlantic region, which could have very important ramifications for our understanding of how this organism affects blue crabs. Studies have shown that at least one species of *Hematodinium*, the species that infects the Norway lobster, is a host generalist (Small et al. 2007a, Hamilton et al. 2007, 2009). If this is also true in the blue crab system, then alternate hosts could serve as reservoirs for the parasite, allowing the parasite to remain in the ecosystem regardless of fluctuations in the blue crab population.

This project provides information about the number of *Hematodinium* species infecting blue crabs over a large geographic range. Previously, only a single study has attempted to show that the same species of *Hematodinium* was present in blue crab

populations in both Virginia and Georgia (Small et al. 2007b); however, as mentioned above, no sequence data was obtained to confirm the findings. Thus it was unknown whether other species of *Hematodinium* occurred in other blue crab populations. Previous studies have shown population differentiation of numerous marine species between the Atlantic coast and Gulf of Mexico using mitochondrial DNA (for review see Avise 1992). Determining whether or not this separation occurs in marine parasites has only been attempted for *Perkinsus marinus* (Reece et al. 2001). *Perkinsus marinus* showed distinct differences in allele frequencies between three regions of the USA: the Northeast Atlantic, Southeast Atlantic, and the Gulf of Mexico. This information was useful in determining the historical expansion of this parasite.

This project also provides novel information about the ploidy, population structure, existence of a sexual stage, and information about multiple infections through the use of microsatellite markers developed specifically for the species of *Hematodinium* that infects the blue crab. Microsatellite data was previously used to examine various life history aspects in free-living dinoflagellates and *Symbiodinium* spp. such as population structure (Kirk et al. 2009, Nagai et al. 2007a, 2007b, 2009), genetic diversity over various geographic scales (Magalon et al. 2006, Howells et al. 2009, Masseret et al. 2009, McCauley et al. 2009), life cycle dynamics (Alpermann et al. 2009), ploidy (Santos and Coffroth 2003), and movement of species (Nagai et al. 2009).

Chapter 1: The role of alternate hosts in the ecology and life history of *Hematodinium* sp., a parasitic dinoflagellate of the blue crab

Abstract

Hematodinium sp. infections are relatively common in American blue crab populations in the western Atlantic Ocean. Outbreaks of disease caused by *Hematodinium* sp. in blue crab populations can be extensive and can cause substantial mortalities at high salinities. Several species of crustaceans were examined to determine if the same species of *Hematodinium* that infects *C. sapidus* infects other crustaceans from the same localities. A total of 1,829 crustaceans were collected from the Delmarva Peninsula, Virginia, and examined for the presence of infections over a two-year period. A portion of the first internal transcribed spacer (ITS1) region of the ribosomal RNA (rRNA) gene complex from *Hematodinium* sp. was amplified. Sequences were compared among 35 individual crustaceans putatively infected with the parasite as determined by a microscopic examination and four crustaceans putatively infected based only on PCR analysis. Of the 18 crustacean species examined, five were infected with *Hematodinium* sp. after microscopic examination and PCR analysis, including three new host species, and an additional species was determined to be positive only via PCR analysis. The ITS1 region sequences of *Hematodinium* sp. from the infected crustaceans were highly similar to each other and that reported from *C. sapidus* (>98%). The similarity among the ITS1 region sequences and similarities in the histopathology of infected hosts is evidence that the same species of *Hematodinium* found in *C. sapidus* infects a broad range of crustaceans along the Delmarva Peninsula. These data indicate that the species of *Hematodinium* found in blue crabs on the East Coast of North America is a host generalist, capable of infecting hosts in different families within the Order Decapoda. Additionally, PCR evidence suggests that it may be capable of infecting crustaceans within the Order Amphipoda.

Introduction

The American blue crab, *Callinectes sapidus*, supports regional fisheries along the eastern seaboard and Gulf of Mexico in the USA. Landings for the fisheries averaged 213 million pounds from 1989 - 1993, with a dockside value of US$137 million in 1994 (Johnson et al. 1998). In Chesapeake Bay, the blue crab fishery had an average dockside value of US$53.1 million dollars between 1990 - 1994 (Rugolo et al. 1998); however, in 2007, the total harvest was 43.5 million pounds, which was the lowest on record since 1945 (Chesapeake Bay Advisory Report 2008). Blue crabs are subject to a number of factors that have potentially contributed to their decline in the Chesapeake Bay including overexploitation, pollution, summer hypoxia, degraded nursery habitat, and disease (Kennedy and Cronin 2007).

One possible reason for the decline of *C. sapidus* populations in high salinity areas of the Chesapeake Bay region may be the presence of a parasitic dinoflagellate, *Hematodinium* sp., which can reach extremely high prevalence in coastal embayments. Newman and Johnson (1975) reported a prevalence of *Hematodinium* sp. in blue crabs of 30% from Florida, while Messick (1994) observed prevalence as high as 90% in samples containing juvenile, post-juvenile, and adult blue crabs from the coastal bays of Maryland and Virginia. Messick and Shields (2000) reported a prevalence of >30% from Florida, South Carolina, Delaware, and Virginia and a prevalence of >50% has been reported from Georgia (Gruebl et al. 2002). The majority of blue crabs naturally infected with *Hematodinium* sp. do not survive for more than 35 days (Messick & Shields 2000), while those experimentally infected live only 40-55 days post-inoculation (Shields and Squyars 2000), indicating that areas with a high prevalence of *Hematodinium* sp. likely suffer significant crab mortality due to this disease.

Infections of *Hematodinium* spp. have been reported from >30 different crustaceans (see Stentiford and Shields 2005). Most of these hosts have been diagnosed using histological analysis of tissues. Comparatively, relatively few studies have used molecular techniques, specifically sequence data, to determine whether or not the same species of *Hematodinium* infects different crustacean species in the same geographic regions. Molecular evidence indicates that a species of *Hematodinium* infects multiple crustacean species throughout the waters of the United Kingdom (UK). Based on ITS1

region sequences, Small et al. (2007a) concluded that one species of parasite infects the Norway lobster, *Nephrops norvegicus*, the edible crab, *Cancer pagurus*, and a hermit crab, *Pagurus bernhardus*, from the UK as well as the snow crab, *Chioneocetes opilio*, from Newfoundland. Similarly, sequence data from the a portion of the rRNA gene complex (SSU-ITS1-5.8s-ITS2) showed that the same species of *Hematodinium* also infects *N. norvegicus*, *C. pagurus*, *Carcinus maenas*, *Liocarcinus depurator*, *Munida rugosa*, *Necora puber*, *P. bernhardus*, and *Pagurus prideaux* from Scotland (Hamilton et al. 2007, 2009). Using small subunit (SSU) rRNA gene sequences of the parasite from eight different host species obtained from three different locations, Jensen et al. (2010) proposed that a single species of *Hematodinium* is capable of infecting multiple crustaceans in the boreal region.

Compared to the research in the UK, less molecular data has been generated to examine the host specificity of *Hematodinium* sp. along the eastern seaboard of the USA. Johnson (1986) reported infections of a species of *Hematodinium* in 13 species of gammarid amphipods collected from the continental shelf from Massachusetts to Maryland. MacLean and Ruddell (1978) reported infections of a species of *Hematodinium* in *Cancer irroratus*, *Cancer borealis*, and *Ovalipes ocellatus* from the coastal waters of New York and New Jersey. Messick and Shields (2000) reported infections of a species of *Hematodinium* in *C. maenus* (8% prevalence from MD coastal bays), *Callinectes similis* (0.7% from Georgia), and three species of mud crabs (8%). They also reported finding infections of a species of *Hematodinium* (8.5%) in one sample of gammarid amphipods from a coastal bay in Maryland. However, none of these studies used molecular work to confirm that these crustaceans were infected with the same species of *Hematodinium* found in *C. sapidus*. Sheppard et al. (2003) stated that the *Hematodinium* sp. in the spider crab, *Libinia emarginata*, and the stone crab, *Menippe mercenaria*, is the same or very similar to that found in blue crabs; however, they analyzed sequences of the highly conserved SSU rRNA gene. A more variable region, such as the ITS1 region, could demonstrate more conclusively that these are the same species of *Hematodinium*.

The purpose of this study was to determine if the same species of *Hematodinium* that infects blue crabs in the Chesapeake Bay region infects other crustaceans from the

same region. To explore this, various crustacean species from multiple sites along the Delmarva Peninsula were collected, with infection status determined via microscopy and diagnostic PCR. The ITS1 region sequences of *Hematodinium* sp. collected from these individuals were compared to ITS1 region sequences of *Hematodinium* sp. from *C. sapidus* from the same locations at the same times as well as to sequences from *Hematodinium* sp. from *C. sapidus* previously deposited in Genbank. The ITS region was used because it is a commonly used molecular marker for distinguishing between species of free-living dinoflagellates (Litaker et al. 2007) and other shellfish parasites (Dungan et al. 2002, Moss et al. 2008). Additionally, histological sections from several of the infected host species were compared to those from infected blue crabs to evaluate whether pathological differences exist between the different hosts. Finally, pilot inoculation trials were conducted to assess the infectivity of the *Hematodinium* sp. from blue crabs in a number of alternate host species. Whether these alternate hosts likely represent reservoir or incidental hosts for this parasite is discussed as well as the potential impacts both of these roles may have on infection dynamics in blue crab populations.

Materials and Methods

Specimen collection

Crustaceans were collected from three primary sites (Chincoteague Bay, Wachapreague Creek, and Fisherman Island) along the Delmarva Peninsula, Virginia in 2008 (May, September-December) and 2009 (May-December, see Figure 1.1). Four additional sites (Pungoteague Creek, Cherrystone Creek, Oyster Creek, and Metompkin Bay) were sampled in 2008. In addition to blue crabs, the following species were collected: mud crabs (*Eurypanopeus depressus*, *Panopeus herbstii*, *Dyspanopeus sayi*, *Hexapanopeus angustifrons*), spider crabs (*Libinia emarginata*, *Libinia dubia*), *Callinectes similis*, *Cancer* spp. (*Cancer irroratus*, *Cancer borealis*), hermit crabs (*Pagurus longicarpus*, *Pagurus pollicaris*, *Clibanarius vittatus*), *Hemigrapsus sanguineus, Portunus spinimanus*, *Portunus gibbesii*, *Ovalipes ocellatus*, *Palaemonetes* spp. (grass shrimp) and caprellid amphipods (*Caprella equilibra*, *Caprella geometrica*). Species identifications were confirmed using Williams (1984) as the primary source as

well as Weiss (1995), McCain (1965), Lippson and Lippson (1997), and internet sources (www.usgs.gov).

Most large crustaceans (spider crabs, portunid crabs, *Cancer* spp., and hermit crabs) were caught in crab pots with blue crabs, though some were also caught while trawling or dredging in the Wachapreague Inlet system. Mud crabs were collected from small metal or plastic cages filled with oyster shell, which were deployed in the same general areas as crab pots. Occasionally additional mud and shore crabs were collected from the oyster flats at Wachapreague Creek by hand or from trawling or dredging within the Wachapreague Inlet system. Grass shrimp (*Palaemonetes* spp.) were caught in mud crab cages from Wachapreague Creek in December 2008. Caprellid amphipods were collected from algae attached to the buoy lines of the mud crab cages.

Infection status

For all decapod crustaceans >10mm carapace width (CW; n=1,344), hemolymph was drawn from the arthrodial membranes with a 27 ga. needle. Hemolymph subsamples were mixed 1:1 with 0.3% Neutral red in crustacean saline (modified from Appleton and Vickerman 1998, see Shields & Squyars 2000) and smears were examined with a light microscope at 100x and 400x magnification for uptake of the dye into the parasite vacuoles (see Stentiford and Shields 2005). As it was difficult to draw hemolymph from the small mud crabs, those <10mm CW (n=269) were dissected and squash preparations of small pieces of gill were stained and examined as above. If *Hematodinium* sp. was viewed on the hemolymph smears, 500µl of hemolymph was obtained and stored in 500µl of 95% ethanol, which was stored at -20°C. If *Hematodinium* sp. was viewed on smears from the <10mm CW crustaceans, whole animals were first frozen, then transferred into 95% ethanol and saved for later molecular analysis. Grass shrimp, *Palaemonetes* spp., were examined solely by histological examination; their bodies were preserved whole in Bouin's solution (Fisher Scientific, Pittsburgh, Pennsylvannia). Caprellid amphipods were examined either by histological examination or PCR analysis. Those kept for histological analysis were preserved whole in neutral buffered formalin, while those kept for PCR analysis were preserved whole in 95% ethanol.

Due to potential differences in the morphologies and life history stages of the parasite, if an organism viewed on the microscope exhibited Neutral red uptake characteristic of *Hematodinium* sp., the host was saved for further processing. When possible, infections from microscopic diagnosis were confirmed as *Hematodinium* sp. by PCR. As recommended by Burreson (2008), an organism was considered infected with the parasite if the parasite was observed in hemolymph smears, tissues smears, or after histological examination and the identity of the parasite was confirmed after sequencing the parasite DNA. An organism was not considered infected if the parasite was only detected via PCR.

DNA extraction and PCR

For hemolymph samples, 200μL of hemolymph in 95% ethanol suspension was centrifuged at 1,500Xg for 1 minute and excess ethanol was removed. The sample was then allowed to sit for ~30 minutes to allow for evaporation of residual ethanol. For whole crabs, several tissues (gills, heart, muscle or percentage of the whole, depending on size) were macerated using a sterile scalpel blade and blotted to remove residual ethanol. Caprellid amphipods were macerated whole with a sterile scalpel blade and blotted to remove residual ethanol.

Following an overnight digestion with proteinase K, DNA was extracted from all samples using a Qiagen Tissue and Blood kit (Qiagen, Valencia, California) following the manufacturer's instructions for animal tissues. All DNA samples were eluted in 100μL of buffer, which was passed over the column twice, and incubated on the column for 5 minutes each time to increase DNA yield (Audemard et al. 2004). All extractions completed within the same day included a blank column extraction, which, when subjected to PCR analysis alongside samples, served as a control for contamination during DNA extraction. The original DNA elutions were stored at -20°C, while separate aliquots (~20μL), which were made to avoid contamination of original DNA elutions, were stored at 4°C. Each DNA sample was quantified using a NanoDrop 2000 (Thermo Scientific, West Palm Beach, Florida).

A general metazoan PCR primer set, nSSU A (5'-AACCTGGRTTGATCCTGATCCTGCCAGT-3') and nSSU B (5'-

GATCCTTCCGCAGGTTCACCTAC-3') (modified from Medlin 1988, see Moss et al. 2007), which targets the SSU rRNA gene, was used to determine if high molecular weight DNA was present in the extracted DNAs as this PCR assay amplifies a large DNA fragment. PCR reactions consisted of 1X PCR Buffer (Invitrogen), 1.5mM $MgCl_2$, 0.2mM dNTP, 1μM each primer, 0.4 mg/mL BSA, 0.025μL *Taq* polymerase (Invitrogen) and water to a final volume of 25 or 10μL. Thermocycling was carried out using a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, Massachusetts) with an initial denaturation of 94°C for 4 minutes followed by 40 cycles of 94°C for 30 seconds, 45°C for 30 seconds, 72°C for 2 minutes, and a final extension of 72°C for 5 minutes. An aliquot of the PCR product (5μL) was electrophoresed on an agarose gel (2% w/v) and visualized under UV light after ethidium bromide staining. The expected fragment size was ~1700 bp.

For all samples collected during both years, a second PCR assay targeting the more specific ITS1 region of the rRNA gene complex of the parasite was used (Small et al. 2007b) to verify the presence of *Hematodinium* sp. These PCR reactions consisted of 1X PCR Buffer (Invitrogen), 1.5mM $MgCl_2$, 0.1mM dNTP, 0.5μM HITS1F (5'-CATTCACCGTGAACCTTAGCC-3'), 0.5μM HITS1R (5'-CTAGTCATACGTTTGAAGAAAGCC-3'), 1μL *Taq* Polymerase (Invitrogen) and water to a final volume of 20 μL or 10μL. Thermocycling was carried out with an initial denaturation of 94°C for 5 minutes followed by 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 90 seconds, and a final extension of 72°C for 5 minutes. The PCR product (5μL) was electrophoresed on a 2% w/v agarose gel and visualized under UV light after ethidium bromide staining. The expected fragment was 302 bp in size. Due to non-specific amplification observed after the caprellid samples were assayed, the ITS1 region PCR assay for the caprellids was further optimized with a final concentration of 1mM $MgCl_2$, an annealing temperature of 58°C, and a total of 40 cycles.

An assay targeting the SSU rRNA gene of *Hematodinium* sp. designed by Gruebl et al. (2002) was used to amplify *Hematodinium* sp. DNA from samples collected in 2008 and all the caprellid amphipods. This assay was used to determine the presence of other species of *Hematodinium*, as this assay targets the SSU rRNA gene which is more

conserved than the variable ITS1 region, increasing the potential for detection of other species if present. The assay was modified so that each reaction contained 1X PCR Buffer (Invitrogen), 1.5mM MgCl2, 0.1mM dNTPs, 0.5μM Hemat-F-1487 (5'-CCTGGCTCGATAGAGTTG-3'), 0.5μM Hemat-R-1654 (5'-GGCTGCCGTCCGAATTATTCAC-3'), 1mg/mL BSA, 1μL *Taq* Polymerase (Invitrogen) and water to a final volume of 20 μL. As above, thermocycling was carried out using a PTC-200 Peltier Thermal Cycler with an initial denaturation of 94°C for 10 minutes followed by 35 cycles of 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 1 minute, and a final extension of 72°C for 10 minutes. An aliquot of PCR product (5μL) was electrophoresed on a 2% (w/v) agarose gel and visualized under UV light after ethidium bromide staining. The expected fragment was 187 bp in size. Due to non-specific amplification in the caprellid samples, the SSU rRNA gene PCR assay for the amphipods was further optimized with a final concentration of 1mM $MgCl_2$, an annealing temperature of 58°C, and a total of 40 cycles. A negative control, consisting of no DNA, was included in all of the diagnostic PCR assays and a positive control, consisting of either DNA extracted from *Hematodinium* sp. culture material or a sample that had reliably amplified in the past, was included in all diagnostic PCR assays.

Cloning and sequencing of parasite DNA

Sequence data was used to determine if the same species of *Hematodinium* infected blue crabs and all other crustaceans. The PCR products from the diagnostic assay for the ITS1 region were cloned and sequenced as described in Moss et al. (2008). Briefly, samples were cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, California) using half of the total volume of cells and vector recommended by the manufacturer's instructions. Plasmid DNA was extracted from individual colonies following a boil-prep method and screened using M13F/R vector primers. An aliquot of the PCR product (5μL) was visualized under UV light on a 2% (w/v) agarose gel after ethidium bromide staining. Fragments of appropriate size (~500 bp) were purified with exonuclease I (EXO) and shrimp alkaline phosphatase (SAP) (USB Corporation, Cleveland, OH). The purified PCR product was used as the template for sequencing with the M13F/R primers and the Big Dye Terminator kit (Applied Biosystems, Norwalk, CT)

using an eighth of the reaction size recommended. All samples were bidirectionally sequenced. The sequencing reactions were precipitated using an ethanol/sodium acetate protocol (ABI User Bulletin, April 11, 2002), resuspended in Hi-Di formamide (Applied Biosystems), and electrophoresed on a 3130xl Genetic Analyzer (Applied Biosystems).

Due to the lack of variation observed in the initially cloned ITS1 region sequences and to reduce costs, the ITS1 region was directly sequenced for positive samples collected after September 2009, excluding amphipods, using the HITS1F and HITS1R primers as described above. When samples were cloned, 1-5 clones per sample were sequenced.

Sequence analysis

Hematodinium sp. sequences from all hosts were edited with CodonCode Aligner (CodonCode Corp, Dedham, Massachusetts). *Hematodinium* sp. sequences obtained from the same host species were aligned and compared using CodonCode Aligner and MacClade 4.07 OSX (Madison and Madison 1992) to examine the similarity within the same host. All identical sequences obtained from the same host were combined to create a consensus sequence. These consensus sequences were then compared to sequences of *Hematodinium* sp. from blue crabs deposited in GenBank (National Center of Biotechnology Information; DQ925230 and DQ925231), then to sequences of *Hematodinium* sp. from blue crabs collected from Chincoteague Bay, Virginia (Genbank Accession JN368147-JN368148), and finally to sequences of *Hematodinium* sp. from the other hosts examined in this study (Genbank Accession numbers JN368149-JN368194). The alignment of *Hematodinium* sp. sequences from blue crab and alternate hosts was analyzed with TCS 1.21 (http://darwin.uvigo.es/software/tcs.html; Templeton et al. 1992), with gaps treated as a fifth state, to determine if host groupings occurred.

Histopathological preparation and examination

For histological preparation, several tissues (hepatopancreas, heart, epidermis, muscle, gills and gonads) from putatively infected *C. sapidus*, *L. emarginata* and *L. dubia* were removed and preserved in Bouin's solution (Fisher Scientific). After 48 hours of preservation in either Bouin's or 10% neutral buffered formalin, tissues were transferred

into 70% ethanol until further processing. Gill tissues, whole *Palaemonetes* spp., and whole *Caprella* spp. were decalcified 24 hours in formic acid-sodium citrate (Luna 1968). All tissues were processed using routine paraffin histological techniques and stained with Mayer's hematoxylin and eosin (Luna 1968). Slides were examined with an Olympus BX51 compound microscope and images were captured using a Nikon DXM1200 digital camera and the ACT-1 computer program (Nikon).

When present, the life history stage and the relative intensity of the parasite were noted. The life history stages of the parasites in the tissues were categorized as filamentous trophonts, ameboid or multinucleated trophonts, clump colonies, prespores, or dinospores. The relative intensity of infection was rated on a semi-quantitative scale: no parasite cells present, light infection (1-5 parasites per microscopic field at 400×), moderate infection (5-20 parasites per field), or heavy infection (20+ parasites per field) (see Wheeler et al. 2007). Morphological changes to the tissues were noted if present.

Inoculation trials

Pilot inoculation trials were conducted to assess the susceptibility of alternate hosts to the *Hematodinium* sp. from *C. sapidus*. Animals used in the inoculation trials were held for one week and their hemolymph was microscopically examined for *Hematodinium* sp., as described above, prior to inoculation. Post-inoculation, all animals had their hemolymph microscopically examined for *Hematodinium* sp. monthly for 3-5 months.

Three separate inoculation trials were conducted with spider crabs, *L. dubia* and *L. emarginata,* at three different times. *Libinia dubia* were collected from pots off the VIMS pier (i.e., York River, 37° 14'45.96" N, 76° 29'59.52 W) and eight *L. emarginata* were collected from Chincoteague Bay, Virginia. Spider crabs were inoculated with 100μL of infected blue crab hemolymph with a heavy infection of ameboid trophonts. For this study, the inoculum consisted of infected blue crab hemolymph with approximately 80-90% *Hematodinium* cells and few host hemocytes remaining. Post-inoculation, the spider crabs were held 1-2 per tank in 38-L tanks at 26-32 psu and 23 °C. Water quality was monitored throughout each experiment and water changes were performed to maintain ammonia, nitrite, and nitrate (Hach Co., Loveland, Colorado)

concentrations within normal limits. The trials with *L. dubia* were conducted for 3-5 months, those with *L. emarginata* for three months. For positive controls, blue crabs (n=10, 15, 10) were inoculated with the same infected hemolymph and held under the same environmental parameters in each trial.

For the infection trials with mud crabs, 20 *Panopeus herbstii* were collected from the VIMS pier and inoculated with 25µL of infected blue crab hemolymph with a moderate infection of ameboid trophonts. The mud crabs were held together in a 38-L tank at 26 psu and 23°C. Water quality was monitored as above. This trial was conducted for three months. For positive controls, blue crabs (n=10) were inoculated with the same infected hemolymph and held under the same environmental conditions.

Host specificity

Two host specificity indices were used to analyze data on the host range of the parasite. The first host specificity index calculates the average taxonomic distance among the host species infected by the parasite,

$$S_{TD} = \frac{\Sigma \Sigma \omega_{ij}}{s\,(s-1)}$$

Equation 1:

(http://www.otago.ac.nz/zoology/downloads/poulin/TaxoBiodiv1.2/; Poulin and Mouillot 2003), where s is the total number of host species, i and j are the different host species, and ω_{ij} is the taxonomic distinctness between the host species. The second host specificity index combines the taxonomic relatedness of the host species with the prevalence of the parasite in each host species,

$$S_{TD^*} = \frac{\Sigma \Sigma \omega_{ij} (p_i p_j)}{\Sigma \Sigma_{i<j} (p_i p_j)}$$

Equation 2:

(http://www.otago.ac.nz/zoology/downloads/poulin/TaxoBiodiv2/; Poulin and Mouillot 2005), where s, i, j and ω_{ij} are defined as above and p_i and p_j are the prevalence of the parasite in different host species i and j. The first host specificity index (S_{TD}) describes

the taxonomic relationships of the included host species, whereas the second host specificity index (S_{TD*}) weights the taxonomic relatedness of the host species by the prevalence of the parasite in the different host species. For both indices, a larger number indicates that the host species are not closely related, while a smaller number indicates that the host species are closely related. Using the classic 5 taxonomic levels, the maximum value either index can obtain is 5, when all hosts belong to different phyla, and the minimum value either index can obtain is 1, when all hosts are congeners.

The ITIS classification system was used to determine relatedness of all the alternate hosts (Table 1; http://www.itis.gov/); however, only the traditional classifications (Phylum, Class, Order, Family, Genus, Species) were used for the host specificity indices. For the second index, overall prevalence was used, including the prevalence for blue crabs, which was calculated from equivalent locations and time frames reported here (Shields et al., unpublished data). In this study, the overall prevalence refers to the prevalence of *Hematodinium* sp. over the entire time frame of the study and includes every site. Due to the fact that the caprellid amphipods were only positive via PCR and parasites could not be visualized histologically, the two indices were conducted with and without this host species. Additionally, a separate host specificity analysis was conducted using all purported crustacean hosts that were reported from locations within the blue crab range (data from Stentiford and Shields 2005). This analysis was conducted separately as there was no means of verifying, via sequence data, that these hosts were infected with the same species of parasite.

Results

Prevalence and intensity

Excluding *C. sapidus*, a total of 1,829 crustaceans were caught and examined for infection by *Hematodinium* sp. over the 2008-2009 sample periods. Several taxa were represented, including two Orders, and six Superfamilies within the Infraorders Brachyura, Anomura, and Caridea (Table 1.1). Thirty-five crustaceans were positive for *Hematodinium* sp. based on both microscopic and PCR analysis, including three new hosts for this parasite: *Libinia dubia*, *Pagurus pollicaris*, and *Eurypanopeus depressus* (Table 1.1). Four *Caprella geometrica* were positive for *Hematodinium* sp. by PCR

analysis; however, infection could not be confirmed, as parasite cells were not observed in the caprellids that were processed via histology. Of the 592 alternate hosts sampled in 2008, six (1.0%) were positive for *Hematodinium* sp. (Table 1.2). Of the 1,198 alternate hosts sampled in 2009, 33 (2.8%) were positive for *Hematodinium* sp. (Table 1.2). There were 15 individuals presumptively infected via microscopic examination that were negative by PCR analysis; they were excluded from the analysis.

For the alternate hosts, the highest overall prevalence in a single host group was observed in spider crabs (4.7%) followed by mud crabs (2.7%) (Table 1.1). Within a species, the highest overall prevalence was observed in the spider crab, *L. dubia* (17.6%), followed by <10mm CW mud crabs (8.2%) (Table1.1).

A significantly higher overall prevalence was seen in the alternate hosts in 2009 when compared to 2008 (χ^2= 4.65, p=0.03). In 2008, the highest prevalence was observed at Cherrystone Creek (4.2%, Table 1.2B). In 2009, a similar prevalence was observed in alternate hosts at Wachapreague Creek (4.1%; Table 1.2A). The prevalence from Wachapreague Creek (Fisher's exact test, t=2, p=0.38, n=2 of 104 in 2008 and n=14 of 339 in 2009), Chincoteague Bay (Fisher's exact test, t=2, p=0.29, n=2 or 265 in 2008 and n=6 of 295 in 2009), and Fisherman Island (Fisher's exact test, t=2, p=0.5, n=1 of 101 in 2008 and n=13 of 597 in 2009) were not significantly different between years.

The lowest intensity infections were seen in two hermit crabs, which were microscopically diagnosed from a single ameboid trophont. The highest intensity infection was a prespore infection observed in one spider crab in October 2008, which resembled the heaviest infections observed in blue crabs. Ameboid trophonts were the most frequent parasite stage observed in all hosts.

Of the six crustaceans positive for *Hematodinium* sp. in 2008, nearly all were caught in fall months (October, n=2; November, n=3). Of the 33 crustaceans positive for *Hematodinium* sp. in 2009, the majority were also caught in fall months (October, n=14; November, n=7). There was no apparent seasonal relationship between prevalence and location or between prevalence and stage of infection.

Monthly prevalence was determined for the three taxa with the largest sample sizes (majids, xanthids, and anomurans; Figure 1.2A-D). These were compared to the monthly prevalence observed in blue crabs from corresponding locations and periods. For

the blue crabs, 9-14% prevalence was observed during September-December 2008 (Figure 1.2A). When compared to 2008, a higher prevalence of *Hematodinium* sp. in blue crabs was observed during June and July 2009, with a second peak in prevalence occurring October-December 2009. The prevalence in majids peaked in October 2008 and again in December 2009 (Figure 1.2B). Unfortunately, the sample sizes of the majids during the summer months were too low to make valid comparisons. A low prevalence was observed in xanthids in 2008 (Figure 1.2C). The prevalence in xanthids increased in 2009, with peaks observed in both July and October, correlating to the time frame of the peaks seen in blue crabs; however, the prevalence observed in the xanthids was lower in magnitude at all times compared to the prevalence observed in blue crabs. For the anomurans, the only two hermit crabs positive for *Hematodinium* sp. were observed in October 2009 (Figure 1.2D).

ITS1 sequence analysis

In total, 66 ITS1 region sequences were generated, from which 48 consensus sequences were generated and used to examine the relationships among the sequences. Ten ITS1 region sequence variants were observed from this species of *Hematodinium*. Sequences of the ITS1 region of *Hematodinium* sp. from alternate hosts had a >98% similarity to the ITS1 region sequences of *Hematodinium* sp. obtained from blue crabs. A single clone of *Hematodinium* sp. sequenced from *L. emarginata,* which differed from sequences of *Hematodinium* sp. from a blue crab by five base pairs (Figure 1.3), was responsible for most of the variation observed among ITS1 region sequences. When this clone sequence was removed from the alignment, the similarity of the *Hematodinium* sp. ITS1 region sequences from the various hosts was >99%. Single nucleotide polymorphisms (SNP) or single base pair deletions among the clones or samples were responsible for the remaining variation observed.

There were no apparent groupings of the parasite sequences by host taxa. In fact, one ITS1 variant comprised 82% (n=40) of the sequences and was found in all host taxa (Figure 1.3). A second ITS1 variant was observed in both *C. sapidus* and *L. dubia*. A third ITS1 variant was observed in two caprellid amphipods. The remaining six ITS1 variants were only observed a single time in an alternate host.

Histology and histopathology

Of the two *L. emarginata* and the two *L. dubia* examined histologically, three were infected with *Hematodinium* sp. (Figure 1.4). One *L. emarginata* and one *L. dubia* had moderate numbers of parasites in the soft tissues but presented a heavy infection in the gonads and gills. The other *L. dubia* had a heavy infection in the hemal sinuses of all tissues. The second *L. emarginata* was not found to be infected via histology and was used as a comparison for histopathology. The cell type of *Hematodinium* sp. varied between crabs and tissue types. One moderately infected *L. emarginata* and one heavily infected *L. dubia* had mostly prespores and dinospores of *Hematodinium* sp., with few ameboid trophonts and clump colonies, while the moderately infected *L. dubia* had mostly ameboid trophonts and a few multinuclear trophonts.

Overall, the histopathological effects of *Hematodinium* sp. infections observed in the *Libinia* spp. crabs were similar to that observed in *C. sapidus* collected from the same locations and times (Figure 1.4). There was a loss of connective tissue from edematous changes to the circulatory system, a loss of hemocytes, and some distortion in the typical architecture of all tissue types due to pressure necrosis from the volume of parasites within the hemal sinuses. In the hepatopancreas, the fixed phagocytes were enlarged and activated. There was also distinct erosion of the epithelial and trabecular cells lining the inner gill cuticle. The largest numbers of parasites were found in the gill stem and towards the distal tips of the lamellae. In addition, the epidermal, gill, and gonadal tissues of the spider crabs had increased numbers of parasites compared to the heart, muscle, and hepatopancreas of these animals. The major difference in pathology observed between the infected spider crabs and blue crabs was that infected spider crabs retained intact reserve inclusion cells, which were present in the spongy connective tissue cells, while the reserve inclusion cells in the blue crabs were generally depleted and atrophied, with these cells showing some variability in infected blue crabs (Wheeler et al. unpubl. data).

No *Hematodinium* infections were observed in the 56 *Palaemonetes* spp. or the 36 *Caprella* spp. examined histologically.

Host specificity

Overall, *Hematodinium* sp. infections were observed in six different crustacean species, which included five genera and four families. As the infections in the caprellid amphipods could not be confirmed with histology, the analysis was conducted with and without *C. geometrica* included. Hermit crabs are separated from the other crustacean hosts at the infraorder level; however, the host-specificity index was only based on the order and family level, not at the level of the infraorder. Based on these data, the species of *Hematodinium* that infects blue crabs is a host generalist with an S_{TD} of 2.8 (stdev = 0.54) without caprellids, and 3.14 (stdev = 0.7) with caprellids included. When taxonomic distinctness is weighted by prevalence, the S_{TD}* was 2.77 without caprellids, and 3.07 with caprellids.

Inoculation trials

Hematodinium sp. infections occurred in 96% (n=43) of the blue crabs used as positive controls. None of the *L. dubia* developed detectable infections in either of the two trials. One *L. emarginata* became infected with *Hematodinium* sp. and sporulation occurred 47 days post-inoculation; however, no other *L. emarginata* developed detectable infections. None of the *P. herbstii* developed detectable infections.

Discussion

Based on the high similarity of the ITS1 region sequences obtained (>98%), and the similarity in morphology and pathology between host species, there is evidence that one species of *Hematodinium* infects a broad range of crustaceans in the Chesapeake Bay region. That is, this species of *Hematodinium* is a host generalist in this system, and probably occurs in a wide range of hosts throughout the temperate western Atlantic. In this study, infections were confirmed in *C. sapidus,* as well as five alternate host species (*L. dubia*, *L. emarginata*, *E. depressus*, *P. herbstii*, and *P. pollicaris*) from the Delmarva Peninsula, Virginia, including three new hosts for this parasite, (*L. dubia*, *E. depressus*, and *P. pollicaris*). PCR evidence for *Hematodinium* sp. infection in the caprellid amphipod, *C. geometrica,* is reported.

These findings of high similarity within the ITS1 region from *Hematodinium* sp. (>98%) are consistent with previously published studies. Small et al (2007b) observed a comparable similarity (99%) when they cloned and sequenced the ITS1 region from two samples of *Hematodinium* sp. from *C. sapidus* from Wachapreague. In addition, Small et al. (2007a) reported a >98% similarity among ITS1 region sequences of *Hematodinium* sp. from *N. norvegicus*, *C. pagurus*, and *P. bernhardus* from the UK as well as *C. opilio* from Newfoundland and Labrador, Canada. Likewise, Hamilton et al. (2009) reported 97-100% similarity among ITS1 region sequences of *Hematodinium* sp. from *C. pagurus*, *N. norvegicus*, *L. depurator*, *M. rugosa*, *N. puber*, *P. bernhardus*, and *P. prideaux* from the Clyde Sea, Scotland.

There was variation in the prevalence and intensity of infections among host species, though the overall prevalence in all alternate hosts was low. While prevalence data for *Hematodinium* sp. was obtained for the various crustaceans collected, the main purpose of this study was to determine the identity of the species of *Hematodinium* infecting these crustaceans. Thus, the collection methods were biased towards species that entered commercial crab pots and mud crab cages or were readily collected by hand. The methods were not necessarily ideal for sampling other potential alternate host species. This may explain the low sample sizes for many of the crustaceans and may underestimate the prevalence data reported here.

Though the overall prevalence in alternate hosts was low, two findings are noteworthy. First, the higher prevalence in *L. dubia* is intriguing, especially considering that the prevalence was substantially lower in the closely related *L. emarginata*. In fact, the prevalence in *L. dubia* (17.6%, n=17) was comparable to that seen in blue crabs sampled at the same times and locations (10.6%, n=4,852), and warrants further investigation as to the reason for the seemingly high prevalence seen in this alternate host. Second, juvenile mud crabs also had a relatively high prevalence, which corresponds with previous studies that have shown a higher prevalence in juvenile blue crabs compared to adults (Messick 1994, Messick & Shields 2000). This finding supports the argument that juveniles may be important in the ecology of infectious diseases of crustaceans and should be more extensively sampled (Pestal et al. 2003, Shields et al. 2005, Stentiford 2008). However, the mud crab, *E. depressus* is capable of achieving

sexual maturity at 6mm CW (Ryan 1956, McDonald 1982) making it possible that some of the small mud crabs were actually adults.

The *Hematodinium* sp. in crustacean hosts from the Delmarva Peninsula is a host generalist capable of infecting organisms in several different families within the Order Decapoda and possibly the Order Amphipoda. Using the host specificity index, the S_{TD} of 2.8 calculated for species of *Hematodinium* in this study (excluding the caprellids) is very similar to the specificity calculated for the species of *Hematodinium* that infects boreal crustacean species (S_{TD}= 2.44; stdev= 0.8) (host data from Jensen et al. 2010, Ryazanova 2008). The S_{TD} calculation assumes that the *Hematodinium* species reported from crabs in Russia is the same species found in other boreal crabs, which partial SSU rRNA gene sequences in Genbank tentatively indicate (H. Small, pers. comm.). If it is assumed that *Hematodinium* sp. infections reported from all purported hosts from the temperate western Atlantic constitute a single parasite species, then the parasite has an S_{TD} of 3.46 (stdev= 0.62, host data from Stentiford & Shields 2005 and this study). The S_{TD} of 3.17 is similar to that for the species of *Hematodinium* that infects Norway lobsters in the UK (S_{TD} = 3.07, stdev = 0.7), indicating that these two parasite species have a similar taxonomic range of hosts (host data from Small 2004, Small et al. 2007a, Hamilton et al. 2009). Poulin and Mouillot (2004) calculated that the average S_{TD} values for various fish parasites were 2.82 ± 0.48 for trematodes, 2.99 ± 0.6 for cestodes, and 3.02 ± 0.57 for nematodes. Thus, *Hematodinium* sp. from the blue crab has a host range comparable to that observed for some generalist fish parasites.

The higher S_{TD} for the parasites with all purported hosts results from the inclusion of caprellid and gammarid amphipods, thus increasing the taxonomic distinctness index. In the UK, Small (2004) reported PCR evidence for the presence of *Hematodinium*-like DNA in total genomic DNA preparations of a small number of scavenging amphipods from the Clyde Sea area, but no histological work was conducted to confirm infection. A more recent study found no evidence of *Hematodinium* species infections in a larger survey of the same amphipod species (Hamilton et al. 2009). Thus, confirmation of *Hematodinium* species infections in these scavenging amphipods is needed. In the blue crab system, Johnson (1986) and Messick and Shields (2000) have previously reported infections of a species of *Hematodinium* in gammarid amphipods via histological

examination; however, it remains to be demonstrated that these infections are from the same species of *Hematodinium* found in this study. The ITS1 region sequence data reported here suggests that the same *Hematodinium* species may be capable of infecting caprellid amphipods, but histological evidence is required to confirm infections (see Burreson, 2008 for discussion of this issue). As these caprellid species are mainly filter feeders (Caine 1974, 1977, 1979), it is possible that *Hematodinium* sp. DNA was amplified from their gut contents. Multiple studies have also amplified *Hematodinium* sp. DNA from the water column (Frischer et al. 2006, Li et al. 2010), so it is also possible that the amphipod samples were contaminated with *Hematodinium* sp. DNA from the environment.

Alternate host – parasite associations

One obvious question to address is: what do all these hosts have in common that makes them susceptible to infection? Not surprisingly, there is great variety between the diets of these hosts, with all being omnivorous to some extent (Roberts 1968, Caine 1975, Aldrich 1976, Gerlach et al. 1976, McDermott and Trautmann 1987, Scully 1978, McDonald 1982, Auster and DeGoursey 1983, Ropes 1989, Mansour 1992, Stachowicz and Hay 1999, Whitman et al. 2001, Donahue et al. 2009). Thus it is unlikely that ingestion or cannibalism is a primary mode of transmission for these other hosts, although it has been suggested as a mode of transmission for *C. sapidus* (Walker et al. 2009, but see Li et al. in review). However, the ingestion of dinospores, either from a food source or through imbibition, cannot be excluded as a mode of entry into hosts. Alternatively, dinospores may be capable of penetrating the softened exoskeleton of a postmolt crustacean (for review see Stentiford and Shields 2005, Frischer et al. 2006). It appears that the majority of these crustaceans molt in the warmer months of the year (Ryan 1956, Van Engel 1958, Hinsch 1972, Terretta 1973, Haefner 1976, Reilly and Saila 1978, Swartz 1978, McDonald 1982, Williams 1984, DeGoursey and Stewart 1985, Hines et al. 1987, Lancaster 1988, DeGoursey and Auster 1992). The molting cycles of the alternate host species are not synchronized among all species, which may be important to the parasite, as variability in molting between host species could serve to guarantee the availability of susceptible hosts throughout the year. Interestingly, hosts

that molt continuously throughout the year (e.g. *Pagurus* spp.) were not necessarily more likely to become infected compared to those that have a terminal molt (e.g., *Libinia* spp.).

Roles of the different hosts

There is an ongoing debate in the literature as to the definition of reservoir hosts. Ashford (1997, 2003) defined a reservoir host as "an ecologic system in which an infectious agent lives indefinitely" and included all intermediate hosts, vectors, and any host populations large enough to sustain the infectious agent indefinitely; however, he excluded liaison or incidental hosts, as these hosts are not necessary for maintenance of the disease. Haydon et al. (2002) defined reservoirs as "one or more epidemiologically connected populations or environments in which the pathogen can be permanently maintained and from which it can be transmitted to the defined target population". The definition given by Hayden et al. (2002) appears to be the most useful as they specified that transmission must occur between the reservoir host population and the target host population and included both maintenance and non-maintenance hosts as important (Small and Pagenkopp 2011).

The data from the present study indicate that the same species of *Hematodinium* infects several host species, thus the ecological role of each of these alternate host species has implications for the critical threshold of host abundance necessary for the parasite to remain in the system (Begon 2008). The blue crab appears to be an important host for this parasite as infections are common and abundant in this host and the host population sustains the parasite at high levels during peaks in prevalence. If the other taxa represent true reservoirs with respect to blue crab populations, then if the blue crab population declined, the parasite would be maintained in the system by using other crustaceans as hosts. If these species represent incidental or spillover hosts, with little transmission back to blue crab hosts, then the parasite might be eradicated from the system once the blue crab population fell below the critical threshold value necessary for transmission.

As only two infected hermit crabs were found over the two-year period, and many were sampled, hermit crabs in this system are probably incidental or spillover hosts and are probably not important for the maintenance of the parasite in the system. On the other hand, the two spider crab species are probably reservoirs because the prespore and

dinospore stages, presumably the infective stage of the parasite (Frischer et al. 2006), were observed in these organisms in the field (*L. dubia*) and the laboratory (*L. emarginata).* Additionally, the prevalence observed in *L. dubia* was comparable to that seen in the blue crab. As *Libinia* spp. are believed to migrate to deeper waters offshore during the winter months (Winget et al. 1974), this species could serve as an overwintering host for the parasite or for transport to other habitats.

The role of the xanthids in this system is less obvious. The juvenile mud crabs may be reservoirs of the parasite, or they may be more susceptible to infection than adults. Additionally, the seasonal peaks of infection in xanthids coincide with the seasonal peaks seen in the blue crabs, leading to the speculation that the xanthid crabs are at least incidental or spillover hosts of the parasite. Neither prespore nor spore stages were ever observed in the infected xanthids, making it difficult to determine their role as spillover or reservoir hosts.

In conclusion, based on the high similarity in the ITS1 sequences, in addition to the similarities in morphology and pathology, the same species of *Hematodinium* that infects blue crabs appears to be capable of infecting a number of other crustacean species, which has implications for the propagation of the parasite in this system.

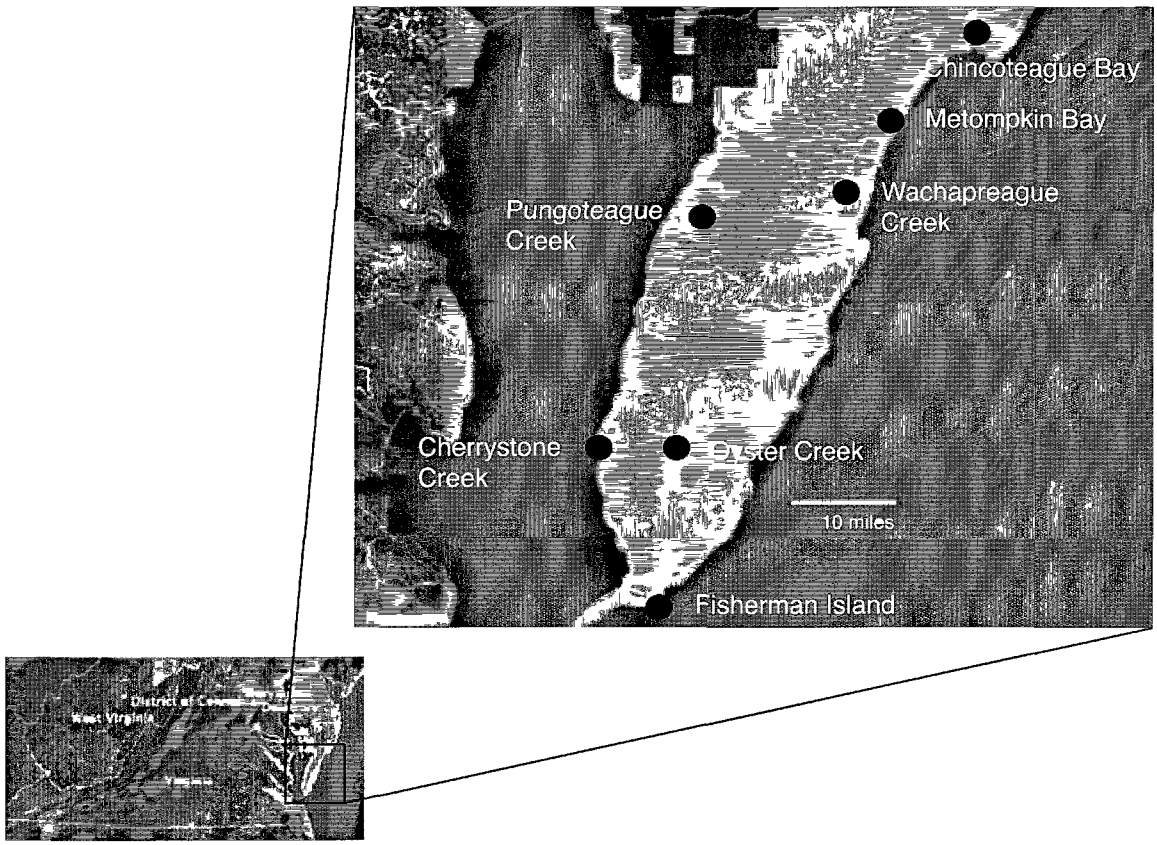


FIGURE 1.1. Sampling locations for this study along the Delmarva Peninsula, Virginia.

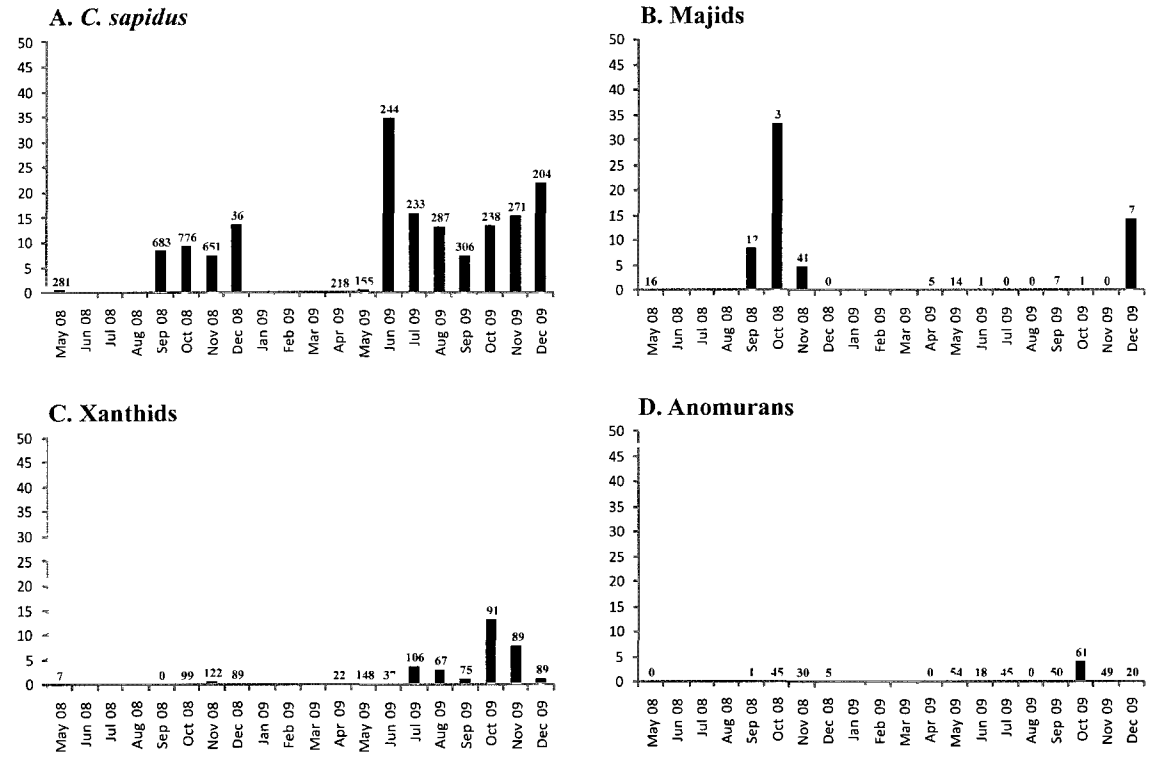


FIGURE 1.2. Monthly prevalence of *Hematodinium* sp., as determined by microscopy and confirmed by PCR, in the four major taxonomic groups collected. Total numbers of specimens collected each month are listed along the x-axis.

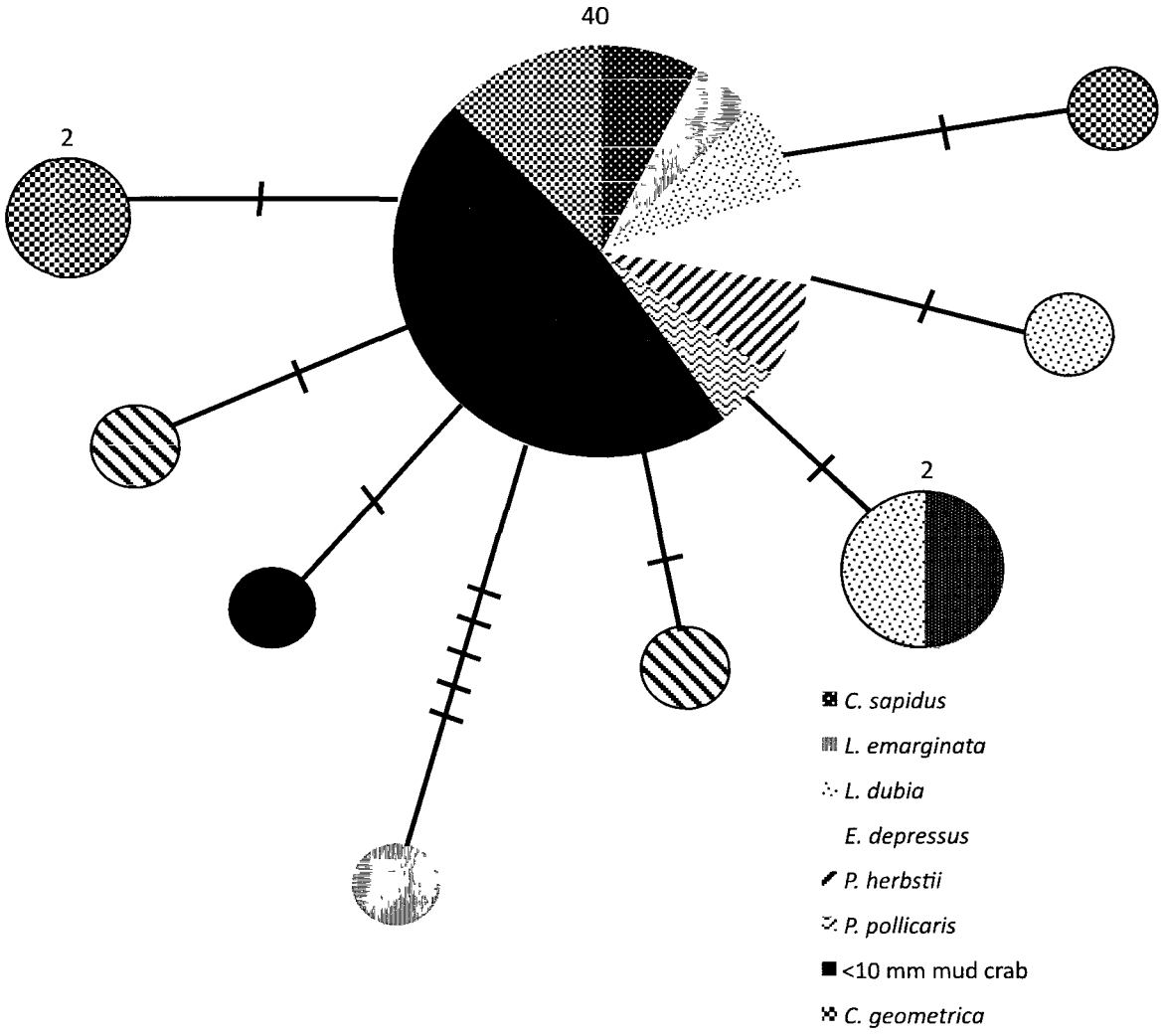


FIGURE 1.3. A modified TCS generated network using the *Hematodinium* sp. ITS1 sequences obtained from the different hosts. Each hash mark represents either a single nucleotide polymorphism or a gap. The number of consensus sequences included in each circle is indicated above the circle if more than one sequence is represented.

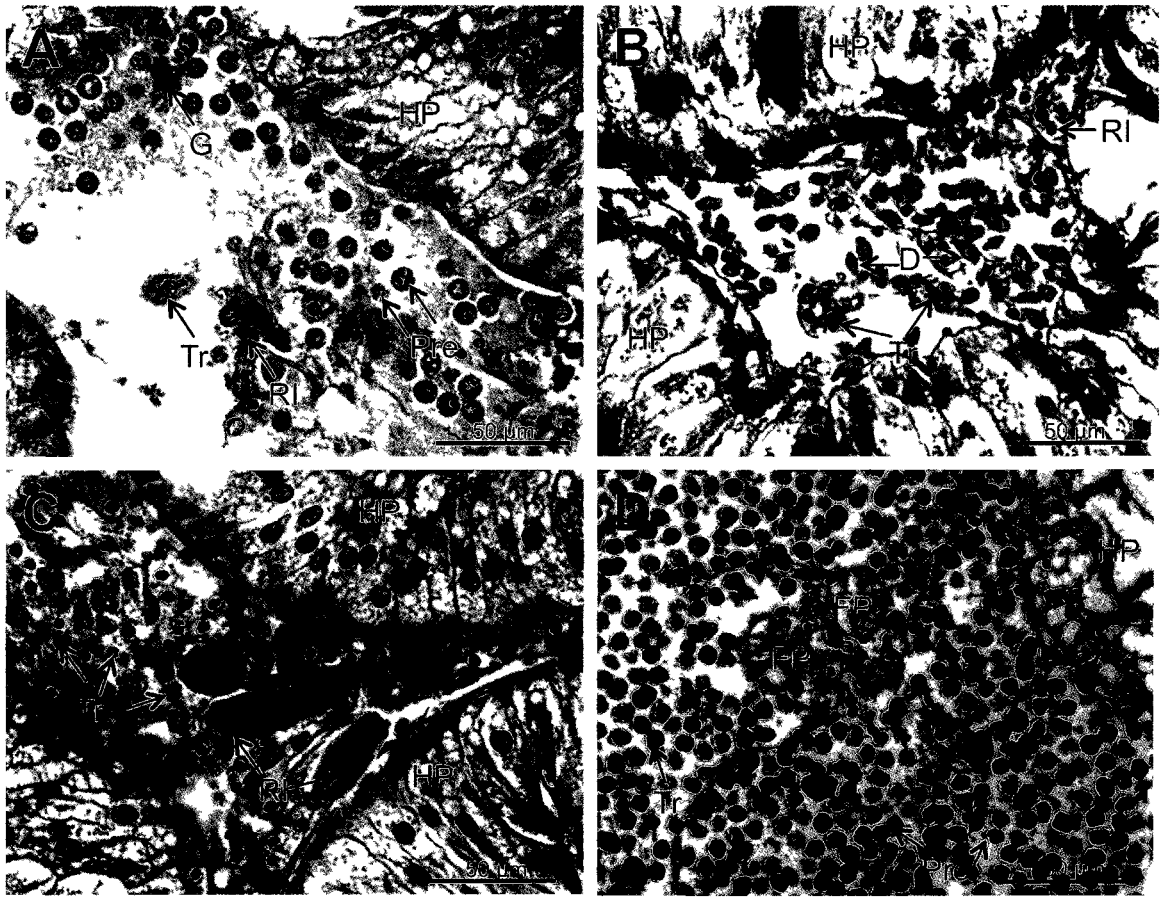


FIGURE 1.4. Comparison of *Hematodinium* sp. infections in the hepatopancreases of *Libinia* spp. and *C sapidus*. (A) *L. dubia* (sample number= EID 2397) with prespores and ameboid trophonts present, reserve inclusion cells reduced but present, loss of connective tissue and edematous hemal sinus. (B) *L. emarginata* (EID 1809) with prespores and ameboid trophonts present, RI cells absent, loss of connective tissue and edematous hemal sinus. (C) *L. dubia* (EID 7766) with trophonts present, RI cells present, and less damage to the connective tissues than in A and B. (D) *C sapidus* (EID 1544) with dinospores & prespores present, RI cells absent, nearly complete necrosis of the connective tissue, edematous hemal sinus. Key: Tr, trophonts; HP, hepatopancreas; RI, reserve inclusion cells; FP, fixed phagocytes; Pre, prespores; G, granulocytes; D, dinospores.

TABLE 1.1. Decapod crustaceans collected for this study with the ITIS classifications, the number of each species positive for *Hematodinium* sp., and prevalence, as determined by microscopy and confirmed by PCR, with the exception of the caprellid amphipods, which were assessed solely via PCR analysis and are noted with an asterisk. Some mud crab species were sometimes difficult to identify and are labeled with question marks.

TABLE 1.1

			Number sampled	Number positive	Prevalence
Order					
Decapoda					
Family	**Panopeidae**				
	<10mm mud crab		269	22	8 2
	Dyspanopeus	*sayi?*	246	0	0 0
	Eurypanopeus	*depressus*	288	3	1 0
	Hexapanopeus	*angustifrons?*	9	0	0 0
	Panopeus	*herbstii*	228	3	1 3
		Total	**1040**	**28**	**2.7**
Family	**Pisidae**				
	Libinia	*dubia*	17	3	17 6
	Libinia	*emarginata*	90	2	2 2
		Total	**107**	**5**	**4.7**
Family	**Cancridae**				
	Cancer	*borealis*	3	0	0 0
	Cancer	*irroratus*	72	0	0 0
		Total	**75**	**0**	**0.0**
Family	**Portunidae**				
	Ovalipes	*ocellatus*	1	0	0 0
	Portunus	*spinimanus*	1	0	0 0
	Portunus	*gibbesii*	1	0	0 0
	Callinectes	*similis*	4	0	0 0
		Total	**7**	**0**	**0.0**
Family	**Varunidae**				
	Hemigrapsus	*sanguineus*	15	0	0 0
		Total	**15**	**0**	**0.0**
Family	**Paguridae**				
	Pagurus	*longicarpus*	59	0	0 0
	Pagurus	*pollicaris*	270	2	0 7
Family	**Diogenidae**				
	Clibanarius	*vittatus*	40	0	0 0
		Total	**369**	**2**	**0.5**
Family	**Palaemonidae**				
	Palaemonetes	sp	56	0	0 0
		Total	**56**	**0**	**0.0**
Order	**Amphipoda**				
Family	**Caprellidae**				
	Caprella	*equilibra*	35	0	0 0
	Caprella	*geometrica*	121	4	3 3
	Caprella	sp	4	0	0 0
		Total	**160**	**4***	**2.5**
		Grand Total	**1829**	**39**	**2.1**

TABLE 1.2. Sample size and prevalence of decapod crustaceans collected at different sites and years. Prevalence is in bold when *Hematodinium* sp. was present. Chincoteague, Fisherman's Island, and Wachapreague were sampled in 2008 and 2009, while Cherrystone, Metompkin, Oyster, and Pungoteague were only sampled in 2008.

TABLE 1.2

	Chincoteague		Fishermans Island		Wachapreague		Cherrystone	Metompkin	Oyster	Pungoteague
	2008	2009	2008	2009	2008	2009	2008	2008	2008	2008
10mm mud crab	0% (0/16)	**11.9%** (5/42)	0% (0/1)	**7.1%** (7/99)	**7.7%** (1/13)	**12.9%** (9/70)	0% (0/8)			0% (0/20)
vspanopeus sayi?	0% (0/169)	0% (0/24)	0% (0/5)	0% (0/6)	0% (0/6)	0% (0/26)				0% (0/10)
rv panopeus depressus		0% (0/156)		**1.5%** (1/65)	0% (0/1)	**3.0%** (2/66)				
exapanopeus igustifrons	0% (0/2)				0% (0/7)					
anopeus herbstii	0% (0/1)			0% (0/12)	0%(0/18)	**1.9%** (3/158)				0% (0/39)
ancer irroratus	0% (0/12)	0% (0/14)	0% (0/2)	0% (0/44)						
ancer borealis			0% (0/2)		0% (0/1)					
binia emarginata	**2.9%** (1/35)	0% (0/15)	0% (0/3)	0% (0/12)	**100%** (1/1)			0% (0/1)	0% (0/22)	
binia dubia		0% (0/1)	**50%** (1/2)	**14.3%** (1/7)			**14.3%** (1/7)			
'ibanarius vittatus			0% (0/1)	0% (0/38)		0% (0/1)				
allinectes similis	0% (0/2)		0% (0/1)					0% (0/1)		
n tunus gibbesii									0% (0/1)	
n tunus spinimanus					0% (0/1)					
valipes ocellatus			0% (0/1)							
emigrapsus sanguineus			0% (0/1)			0% (0/14)				
agurus pollicaris	0% (0/3)	0% (0/1)	0%(0/59)	**1.1%** (2/189)			0% (0/1)	0% (0/11)	0% (0/6)	
agurus longicarpus				0% (0/57)		0% (0/2)				
alaemonetes sp					0% (0/56)					
aprella equilibra	0% (0/12)	0% (0/7)	0% (0/14)			0% (0/2)				
aprella geometrica	**7.6%** (1/13)	**3.1%** (1/32)	0% (0/9)	**3.0%** (2/67)						
aprella spp		0% (0/3)		0% (0/1)						
otal	**0.8%** (2/265)	**2.0%** (6/295)	**0.9%**(1/101)	**2.2%**(13/597)	**1.9%**(2/104)	**4.1%** (14/339)	**6.3%** (1/16)	0% (0/14)	0% (0/29)	0% (0/69)

Chapter 2. Conservation in first internal transcribed spacer (ITS1) region sequences of *Hematodinium* sp. from blue crabs

Abstract

Blue crabs support economically viable commercial fisheries along the eastern seaboard and the Gulf of Mexico of the USA from Delaware to Texas; however, many of these fisheries have experienced substantial declines in landings during the last few decades. A parasitic dinoflagellate, *Hematodinium* sp., has been reported from blue crab populations from New Jersey to Texas and has been found to cause mortality in crabs in high salinity areas; however, there was little molecular evidence to determine if the same parasite species was infecting blue crabs throughout this range. In order to determine if the same species of *Hematodinium* was infecting blue crabs over a broad geographic range, 317 blue crabs collected from Massachusetts, Virginia, Georgia, Florida, Louisiana, and Texas were tested for the presence of *Hematodinium* sp. using a PCR assay targeting a portion of the first internal transcribed spacer (ITS1) region of the ribosomal RNA (rRNA) gene complex of the parasite. Partial sequences of the ITS1 region from the parasite were compared to previously published ITS1 region sequences of *Hematodinium* sp. from blue crabs and to ITS1 region sequences of *Hematodinium* sp. from alternate hosts. In total, 29 distinct ITS1 sequence variants of *Hematodinium* sp. were identified from blue crabs and 33 distinct ITS1 variants of *Hematodinium* sp. were identified with alternate hosts included. However, a single ITS1 variant appeared in all geographic regions, hosts, and blue crabs sampled from a previous study. Based on the high similarity among all the ITS1 region sequences examined (>99%) and the observation of a single ITS1 variant over a large geographic area, it appears that a single species of *Hematodinium* infects blue crabs from Virginia to Texas and multiple alternate host species in Virginia.

Introduction

Economically important blue crab fisheries occur in eleven states in the USA from Delaware south to Texas (Milliken and Williams 1984). Within the last decade, there have been major declines in many blue crab fisheries in the USA. In 2007, the Chesapeake Bay had a total harvest of 43.5 million pounds, which was the lowest on record since 1945 (Chesapeake Bay Advisory Report 2008). Along coastal Georgia, blue crab landings have dropped to 1.8 million pounds, well below the 45-year average of 3.9 million pounds (Lee and Frischer 2004). Overexploitation, pollution, summer hypoxia, degraded nursery habitat, and disease are all thought to affect blue crab populations (Kennedy and Cronin 2007).

One potential explanation for the decline of *C. sapidus* populations in high salinity waters may be the parasitic dinoflagellate, *Hematodinium* sp., which can reach high prevalence in blue crabs in high salinity waters. To date, *Hematodinium* sp. infections in adult blue crabs have been reported from New Jersey south to Texas (Messick and Shields 2000), with prevalence ranging from 9 - 98% in some blue crab populations (Newman and Johnson 1975, Messick 1994, Messick and Shields 2000, Lee 2000, Gruebl et al. 2002). Data indicate that the majority of blue crabs naturally or experimentally infected with *Hematodinium* sp. only survive 35- 55 days (Messick and Shields 2000), suggesting that areas with a high prevalence of *Hematodinium* sp. likely suffer significant crab mortality due to disease.

Many of the reports of *Hematodinium* sp. in blue crabs have used only histological examination of tissues or hemolymph smears. However, morphological characteristics are insufficient for species determinations of this parasite, making molecular data necessary for parasite species identification (Stentiford and Shields 2005). While the current molecular evidence suggests a species of *Hematodinium* parasite infects multiple crustacean species throughout the waters of the United Kingdom (UK) (Small et al. 2007a, Hamilton et al. 2009, 2010) and the Bering Sea (Jensen et al. 2010), comparatively less molecular work has been done to examine the range of *Hematodinium* sp. in blue crabs. Previous work demonstrated that a single species of *Hematodinium* appears to be present throughout the Delmarva Peninsula, Virginia, because of a high similarity in ITS1 region sequences from *Hematodinium* sp. obtained from a variety of

infected hosts (see Chapter 1). However, the geographic range of this species of *Hematodinium* is still unknown. Additionally, the findings of Small et al. (2007b) suggest the same species of *Hematodinium* occurs in Virginia and Georgia waters. This assumption was based on the results of a restriction fragment length polymorphism (RFLP) assay, which showed that the *Hematodinium* sp. obtained from blue crabs from Virginia and Georgia had identical profiles on a gel; however, no sequence data was obtained to substantiate this finding.

The purpose of this study was to determine the number of species of *Hematodinium* in blue crab populations over a wide geographic range. Sequence data from a portion of the ITS1 region of the rRNA gene complex of *Hematodinium* sp. was used to determine the number of species of *Hematodinium* present in blue crabs collected from Massachusetts, Virginia, Georgia, and the Gulf of Mexico. The ITS region was chosen as it is a commonly used molecular marker for distinguishing between closely related species of dinoflagellates (Litaker et al. 2007) and shellfish parasites (Dungan et al. 2002, Casas et al. 2004, Dungan and Reece 2006, Moss et al. 2008). Additionally, it was the most variable marker available for this species. The sequences obtained for this study from the ITS1 region of *Hematodinium* sp. were compared to previously published ITS1 region sequences of *Hematodinium* sp. from blue crabs and ITS1 region sequences of *Hematodinium* sp. obtained from alternate hosts to determine the range and temporal stability of the parasite.

Materials and Methods

Sample collection

Blue crabs were sampled from 19 different locations in the USA (Figure 2.1; Table 2.1). Blue crabs from the Delmarva Peninsula, Virginia were collected via crab pots from three primary seaside sites (Chincoteague Bay, Wachapreague Creek, and Fisherman's Island) in 2008 (May-December) and 2009 (April-December) using commercial crab pots. An additional three sites (Cherrystone Creek, Oyster Creek, and Metompkin Bay) were sampled only in 2008. Alternate hosts were collected as described in Chapter 1.

Extracted DNA samples from the muscle tissues of blue crabs collected from five sites in Massachusetts (n= 112) and from seven sites in the Gulf of Mexico (n= 155) during 2002 were obtained from Dr. Allen Place (Center of Marine Biotechnology, UMBI). Extracted DNA samples of blue crabs infected with *Hematodinium* sp. from coastal Savannah, Georgia (n=8) collected between 2000-2004 were obtained from Dr. Marc Frischer and Dr. Richard Lee (Skidaway Institute of Oceanography). Samples of blue crab hemolymph stored in 95% ethanol from Corpus Christi Bay, Texas (n=7) collected between 2008-2009 were obtained from Jon Gain (Texas A&M University).

Infection status

Infection status for blue crabs and other large crustaceans collected from Virginia was assessed as described in Chapter 1. Briefly, infection status was first assessed microscopically. Hemolymph smears were stained 1:1 with 0.3% Neutral red and examined with a light microscope at 100x and 400x magnification for uptake of the dye (see Stentiford and Shields 2005). Mud crabs <10mm CW were dissected and small pieces of gill were stained and examined as above. If *Hematodinium* sp. was observed microscopically, then samples were saved for molecular analysis.

The infection status of the blue crab samples obtained from Massachusetts, Georgia, and the Gulf of Mexico was assessed solely via PCR (see below) as only DNA was obtained for those samples.

PCR amplification and sequencing

All DNA extractions, ITS1 amplifications, and sequencing protocols were conducted as described in Chapter 1. Briefly, 200µL of hemolymph in 95% ethanol was extracted with a Qiagen Tissue and Blood kit (Qiagen, Valencia, California) following the manufacturer's protocol for animal tissue. All extractions completed within the same day included a blank column extraction, which, when subjected to PCR analysis alongside samples, served as a control for extraction contamination. After extraction, the DNA was aliquoted (~20µL) to avoid contamination of the entire stock of DNA. The remaining stock DNA (~80µL) was stored at -20°C and the 20µL aliquot was stored at

4°C. Extracted DNAs were quantified using a NanoDrop 2000 (Thermo Scientific, West Palm Beach, Florida).

To confirm the presence of high molecular weight DNA in extractions, a general metazoan primer set, nSSU A and nSSU B (modified from Medlin 1988) was first used to amplify DNA extracted from samples. A second PCR assay targeting the ITS1 region of the parasite was used (Small et al. 2007b) to confirm infection status. A third PCR assay, a modified version of the Gruebl et al. (2002) assay, which amplifies a fragment of the SSU rRNA gene, was applied to blue crab samples from Massachusetts and the Gulf of Mexico to ensure that all infections were identified due to the possibility of encountering other *Hematodinium* species in these locations. A negative control, consisting of no DNA, was included in all of the diagnostic PCR assays, and a positive control, consisting of either DNA extracted from a culture of *Hematodinium* sp. or a sample that had reliably amplified in the past, was also included in all diagnostic PCR assays.

The ITS1 region PCR products were cloned and sequenced as described in Moss et al. (2008) or directly sequenced. Briefly, ITS1 region amplicons were cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, California) using half of the total volume of cells recommended by the manufacturer's instructions. Plasmid DNA was extracted from individual colonies following a boil-prep method and colonies were screened using the M13F/R vector primers provided in the cloning kit. An aliquot of the PCR products (5μL) was visualized under UV light on a 2% (w/v) agarose gel after ethidium bromide staining. Colonies with inserts of appropriate size (~500 bp) were purified with exonuclease I (EXO) and shrimp alkaline phosphatase (SAP). The purified PCR product was used as the template for sequencing with the M13 primers and the Big Dye Terminator kit (Applied Biosystems, Norwalk, Connecticut) using an eighth of the reaction size recommended by the manufacturer. All samples were bidirectionally sequenced. The sequence reactions were precipitated using an ethanol/sodium acetate protocol (ABI User Bulletin, April 11, 2002). The precipitated sequencing products were resuspended in Hi-Di formamide (Applied Biosystems) and electrophoresed on a 3130xl Genetic Analyzer (Applied Biosystems).

For the samples from Georgia, 3-4 clones per sample were sequenced. For the samples from the Gulf of Mexico, 1-5 clones per sample were sequenced. For the seven

samples from Texas, 7-8 clones per sample were sequenced. For the six samples from Virginia that were cloned, 1-4 clones per sample were sequenced. Due to the lack of variability seen previously and to reduce the cost per sample, the remaining samples from Virginia were directly sequenced.

Sequence analysis

Sequences were edited in CodonCode Aligner (CodonCode Corp, Dedham, Massachusetts) and were aligned and compared using CodonCode Aligner and MacClade 4.07 OSX (Madison and Madison 1992) to determine similarity. All identical sequences obtained from the same host were combined to create a consensus sequence. The consensus sequences from this study were then compared to *Hematodinium* sp. sequences from blue crabs collected in 2005, which were deposited in GenBank (National Center of Biotechnology Information; DQ925227-DQ925236) to assess any change of this parasite between 2005 and 2008 in Virginia coastal waters. An alignment with all of the ITS1 region and consensus sequences of *Hematodinium* sp. from blue crabs was analyzed with TCS 1.21 (http://darwin.uvigo.es/software/tcs.html; Templeton et al., 1992), with gaps treated as a fifth state, to determine if any geographic groupings occurred.

A second TCS 1.21 analysis was conducted as described above, which combined the ITS1 region sequences of *Hematodinium* sp. obtained from blue crabs described here and the ITS1 region sequences of *Hematodinium* sp. obtained from alternate hosts, to determine if any host or geographic groupings occurred with the additional hosts included.

Lastly, ITS1 region sequences from *Hematodinium perezi* were downloaded from GenBank (National Center of Biotechnology Information; EF065708-EF065716, EF153724-EF153727) and added to the alignment of ITS1 region sequences of *Hematodinium* sp. from blue crabs and alternate hosts. This alignment was used to calculate the average genetic distances between the sequences in MEGA 5.0 (Kumar et al. 2004). For this calculation, the ITS1 region sequences were combined into three groups: ITS1 region sequences from *H. perezi* from Genbank, ITS1 region sequences from *Hematodinium* sp. from alternate hosts, and ITS1 region sequences from

Hematodinium sp. from *C. sapidus*. Genetic distance was calculated with gaps as missing data and a p-distance model.

Results

In total, 152 ITS1 region sequences of *Hematodinium* sp. from blue crabs were obtained from ten locations (Table 2.1), from which 91 consensus sequences were generated (National Center of Biotechnology Information, JN368147-JN368148, JN380091- JN380179). *Hematodinium* DNA was not amplified from any blue crabs from Massachusetts using either the ITS1 or the SSU PCR assays. *Hematodinium* sp. DNA was amplified from all of the blue crabs from Georgia, seven blue crabs from Texas, and eleven blue crabs from the Gulf Coast of Florida using the ITS1 PCR assay. *Hematodinium* sp. DNA was amplified from one sample from Florida with the SSU PCR assay, but not with the ITS1 PCR assay. Although weak amplicons were observed, ITS1 region sequences of *Hematodinium* sp. could not be obtained from two additional samples from Florida. These three samples from Florida were excluded from the analysis.

Of those *Hematodinium* sp. sequences obtained from blue crab hosts, 29 ITS1 sequence variants were observed; however, a single ITS1 variant was observed in all geographic regions and sequences previously deposited in Genbank (n=70, Figure 2.2). Three additional ITS1 variants were observed in multiple locations. One *Hematodinium* sp. ITS1 variant was obtained from a blue crab from Texas and another blue crab from the Gulf Coast of Florida. One *Hematodinium* sp. ITS1 variant was observed in one blue crab from Georgia, one blue crab from Texas, and two blue crabs from the Gulf Coast of Florida. Another *Hematodinium* sp. ITS1 variant was observed in one blue crab from Georgia and another blue crab from Texas. The site with the most unique ITS1 variants was Georgia (n=11), while only one unique ITS1 variant was observed from the Gulf Coast of Florida.

When ITS1 region sequences of *Hematodinium* sp. from alternate hosts were combined with those from blue crabs, a total of 33 ITS1 sequence variants were observed. As before, a single ITS1 variant was observed in all geographic locations, all hosts examined, and sequences previously deposited in Genbank (n=107, Figure 2.3). Six additional ITS1 variants were observed either in multiple locations or in multiple host

species. One ITS1 variant from *Hematodinium* sp. was obtained from both a blue crab from Virginia and a spider crab, *Libinia dubia,* from Virginia. Another ITS1 variant from *Hematodinium* sp. was obtained from a blue crab from Georgia and a spider crab from Virginia. A third ITS1 sequence variant from *Hematodinium* sp. was obtained from two blue crabs from the Gulf Coast of Florida, a <10 mm mud crab from Virginia, a blue crab from Georgia, and a blue crab from Texas. A fourth ITS1 variant from *Hematodinium* sp. was obtained from a blue crab from Georgia and a blue crab from Texas. A fifth ITS1 variant from *Hematodinium* sp. was obtained from a blue crab from the Gulf Coast of Florida and a blue crab from Texas. A sixth ITS1 variant from *Hematodinium* sp. was obtained from a blue crab from Texas and a caprellid amphipod from Virginia. Overall, the ITS1 region sequences of *Hematodinium* sp. obtained from blue crabs exhibited >99% similarity over a large geographic range and >98% similarity from all of the various host species examined (Figure 2.2).

The average genetic distance between ITS1 region sequences of *Hematodinium* sp. obtained from *C. sapidus* and the alternate hosts was only 0.002 (99.8% similarity; Table 2.2). The average genetic distances between ITS1 region sequences from *H. perezi* from its type host and ITS1 region sequences from *Hematodinium* sp. obtained from *C. sapidus* and alternate hosts was 0.038 (96.2% similarity) and 0.037 (96.3% similarity), respectively (Table 2.2).

Discussion

Based on the high similarity (>99%) among the ITS1 region sequences obtained in this study, it appears that a single species of *Hematodinium* infects blue crabs from Virginia to Texas and this same species of *Hematodinium* infects other crustaceans in the coastal waters of Virginia. The average genetic distance between ITS1 region sequences from *H. perezi* and the *Hematodinium* sp. from the eastern seaboard and the Gulf of Mexico, USA was an order of magnitude larger than the average genetic distance between ITS1 region sequences obtained for this study from *Hematodinium* sp. collected from the US eastern seaboard and the Gulf of Mexico, USA. This supports the assertion that the same species of *Hematodinium* was found in all of the locations examined in this study. Additionally, it appears that the same species of *Hematodinium* has been infecting

blue crabs in the coastal waters of Virginia since at least 2005. The level of variation reported in this study is consistent with what has been reported for other species of *Hematodinium* (Small et al. 2007a, 2007b, Hamilton et al. 2007, 2009).

Using mitochondrial DNA (mtDNA) sequences, previous studies have discovered population differentiation in five marine or estuarine species between the Atlantic coast and Gulf of Mexico, USA (for review see Avise, 1992), which was used to provide insight into the historical expansion and contraction of the ranges of those species. Examining population differentiation between the Atlantic coast and Gulf of Mexico, USA has only been attempted for one marine parasite, *Perkinsus marinus* (Reece et al. 2001). In that study, researchers reported different allele frequencies between three regions of the USA: the Northeast Atlantic, Southeast Atlantic, and the Gulf of Mexico. The range of *P. marinus* had expanded northward along the Atlantic seaboard in the early 1990s (Ford 1996). This expansion was evident in the molecular data as Reece et al. (2001) found the northeast region was dominated by a single composite genotype of *P. marinus*, possibly indicating that this genotype was better suited for expansion. When examining the blue crab host, McMillen-Jackson and Bert (2004) did not find any geographic structure in blue crabs from the Atlantic seaboard and Gulf of Mexico, USA based on RFLP analysis of mtDNA, but they did find a significant decline in genetic variation with increasing latitude along the eastern seaboard of the USA. They attributed the lack of geographic structuring to a sudden expansion of the species and the decrease in genetic variation with increasing latitude was attributed to post-Pleistocene range expansion. However, no population differentiation between the Atlantic and Gulf of Mexico, USA was observed using the ITS1 region from *Hematodinium* sp. in the blue crab.

As the same species of *Hematodinium* appears to occur from Virginia to Texas, there are several possible mechanisms to explain how the parasite dispersed over that range. First, it is possible that infected blue crabs demonstrate sufficient migration to maintain the gene flow of the parasite between these areas; however, most studies have shown that the majority of adult blue crabs tend to remain within their home estuary and only a small fraction of adult crabs will migrate to neighboring estuaries (for review see Hines 2007). Based on a tag and recapture study with blue crabs from North Carolina,

Judy and Dudley (1970) showed that only 6.2% of female blue crabs and 2.2% of male blue crabs moved > 24 km from the release site and only 1% of females migrated to South Carolina. Another tag and recapture study from North Carolina showed that only 0.3% of crabs migrated north into other estuaries and only 2% migrated south to other estuaries (Schwartz, 1997). While the majority of studies have shown that both male and female crabs tend to remain within their home estuaries, tag and recapture studies have demonstrated that some female crabs from the west coast of Florida can migrate substantial distances of > 322 km from their home estuary (for review see Hines 2007). A second possible mechanism would be transportation of the parasite via infected blue crab megalopae; however, to date, there have been no published findings of infection of *Hematodinium* sp. in blue crab megalopae.

A third possibility is that *Hematodinium* sp. dinospores, presumably the transmissive stage (Frischer et al., 2006; Li et al., 2010), are capable of surviving in the water column long enough to be spread along the eastern seaboard and Gulf of Mexico, USA via ocean currents. Lee and Frischer (2004) state that they were able to detect *Hematodinium* sp. for three weeks after an infected crab died and was removed from a tank. Li et al. (2010) reported the estimated life span of *Hematodinium* sp. dinospores in aquaria to be 4-7 days post sporulation. These life span estimates are shorter that what has been reported for other *Hematodinium* species in boreal climates. Meyers et al. (1987) reported that macrospores from *Hematodinium* sp. from *Chionoecetes bairdi* survived 73 days in sterile seawater and microspores survived 52 days. Appleton (1996) reported that *Hematodinium* sp. macrospores and microspores obtained from an infected Norway lobster (*Nephrops norvegicus*) survived between 4-49 days and 32-63 days in seawater, respectively.

Another possible explanation for the spread of *Hematodinium* sp. is that the parasite is spread by migration of infected alternate host species or blue crabs with chronic infections. Shields and Squyars (2000) reported that most of the crabs in their study (86%) developed acute infections that led to host mortality within 40 days; however, a few of the crabs (10%) developed chronic infections and survived up to 90 days. Experimental inoculations of spider crabs have shown that spider crabs may be refractory to infection or may be asymptomatic carriers of the parasite (see Chapter 1).

As spider crabs are believed to migrate to deeper waters during the winter months (*Libinia* spp.; Winget et al. 1974), it is possible that they are capable of carrying and disseminating the parasite. Alternately, it is possible that the overlapping distributions or shorter migrations between neighboring estuaries of the different host species allows for continuous gene flow along the eastern seaboard and into the Gulf of Mexico, USA. However, to date, it is unknown whether or not transmission occurs between these different host populations or if there are different strains of *Hematodinium* sp. infecting different hosts.

In conclusion, ITS1 region sequences of *Hematodinium* sp. obtained from blue crabs from various geographic locations and alternate hosts indicate that the same parasite species is infecting blue crabs from Virginia to Texas, has been in the coastal waters of Virginia since at least 2005, and is capable of infecting a variety of hosts.

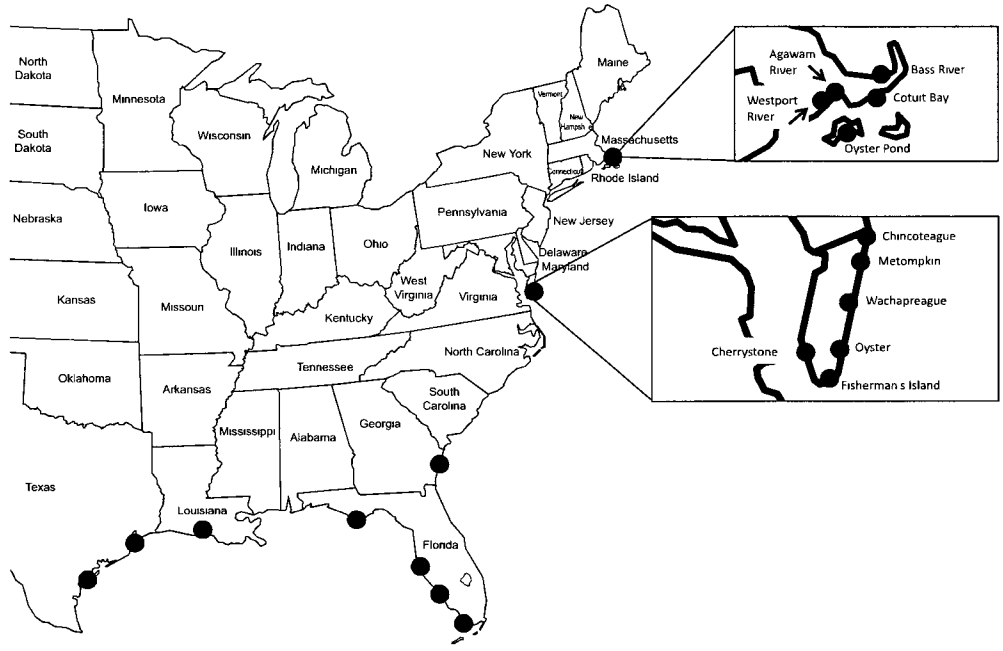


FIGURE 2.1. Locations where samples of blue crabs were collected for this study.

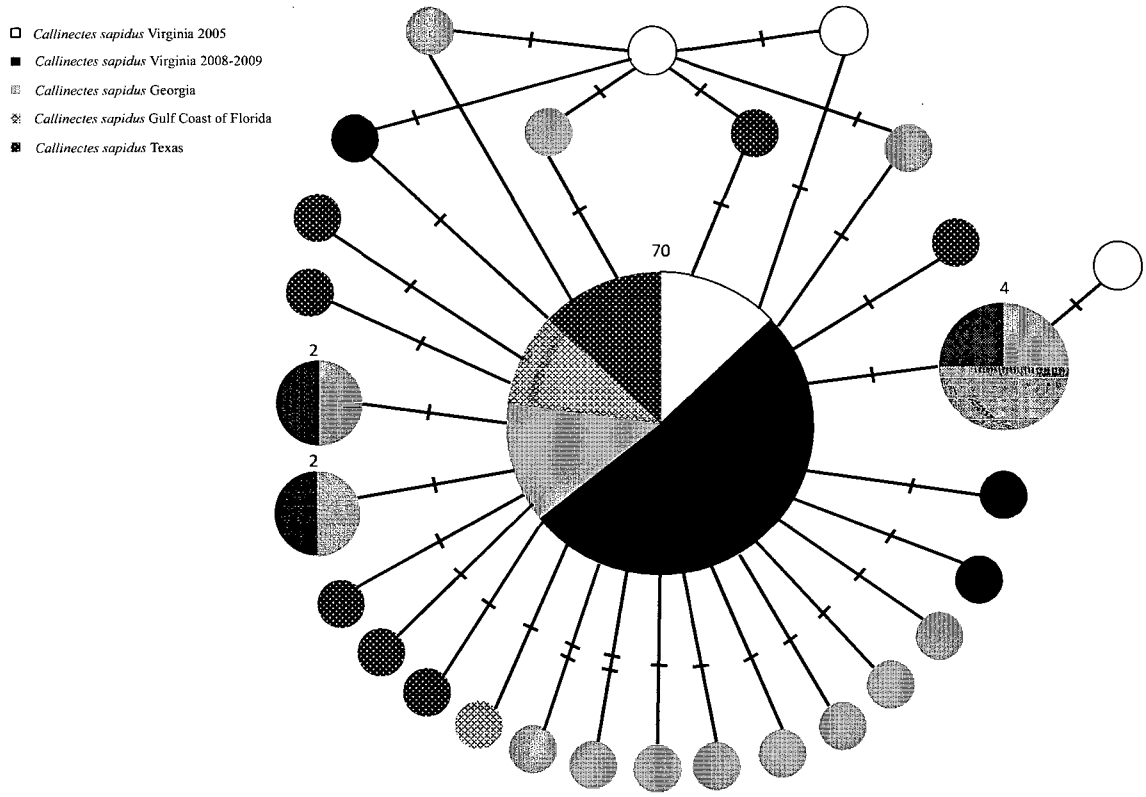


FIGURE 2.2. A modified TCS generated network using the ITS1 region sequences of *Hematodinium* sp. obtained from infected blue crabs. Each dash indicates a single nucleotide polymorphism or a gap. Numbers above the circles indicate the number of consensus sequences represented. Those circles without numbers represent only a single consensus sequence. The center circle represents an ITS1 variant common to all locations.

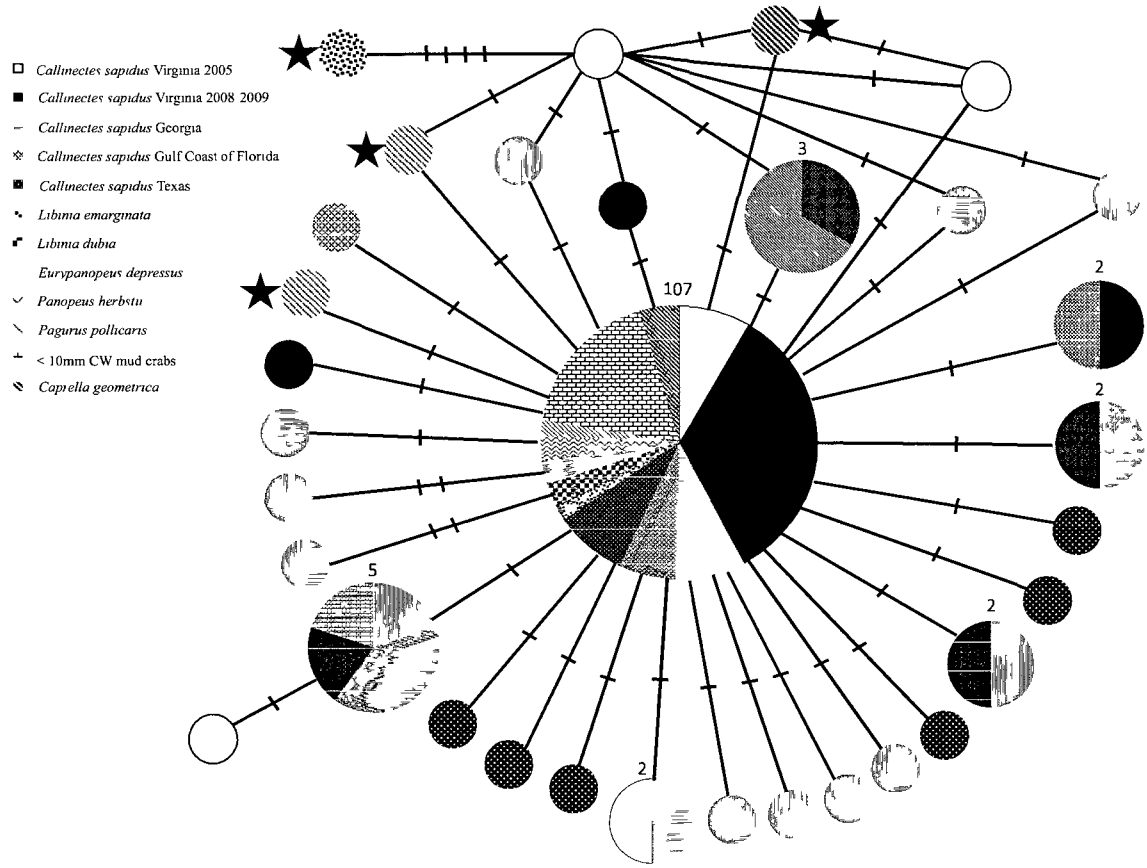


FIGURE 2.3. A modified TCS generated network using the ITS1 region sequences of *Hematodinium* sp. obtained from infected blue crabs and various alternate host species. Each dash indicates a single nucleotide polymorphism or a gap. Stars indicate the four ITS1 variants observed only in alternate host species. Numbers above the circles indicate the number of consensus sequences represented. Those circles without numbers represent only a single consensus sequence. The center circle represents an ITS1 variant common to all locations and host species.

Location Sampled		Number of blue crabs screened	Number PCR positive for *Hematodinium* sp.	Number of *Hematodinium* sp. ITS1 sequences	Number of unique *Hematodinium* sp. ITS1 sequences	Genbank Accession Numbers
Martha's Vineyard, Oyster Pond	Massachusetts	52	0	0	0	
Cociut Bay Estuary	Massachusetts	15	0	0	0	
Bass River	Massachusetts	15	0	0	0	
Agawam River	Massachusetts	15	0	0	0	
Westport River	Massachusetts	15	0	0	0	
Cherrystone Creek	Virginia	6	6	6	0	JN380146-JN380147, JN380157, JN380169, JN380091
Oyster Creek	Virginia	5	5	5	0	JN380148-JN380149, JN380162-JN380163, JN380166
Metompkin Bay	Virginia	5	5	5	0	JN380150, JN380158-JN380160, JN380165
Chincoteague Bay	Virginia	8	8	16	3	JN380144, JN380152-JN380155, JN380172-JN380176
Wachapreague Creek	Virginia	6	6	9	0	JN380151, JN380161, JN380164, JN380170-JN380172
Fisherman's Island	Virginia	6	6	6	0	JN380156, JN380167-JN380168, JN380177-JN380179
Savannah	Georgia	8	8	30	11	JN380092-JN380113
Goodland	Florida	16	2	6	0	JN380114-JN380116
Port Charlotte	Florida	3	0	0	0	
Tampa	Florida	17	1	2	0	JN380124
Panama City	Florida	21	5	13	1	JN380117-JN380123
New Iberia	Louisiana	31	0	0	0	

TABLE 2.1. All blue crabs collected or obtained for this study, as well as the number of blue crabs positive for *Hematodinium* sp. via PCR analysis and from which ITS1 sequences were generated, the number of *Hematodinium* sp. ITS1 sequences that were generated from each location, and the number of unique ITS1 sequences generated from each location.

Host species	Number PCR positive for *Iematodinium* sp.	Number of *Hematodinium* sp. ITS1 sequences	Number of unique *Hematodinium* sp. ITS1 sequences	Genbank Accession Numbers
Libinia emarginata	2	6	1	JN368149-JN368151
Libinia dubia	3	7	0	JN368152-JN368156
Pagurus pollicaris	2	8	2	JN368157-JN368160
Eurypanopeus depressus	2	3	0	JN368161-JN368163
Panopeus herbstii	3	5	0	JN368164-JN368166
Caprella geometrica	3	8	1	JN368187-JN368194
<10 mm mud crabs	22	26	0	JN368167-JN368186
Total	**37**	**63**	**4**	

TABLE 2.2: The alternate hosts that were positive for *Hematodinium* and were included in the second TCS analysis as well as the total number and the number of unique *Hematodinium* ITS1 sequences generated from each host.

	Hematodinium perezi	*Hematodinium* sp. from alternate hosts	*Hematodinium* sp. from *C. sapidus*
Hematodinium perezi			
Hematodinium sp. from alternate hosts	0.037		
Hematodinium sp. from *C. sapidus*	0.038	0.002	

TABLE 2.3. The average genetic distances calculated between three groups: *Hematodinium perezi*, *Hematodinium* sp. from alternate hosts, and *Hematodinium* sp. from *C. sapidus*. Genetic distance calculations were performed using MEGA 5.0 with no variation estimation method, the p-distance model, and gaps as missing data.

Chapter 3. Development of microsatellite markers for the dinoflagellate, *Hematodinium* sp., a parasite of *Callinectes sapidus*

Abstract

Eleven microsatellite markers were developed for *Hematodinium* sp., a parasitic dinoflagellate of the blue crab, *Callinectes sapidus*. Nine of the targeted microsatellite loci had dinucleotide repeats, one a tetranucleotide repeat and the other a pentanucleotide repeat. Perfect repeats were found at five of the loci (four dinucleotide and one pentanucleotide). The number of alleles identified per locus ranged from 1-7 and none of the markers were in linkage disequilibrium. These markers provide useful tools for elucidating aspects of the genotypic variation and population structure of this parasite.

Introduction

Hematodinium sp. is a parasitic dinoflagellate that infects blue crabs from Virginia south to Texas (Messick and Shields 2000, Chapter 2). Prior to this study, the only molecular markers available for this parasite were from the ribosomal RNA (rRNA) gene complex. Sequences from the first internal transcribed spacer (ITS1) region of the rRNA gene complex, a more variable segment of this complex, showed little variation over a large geographic region (Chapter 2). Thus, it was concluded that there is probably one species of *Hematodinium* infecting blue crabs from Virginia to the Gulf of Mexico (Small et al. 2007b, Chapter 1, 2) and that more variable molecular markers were needed to assess intra-specific variation for this parasite.

In order to examine genotypic variation and population structure of this parasite, eleven microsatellite markers were developed for the *Hematodinium* sp. that infects blue crabs using enriched microsatellite library protocols (Glenn and Schable 2005, Hamilton et al. 1999) and a non-clonal culture of *Hematodinium* sp. derived from an infected blue crab from Virginia.

Materials and Methods

Hematodinium sp. *culture collection and preparation*

A non-clonal primary culture of *Hematodinium* sp. was made from a blue crab collected via a crab pot from Wachapreague, Virginia (37° 37'38.94"N, 75° 40'40.80"W). *Hematodinium* sp. cells, consisting mainly of ameboid trophonts, were isolated from an infected blue crab. The infected hemolymph (1mL) was mixed with 9mL of modified Appleton medium (MAM) buffer, as described in Small et al. (2007c), in a sterile 75 cm^2 culture flask (Corning, Corning, New York). This suspension was incubated at room temperature (~23°C) for 30 minutes in order to allow time for the crab hemocytes to adhere to the flask wall, leaving *Hematodinium* sp. cells in suspension. After 30 minutes, the parasite-containing fluid was transferred to a new sterile flask and incubated in the same manner for another 30 minutes. This was repeated a third time in order to remove ~95-99% of the crab hemocytes from the culture. The culture was then allowed to incubate at room temperature for 15 days in MAM +FBS buffer, which was prepared according to Small et al. (2007c).

Prior to DNA extraction, the *Hematodinium* sp. culture was visually examined with an Olympus IX50 microscope for the presence of blue crab hemocytes, which were not found. No cell counts of *Hematodinium* sp. cells were obtained. *Hematodinium* sp. cells were pelleted by centrifugation for 5 minutes at 40Xg. The supernatant was removed and the cells were rinsed with 5mL of sterile filtered artificial seawater (ASW) at the same salinity as the culture media (28 psu). The cells in AWS were slowly mixed, then centrifuged again for 5 minutes at 40Xg, and the supernatant was carefully removed.

Microsatellite library construction

DNA Extraction and restriction enzyme digestion

High molecular weight DNA was extracted from the *Hematodinium* sp. cell pellet following a standard phenol-chloroform extraction protocol (Sambrook and Russell 2001). The extracted DNA was quantified using a GeneQuant Pro (Amersham Biosciences, Piscataway, New Jersey). The genomic DNA was digested separately with the restriction enzymes *Rsa* I and *Alu* I (New England Biolabs, Ipswich, Massachusetts) according to the manufacturer's recommendation and incubated for three hours at 37°C.

To ensure that all DNA was digested and of appropriate length, an aliquot of the digested DNA (4μL) was electrophoresed on a 1% w/v agarose gel and visualized under UV light after ethidium bromide staining. The fragment smear was between 400-600bp. The two digests were then combined for the remaining procedures.

The digested DNA was cleaned with a QIAquick PCR Purification Kit (Qiagen, Valencia, California) with a final elution volume of 30μL. This step removes all fragments < 100bp, which are less likely to contain the targeted microsatellite regions with sufficient flanking regions, and all fragments >10kb, which were likely to be regions that were not successfully digested. The final elution was passed over the column three times to increase DNA concentration in the final elution.

Linker ligation

The digested DNA was ligated to the universal SNX "Super" linkers described in Hamilton et al. (1999): SuperSNX24 F (5'GTTTAAGGCCTAGCTAGCAGAATC) and SuperSNX24+4P R (5'pGATTCTGCTAGCTAGGCCTTAAACAAAA). The double stranded (ds) SuperSNX linkers were created by mixing identical volumes of equal molar amounts of SuperSNX24 and SuperSNX24+4p primers (100μL of 10μM each). Salt was then added to a final concentration of 100mM (i.e. 4μL of 5M NaCl for 200μL total of the primers). The mixture was heated to 95°C, then allowed to slowly cool to room temperature.

To ligate the ds SuperSNX linkers to the digested DNA, the following reagents were combined: 17.5mM ds SuperSNX linker mix, 0.5units/μL *Xmn* I (New England Biolabs), 20 cohesive ends/μL T4 DNA ligase (New England Biolabs), 1X T4 DNA ligase buffer (New England Biolabs), 0.0625μM BSA, 0.0313 M NaCl, 1.5μL PCR quality ddH20, and 24μL of digested, cleaned genomic DNA. The ligation mix was allowed to incubate overnight at 16°C.

The success of the linker ligation was tested via PCR using 1X PCR Buffer (Invitrogen, Carlsbad, CA), 25μg/mL BSA, 0.5μM Super SNX-24F, 150μM dNTPs (Invitrogen), 2.0mM $MgCl_2$, 5units/μL Taq (Invitrogen), 2.0μL linker ligated DNA, and water to a final volume of 25μL. Thermocycling was performed using a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, Massachusetts) with an initial denaturation of

95°C for 2 minutes followed by 30 cycles of 95°C for 20 seconds, 60°C for 20 seconds, and 72°C for 90 seconds. An aliquot of the PCR product (4μL) was electrophoresed on a 1.5% (w/v) agarose gel and visualized under UV light after ethidium bromide staining. The fragment smear was between 300-600bp.

Hybridization

The ligation mix was hybridized with two oligonucleotide mixes in two separate reactions. The first consisted of a single oligonucleotide strand (GATA), and the second was mix #2 described by Glenn and Schable (2005)($(AG)_{12}$, $(TG)_{12}$, $(AAC)_6$, $(AAG)_8$, $(AAT)_{12}$, $(ACT)_{12}$, $(ATC)_8$). The hybridization conditions were as follows: 10μL of 1μM of each oligonucleotide, 25μL of 2X hybridization solution (12X SSC, 0.2% SDS; Glenn and Schable 2005), and 15μL of linker-ligated *Hematodinium* sp. DNA. Due to the low concentration of DNA used, no water was added for the hybridization. On a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, Massachusetts), both of the hybridizatons were performed using the OLIGOHYB program described in Glenn and Schable (2005).

Prior to use, streptavidin paramagnetic particles (i.e. beads; Promega, Madison, Wisconsin) were washed according to the manufacturer's recommendations. The hybridization mixtures were mixed with 3.5 times the recommended amount (350μL) of beads and incubated overnight at 42°C.

After the incubation, the beads with the hybridized DNA were washed in order to remove any weakly hybridized DNA strands and to enrich for strands containing microsatellites. Following Hamilton et al. (1999), the ligation mix and beads were placed in a magnet and allowed to sit for 1 minute, until the beads were on one side. The hybridization solution was then carefully removed with a pipette. The beads were washed twice for 5 minutes each time with 200μL of room temperature 2x SSC, 0.1% SDS. Between these two washes, the beads were resuspended then returned to the magnet for separation. At each step, the stringency of the washes was increased in order to remove DNA fragments that were not complimentary with the oligonuclotides. The beads were washed two additional times with 200μL of 2X SSC, 0.1% SDS for 5 minutes with the temperature at 45°C. As before, the beads were resuspended between washes and

returned to the magnet for separation. The final two washes were done with 200μL of 1X SSC, 0.1% SDS for 5 minutes at 45°C. This final bead-washing temperature was chosen because both oligonucleotide mixes were AT rich.

DNA was eluted from the beads by adding 200μL of preheated (95°C) T.E (10mM Tris-HCL, pH 8.0; 0.1mM EDTA, pH 8.0; Hamilton et al. 1999) and allowing the tubes to incubate in the T.E buffer at 95°C for 10 minutes. The eluted DNA was then transferred to a clean microcentrifuge tube.

Following Glenn and Schable (2005), 22μL of NaOAc/EDTA solution was added to and mixed with the eluted DNA. The DNA was then mixed with 444μL of 95% cold ethanol from the -20°C freezer and this mixture was incubated at -20°C for two hours to ensure precipitation of nucleic acids. The mixture was centrifuged at 20,000Xg for 10 minutes. The supernatant was discarded and 0.5mL of 70% EtOH was added. The mixture was centrifuged again at 20,000Xg for one minute. The supernatant was carefully removed and the remaining pellet was placed in a speed vac for 10 minutes to remove all remaining ethanol. The pellet was resuspended in 25μL of TE buffer (10 mM Tris pH8, 2 mM EDTA) and allowed to rehydrate overnight. The resuspended pellet is what Glenn and Schable (2005) refer to as "pure gold".

As no pellet was observed after the 10 minute centrifugation of the first enrichment described above, a double enrichment of the final product was performed. The second enrichment was performed in the same manner as the first using the same DNA/probe hybridization with the same oligonucleotide probe mixes, but the recommended amount of beads was used for the bead hybridization, the bead hybridization was only for 3 hours at 43°C, and the final wash temperature was raised to 50°C.

After both the single and double enrichment, the enriched DNA was subjected to PCR as described by Glenn and Schable (2005) in order to increase DNA yield. Briefly, the PCR reactions consisted of 1X PCR buffer, 25μg/mL BSA, 150μM dNTPs, 0.5μM SuperSNX-24, 2.0mM $MgCl_2$, 5 units/μL of Taq polymerase, 2.0μL of DNA fragments ("pure gold") and water to a final volume of 25μL. Thermocycling was performed using a PTC-200 Peltier Thermal Cycler (MJ Research) with an initial denaturation of 95°C for 2 minutes followed by 25 cycles of 95°C for 20 seconds, 60°C for 20 seconds, and 72°C

for 90 seconds, then 72°C for 30 minutes. An aliquot of the PCR product (4μL) was electrophoresed on a 1.5% (w/v) agarose gel and visualized under UV light after ethidium bromide staining. The fragment smear was between 400-600bp.

Cloning and sequencing

Both the singly enriched and doubly enriched final products were cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. Plasmid DNA was extracted, amplified, and sequenced as described in Chapter 1. Briefly, plasmid DNA was extracted from individual colonies following a boil-prep method, and colonies were screened using M13F (5'-GTAAAACGACGGCCAG-3') and M13R (5'-CAGGAAACAGCTATGAC-3') primers. An aliquot of the PCR products (5μL) was visualized under UV light on a 2% (w/v) agarose gel after ethidium bromide staining. PCR fragments of the appropriate size (~300-700 bp) were purified with exonuclease I (EXO) and shrimp alkaline phosphatase (SAP). The purified PCR product was used as the template for sequencing with the M13F/R primers and the Big Dye Terminator kit (Applied Biosystems, Norwalk, Connecticut) using an eighth of the of the reaction size recommended. All samples were first sequenced in the forward direction only. The sequence reactions were cleaned using an ethanol/sodium acetate protocol (ABI User Bulletin, April 11, 2002). The precipitated sequences were resuspended in Hi-Di formamide (Applied Biosystems) and electrophoresed on a 3130xl Genetic Analyzer (Applied Biosystems). All sequences were edited with CodonCode Aligner (CodonCode Corp, Dedham, Massachusetts).

After screening 161 colonies and sequencing 128 PCR fragments, 25 useful microsatellite loci were identified. These sequences contained microsatellite repeats with sufficient flanking regions on each side to design PCR primers. For these 25 microsatellites, the reverse direction was sequenced so that the forward and reverse sequences could be confirmed prior to primer design.

Sample collection, Infection status, and PCR amplification

Blue crabs were collected in 2008 and 2009 from Wachapreague Creek using commercial crab pots. Infection status was first assessed microscopically. Hemolymph

was drawn from the arthrodial membranes with a 27 ga. needle. Hemolymph smears were stained 1:1 with 0.3% Neutral red and examined with a light microscope at 100x and 400x magnification for uptake of the dye (Stentiford and Shields 2005). If *Hematodinium* sp. cells were observed microscopically, 500µL of hemolymph was mixed with 500µL of 95% ethanol and stored at -20°C for subsequent molecular analysis.

All DNA extraction, PCR amplifications, and sequencing protocols were performed as described in Chapter 1. Briefly, 200µL of hemolymph in 95% ethanol suspension was used for extraction with a Qiagen Tissue and Blood kit (Qiagen, Valencia, California) following the manufacturer's instructions for animal tissue. All extractions completed within the same day included a blank column extraction, which was subjected to PCR analysis alongside samples to serve as a control for extraction contamination. After extraction, separate aliquots (20µL) of eluted DNA were made to avoid contamination of original DNA stock material. The original DNA elutions were stored at -20°C, while separate aliquots (20µL) were stored at 4°C. Extracted DNA was quantified using a NanoDrop 2000 (Thermo Scientific, West Palm Beach, Florida).

To determine if high molecular weight DNA was extracted from the samples, a general metazoan primer set, nSSU A and nSSU B (modified from Medlin 1998, as described in Moss et al. 2007) was first used to amplify host DNA. A second PCR assay based on the ITS1 region of the parasite was used (Small et al 2007b) to confirm the presence of amplifiable *Hematodinium* sp. DNA. Only samples that were positive for both of these PCR assays were subjected to PCR amplification with the microsatellite primers (see below).

Microsatellite PCR amplifications

Both Primer3 (http://frodo.wi.mit.edu/primer3/; Rozen and Skaletsky 2000) and MacVector 8.1.2 (Accelrys Inc., San Diego, California) were used to design microsatellite primers to target the flanking regions of microsatellite regions. To assess if there was any difference in the amplification success of the primers given by the two programs, the best primer set suggested by each program was tested for the first four markers. As no difference was detected in the ability of either program to produce

primers that successfully amplified fragments from the samples, only the best primer set suggested by either program was used.

In order to determine the optimal annealing temperature for each primer set, microsatellite PCR assays were first subjected to an annealing temperature gradient using a Peltier MJ Thermocycler. The initial sample panel consisted of three samples from blue crabs infected with *Hematodinium* sp., as confirmed by PCR, and one sample from a blue crab that was not infected with *Hematodinium* sp., as confirmed by PCR. The uninfected blue crab sample was used as a negative control to ensure that the microsatellite primers did not amplify blue crab DNA at any temperature. Thermocycling was carried out using the following final concentrations: 1X PCR Buffer (Invitrogen), 1.5mM MgCl2, 0.2mM dNTP (Invitrogen), 0.25μM of the T3 labeled forward primer, 1μM of the reverse primer, 0.025units/μL of Taq polymerase (Invitrogen), 0.1μM of T3 fluorescent primers (Applied Biosystems), 0.5μL of DNA (between 16.4-526.1 ng/μL) with water added up to 5μL. Thermocycling conditions were as follows: initial denaturation at 94°C for 5 minutes, 35 cycles of 94°C for 30 seconds, gradient from 50-62°C for 30 seconds, 72°C for 1 minute, and a final extension of 5 minutes at 72°C.

After PCR amplification, 1.3μL of microsatellite PCR was combined with 8μL of HiDi formamide and microsatellites were run on a 3130xl Genetic Analyzer (Applied Biosystems, Norwalk, Connecticut) with a GeneScan 500 LIZ size standard (Applied Biosystems). The microsatellites were scored using GeneMarker 1.75 (SoftGenetics LLC, State College, Pennsylvania).

For all the primers that amplified DNA fragments from blue crabs infected with *Hematodinium* sp. and not the uninfected blue crab, a panel of 15 samples was subjected to amplification with all microsatellite markers to assess the amplification success, ease of scoring, and level of polymorphism for each marker. All markers that were easy to score and demonstrated reliable amplification were used to amplify all 40 samples from Wachapreague Creek.

Linkage disequilibrium was calculated with Genepop (http://genepop.curtin.edu.au/; Raymond and Rousset 1995) using only those samples that showed only a single allele for every microsatellite marker with 10,000 iterations per batch, 1,000 batches, and 10,000 dememorizations.

Cloning and sequencing of microsatellite DNA fragments

In order to verify that the microsatellite primers were amplifying the appropriate microsatellites, two samples (one sample showing one allele per locus and one sample showing more than one allele per locus) for every polymorphic marker and one sample for each monomorphic marker were cloned as described above. For the samples showing more than one allele per locus, 8-16 clones were sequenced. For the samples that showed only a single allele per locus, 4-8 clones were sequenced. The sequences obtained were compared to the sequences used to design the primers for each microsatellite marker. Primers were discarded if they amplified sequences that were different from that of the original sequence that were within the size range of the microsatellite, and had sufficient amplification that they could be scored, as these primers were suspected of amplifying multiple loci. Primers were deemed acceptable if they amplified sequences different from the original sequence, but these sequences were either much larger or much smaller than the size range of the microsatellite or the sequences did not correspond to any scoreable peaks on the microsatellite electopherogram, as only the loci of interest would be scored. All primers that amplified sequences that matched the sequence used to create the primers were deemed acceptable.

Results

Of the 24 microsatellite loci identified, primers of 21 of the loci were successfully used to amplify microsatellites from infected crab hemolymph. Of those, eleven microsatellite loci were sufficiently easy to score and sequencing demonstrated that the appropriate region was being amplified (Table 3.1). The other thirteen microsatellite loci were excluded due to poor amplification success, evidence of amplifying multiple loci, or difficulty in scoring.

Eleven microsatellite markers were designed and optimized for the species of *Hematodinium* infecting *C. sapidus*. None of the microsatellite primers amplified DNA from uninfected blue crab samples, indicating that these microsatellite primers are most likely amplifying *Hematodinium* sp. DNA and not the host DNA. For further confirmation, a BLAST search of the NCBI databases with the microsatellite sequences was conducted and no matches to any published sequences were indicated. To ensure that

the same sequence used to create the primers was being amplified, 301 clones were sequenced. Of those, only 5% (n=15) were not the correct sequence; however, in every case these other sequences were either too large or two small to be scored and none ever produced a sufficient allelic signal to be scored on the electropherogram. The other sequences confirmed that the alleles being amplified from infected blue crab hemolymph did match the original sequences used to design the microsatellite primers (National Center of Biotechnology Information, JN403088-JN403371).

Nine of the microsatellite loci had dinucleotide repeats, one marker had a tetranucleotide repeat, and another had a pentanucleotide repeat. Only five of the loci (four dinucleotide and the one pentanucleotide) had perfect repeats. Eight of the markers were polymorphic with a range of 2-7 alleles. Three markers were monomorphic. The monomorphic markers were included as the global variation in this parasite is unknown and it is possible that these markers, though monomorphic in *Hematodinium* sp. from blue crabs in Virginia waters, may be useful for elucidating differences in this parasite between wider geographic regions, such as Georgia or the Gulf of Mexico.

After Bonferroni correction, none of the markers were in linkage disequilibrium. This indicates that the loci appear to be randomly assorted and are probably not on the same chromosomes.

Discussion

Eleven microsatellite markers were successfully used to amplify microsatellites of *Hematodinium* sp. from total genomic DNA extracted from the hemolymph of infected blue crabs. The numerous sequences generated verified that the primers for these eight markers are amplifying the appropriate microsatellite regions and further testing indicated that the DNA being amplified most likely belongs to the parasite and not the host. Additionally, none of the markers were found to be in linkage disequilibrium, which is further evidence that these markers will be useful for assessing the intra-specific variation of the species of *Hematodinium* that infects the blue crab. These markers will be used to elucidate aspects of the population structure and genotypic variation of this parasite in the blue crab host, as well as provide information on aspects of the life history traits of this

parasite including ploidy, multiple infections, and whether the parasite has a sexual or asexual population structure.

TABLE 3.1. PCR primers developed for amplifying the eleven microsatellite loci identified in *Hematodinium* sp., a parasitic dinoflagellate that infects *C. sapidus* from Virginia. T_a refers to the annealing temperature used for PCR amplification of each locus.

TABLE 3.1

Locus	Repeat motif	Primer Sequence (5'-3')	T_a (°C)	No. of non-amplifying samples	No. of alleles	Size range (bp)	Genbank Accession No.
HG2_45	$(CA)_8(CG)_1(CA)_5(CG)_1(CA)_2$	F:T3-AAAGGGTGCATCTCTATACTGC R: ACCCGTGAAATCACGAAAGG	53	0	7	213-225	JN403077
H21_1&5	$(GAGAA)_{10}$	F: T3-AATAGTATGCGACGGAAGACG R: CCACCTCATCCCTTCTCG	52	3	7	205-235	JN403078
HG2_40	$(GT)_{13}$	F: T3-GTGTGCTATTGCTGTGAATGC R: GACGTTTTGGCCAGACAGAG	55	0	6	166-174	JN403079
HG2_6	$(GATA)_{12}(CATA)_8$	F: T3-TCTCCTCCTCGTGTTTTGTTTTTC R: CAACTCCATTCATATCTTCCTGGC	55	0	5	337-363	JN403080
HG2_33	$(GC)_1(GT)_8$	F: T3-CAGTGGGGTGATTAGCATGAGTG R: CGCCTGCCTTTATCGTGAGATAC	61	5	2	438-441	JN403081
HG2_10&11	$(GT)_5(GC)_1(GT)_9$	F: T3-GCTCTCGTGATGTGCATTGAGG R: TCTGGGCGAATAAGTGTGTTCAC	55	4	2	229-236	JN403082
HG2_46	$(CA)_7(TA)_1(CA)_7$	F: T3-CTGTAAGTGTTAGAGGCGATAGG R: CGCGAGAGAAAATACATGAGG	53	0	3	236-240	JN403083
H22_46	$(GT)_1(GC)_1(GT)_{18}$	F: T3-AACCATAGAAGCGGTGCAGCAAGC R: ACCTATTCAGGAATGAGGGAACG	58	1	5	217-225	JN403084
H22_5_2	$(CA)_7$	F: T3-CTACAACACCAAGGAGGTTGC R: TCAGGAAATGCGCTTTTAGG	55	1	1	222	JN403085
H21_9	$(GT)_6$	F: T3-TGTCGTAAACTTTTCGTG R: TTTTGTTCACGCGACACTTC	52	1	1	173	JN403086
H21_1	$(GT)_7$	F: T3-AGAAGGACGGCAGGTCAAC R: TCAAGGATGGATGGAAACAG	55	2	1	222	JN403087

Chapter 4. Microsatellite allele frequencies of *Hematodinium* sp. along the Delmarva Peninsula, Virginia

Abstract

Hematodinium sp. is a parasitic dinoflagellate that has been reported in blue crab populations in the USA along the eastern seaboard and Gulf of Mexico. In order to gain insight into the ecology and evolution of this parasite, primers for eleven microsatellite markers were used to amplify DNA fragments of *Hematodinium* sp. from infected blue crabs collected from six sites along the Delmarva Peninsula, Virginia between 2008-2009. A comparison of the allele frequencies showed mixing between sites, as most sites were dominated by the same one or two alleles per locus. Five of the six sites had a few private alleles, which were most likely due to insufficient sample sizes. For three sites that were sampled in both 2008 and 2009, no major shifts in allele frequencies were observed. Analysis of the number of alleles obtained per locus indicated that the life stages of *Hematodinium* sp. observed in the host hemolymph are most likely haploid as the majority of the samples showed only a single allele at all eight polymorphic loci examined. At the same time, there was a high occurrence of multiple infections occurring in this system, with a maximum of seven alleles amplified from one locus in the parasite DNA from a single host. This is the first evidence indicating that the parasite cells observed in the hemolymph are haploid and that multiple infections occur in this system.

Introduction

Hematodinium sp. is a parasitic dinoflagellate that infects the American blue crab, *Callinectes sapidus,* most often in high salinity waters (for review, see Stentiford and Shields 2005). Infections in blue crab populations have been reported from Delaware to Texas (Newman and Johnson 1975, Messick 1994, Messick and Shields 2000, Gruebl et al. 2002) and sequence data from the first internal transcribed spacer (ITS1) region of the

ribosomal RNA (rRNA) gene complex of the parasite genome indicate that the same species of *Hematodinium* infects blue crabs from at least Virginia to Texas (Chapter 2). As the majority of blue crabs naturally and experimentally infected with *Hematodinium* sp. do not survive (Messick and Shields 2000, Shields and Squyars 2000), it is likely that areas with a high prevalence of *Hematodinium* sp. probably suffer significant crab mortality due to disease.

Most research on *Hematodinium* sp. has focused on pathological and physiological impacts of the parasite on the blue crab host and epizootiology studies of this parasite in different blue crab populations. While this work has been essential to our understanding of how *Hematodinium* sp. affects blue crabs, some fundamental questions remain, such as whether the parasite is haploid or diploid (ploidy), the complete life cycle in the host as well as in the environment, whether or not simultaneous infections of multiple genetic variants occur within the same host individual, and the level of genotypic variation for this parasite species. Previous work has shown that the same species of *Hematodinium* is capable of infecting multiple crustacean hosts along the Delmarva Peninsula and occurs in blue crab populations from Virginia to Texas (Chapters 1, 2). However, population level variation within this species could not be assessed with the previous work, because sequences from the ITS1 region did not show sufficient variation to separate populations or genetic strains of the parasite.

Research has demonstrated that sequence data from the ITS1 region, and other portions of the rRNA gene complex, were not sufficient for assessing intra-specific variation in other dinoflagellate species, so more variable markers, such as microsatellites, were needed (Nagai et al. 2009, Masseret et al. 2009, McCauley et al. 2009). Microsatellites are simple sequence repeats that are generally found in the nuclear genome and are characterized by high levels of polymorphism resulting from high mutation rates (for review, see Li et al. 2002, Ellegren 2004). Microsatellites are believed to be selectively neutral and have been used to examine life history characteristics and population structure in a variety of organisms. For example, Nagai et al. (2009) used ten microsatellite markers to genotype *Cochlodinium polykrikoides* from eleven sites around Japan and Korea to demonstrate the presence of three distinct populations that were not differentiated with rRNA gene sequences. Masseret et al. (2009) reported that rRNA

sequence data from *Alexandrium catenella* from the French Mediterranean confirmed previous reports that it originated in Asia. However, twelve microsatellite loci revealed much higher levels of genetic diversity in this dinoflagellate and indicated that the population in the French Mediterranean probably did not originate in Asia. McCauley et al. (2009) used the large subunit (LSU) rRNA gene and ITS1 region sequences to partition *Alexandrium minutum* populations into a "Global" clade and a "Pacific" clade. However, with 12 microsatellite markers that amplified *A. minutum* from the "Global" clade, these authors were able to genetically distinguish *A. minutum* genotypes from different geographic locations.

Likewise, sequence data from the rRNA gene complex has been useful for designating eight clades (A-H designations) of *Symbiodinium* spp. (Baker 2003, LaJeunesse 2001, 2005, Sampayo et al. 2009), zooxanthellae that inhabit the soft tissue of various cnidarians and other invertebrates. However, microsatellite markers were necessary for the examination of genotypic variation and population structure within the clades of *Symbiodinium* spp. Kirk et al. (2009) used six polymorphic microsatellite loci to show that *Symbiodinium* type B1/B184 inhabiting *Gorgonia ventalina* had population differentiation, due to low levels of gene flow, between 14 locations and deep and shallow sites on the same reef within the Florida Keys. Thornhill et al. (2009) used three microsatellite loci for *Symbiodinium* type B1 inhabiting *Montastraea faveolata* and *M. annularis* to show little dispersal of this symbiont among five reefs in the Florida Keys and the Bahamas. Magalon et al. (2006) used two microsatellite loci to determine that *Symbiodinium* sp. inhabiting *Pocillopora meandrina* in Polynesia only had genetic differentiation on a large geographic scale (>2000 km).

The use of microsatellite markers has also led to insight into life history aspects of dinoflagellate and parasite species, such as ploidy and whether or not simultaneous infections by multiple genetic strains can occur. Ploidy can be inferred based on the results of microsatellite analyses. If one or two alleles are amplified with multiple microsatellite markers, each of which is assumed to target a single-copy locus, from the majority of samples, then the organism is likely diploid at that life history stage. Likewise, if only one allele is amplified at all loci examined from the majority of samples, assuming that multiple markers are used, then the organism is probably haploid

at that life history stage. Consequently, multiple infections occur when more alleles are observed at a particular locus than would be expected based on the ploidy of that organism. Microsatellites have been used to infer that *Symbiodinium* spp. inhabiting gorgonian corals are haploid (Santos and Coffroth 2003). Microsatellites were also used to show that cysts of *A. catenella* were four times more likely to be heterozygotes than cell cultures created from the planktonic cell form, adding evidence to the hypothesis that the cyst stage for this dinoflagellate is diploid (Masseret et al. 2009). Additionally, microsatellites have been used to confirm ploidy and multiple infections for numerous protozoan parasites (Babiker and Walliker 1997, Oliveira et al. 1998, Anderson et al. 2000, Ajzenberg et al. 2002, Vardo-Zalik et al. 2009, Balmer et al. 2009).

In this study, primers designed for eleven microsatellite markers were used to amplify DNA fragments from *Hematodinium* sp. from infected blue crabs collected from six sites along the Delmarva Peninsula. Data obtained from the microsatellite loci was used to elucidate some fundamental life history aspects of the parasite including ploidy and whether or not multiple infections occur. Additionally, allele frequencies were calculated and allele distributions were examined for trends or differences among samples collected along the Delmarva Peninsula, Virginia.

Materials and Methods

Infected blue crabs were collected from six sites along the Delmarva Peninsula, Virginia as described in Chapter 1 (Figure 4.1). Infection status was ascertained and hemolymph samples from infected crabs were procured and handled as described in Chapters 1 and 2. Infection intensity and parasite life stages were assessed as described in Chapter 1.

ITS1 and microsatellite PCR amplification

All DNA extractions were conducted as described in Chapter 1. Briefly, total DNA was extracted from 200µL of hemolymph in 95% ethanol suspension with a Qiagen Tissue and Blood kit (Qiagen, Valencia, California) following the manufacturer's instructions for animal tissue. All extractions completed within the same day included a blank column extraction, which was subjected to PCR analysis alongside samples to

serve as a control for extraction contamination. After extraction, separate aliquots (~20 μL) were made to avoid contamination of original DNA stock material. Stock DNA was stored at -20°C and aliquot DNA stored at 4°C. DNA extractions were quantified using a NanoDrop 2000 (Thermo Scientific, West Palm Beach, Florida).

Before samples were subjected to amplification with the microsatellite PCR assays, all DNA samples were subjected to amplification as described in Chapter 1 using both the nSSU PCR assay (modified from Medlin 1988) and the *Hematodinium* sp. ITS1 PCR assay (Small et al. 2007b) to ensure that the DNA was of high quality and that amplifiable *Hematodinium* DNA was present. Only samples that were positive for both of these PCR assays were subjected to amplification with the microsatellite PCR assays.

Microsatellite PCR conditions were conducted as described in Chapter 3, with the exception that Platinum Taq (Invitrogen, Carlsbad, California) was used if amplification with Taq polymerase (Invitrogen) was unsuccessful or had an insufficient signal on the electropherogram. Some additional alleles were cloned and sequenced as described in Chapter 3 to confirm that target loci were amplified. As discussed in Chapter 3, only the microsatellite primers that amplified the same microsatellite region that the primers were developed from, as demonstrated via cloning and sequencing, were used in this study.

Microsatellite data analyses

Allele sizes were resolved using a 3130xl Genetic Analyzer (Applied Biosystems, Norwalk, Connecticut). Allele size determinations were made using GeneMarker 1.75 (SoftGenetics LLC, State College, Pennsylvania). The minimum detectable peak height was set at 100 arbitrary fluorescence units (FU) and all alleles were verified by visual inspection. Due to the low signal intensity observed from many of the samples, issues with stutter bands, and the frequency of more than two alleles being present, the majority of samples were amplified at least twice and up to five times per marker. To ensure consistency in scoring, the shape and signal strength of the electropherograms was evaluated by visual inspection for each scoring attempt. To ensure consistency in scoring, a single person scored the microsatellites every time. In an effort to obtain the most accurate allele scores, all of the electropherograms for each marker per sample were

compared simultaneously and a final allele score was determined before conducting data analyses.

To calculate error rates for microsatellite scoring, 20% (n=47) of the samples were amplified an additional time using the PCR amplification described above with the exception that Platinum Taq (Invitrogen) was used for all markers. Error rates were calculated as the percentage of re-run samples that did not have matching allele scores compared to previous runs. In order to determine a range in error rates, the error rates were calculated two different ways. The first calculation included the allele scores from the re-run samples compared to the initial allele scores for those samples. The second calculation included the allele scores from the re-run samples compared to the final allele scores, which were determined only after all the alleles had been rescored using all available electropherograms for each marker per sample.

Allele frequencies were calculated manually in Microsoft Excel. The allele frequencies were calculated by assuming that all discernable peaks viewed in an electropherogram represented the total number of alleles present (i.e. each peak = 1 allele), which is a biased measure because it underestimates the more frequent alleles. Allele frequencies were calculated independently for the three sites sampled in both 2008 and 2009 (Chincoteague Bay, Wachapreague Creek, and Fisherman Island), so allele frequencies could be compared between years. In this study, a private allele was defined as an allele that was only observed in samples from a single site and a rare allele was an allele that was observed 10 times or less among all sites.

Life stage of the parasite and intensity of infection were compared to the number of alleles and tested with a χ^2 analysis to determine any trends. The life stages of the parasite and the semi-quantitative scale for parasite intensity were described in Chapter 1. As multiple life history stages were often observed, that additional category was added to the analyses for this study.

Results

In total, 286 infected blue crabs were collected from six sites along the Delmarva Peninsula between 2008 and 2009. *Hematodinium* sp. DNA was not amplified from nine samples using the ITS1 PCR assay and five samples using the nSSU PCR assay; thus,

these samples were excluded from further analyses. Of the 272 DNA samples that were initially subjected to microsatellite amplification, an additional 45 samples were excluded for failure to amplify at more than 6 of the 11 microsatellite loci, leaving a total of 227 samples that were used in the data analysis. Of the 59 samples excluded, three of the samples did not include notations of whether or not the host was alive or dead when collected, though one of those samples was listed as a light infection. Additionally, of those samples with notations about the status of the host or the intensity of the infection, 73% (n=41) of the samples that were excluded from the analysis were listed as either light infections or the blue crab was dead when the hemolymph was collected.

Of the eleven microsatellite markers used, three were monomorphic for all samples examined and were not included in the subsequent analyses. The total number of alleles detected was 9, 8, 6, 7, 3, 4, 3, and 7 for the eight polymorphic loci, HG2_45, H21_1&5, HG2_40, HG2_6, HG2_33, HG2_10&11, HG2_46, and H22_46, respectively (Table 4.1, Figure 4.2).

A single allele was observed at all eight polymorphic loci examined for 58% of the 227 samples that were used in the analyses (Table 4.2), which is significantly more single allele infections than expected if the samples were evenly distributed with respect to the number of alleles (χ^2= 27.83, df=2, p<0.0001). Additionally, 21% showed two alleles at some of the loci examined and 21% showed three or more alleles at some loci. The maximum number of alleles observed for a single locus was seven. Cherrystone Creek had the highest percentage (77.8%) of samples with only a single allele at each locus (Table 4.2). Wachapreague Creek had the highest proportions of samples with 1-2 alleles (28.3%) and also of samples with 1-3 or more alleles (30%; Table 4.2).

All life history stages (Table 4.3) and intensities (Table 4.4) were observed in every allele number category. Regardless of the life history stage, most of the samples had single allele infections; however, there was no significant difference between the number of alleles and the life history stage (χ^2= 5.22, df=4, p=0.266). Regardless of the parasite intensity, most samples had single allele infections. In this instance significantly more single allele infections were observed in those samples with light infections (χ^2= 13.152, df=4, p=0.011).

There was no obvious pattern in allele frequencies among the six sites. Five microsatellite loci (HG2_6, HG2_33, HG2_10&11, HG2_46, and H22_46) had one or two alleles most often observed, regardless of the collection site (Figure 4.2). While most of the alleles observed were found in all of the sites sampled, there were some rare alleles and these rare alleles were not always observed in neighboring sites. For HG2_45, three rare alleles were observed (Figure 4.2A). For H21_1&5, two rare alleles were observed (Figure 4.2B). For HG2_40, two rare alleles were observed (Figure 4.2C). For HG2_33, one rare allele was observed (Figure 4.2E). For HG2_10&11, one rare allele was observed (Figure 4.2F). For HG2_46, one rare allele was observed (Figure 4.2G). For H22_46, three rare alleles were observed (Figure 4.2H).

At least one private allele was observed at each site, with the exception of Oyster Creek (Figure 4.2). Chincoteague Bay had two private alleles: 352 from HG2_6 and 227 from HG2_10&11. Metompkin Bay had three private alleles: 176 and 164 from HG2_40 and 355 from HG2_6. Wachapreague Creek had two private alleles: 221 from HG2_45 and 225 from H22_46. Fisherman Island had two private alleles: 227 from HG2_45 and 227 from H22_46. Cherrystone Creek had one private allele: 215 from H22_46.

There were no major shifts in the allele frequencies when compared between years at the three sites where samples were taken in 2008 and 2009 (Figure 4.3). In some cases, alleles were observed in one year but not the other year at a particular site; however, in most of these instances the allele in question was seen at low frequencies in only one of the two years, and the lack of detection in the other year was likely due to insufficient sample size (see Chapter 5).

Some obvious trends appeared when the error rates were calculated. First, the error rate calculated for the final allele scores was generally lower than the error rate calculated from the original allele scores. Second, error rates were solely driven by the errors associated with allele scoring for those samples that showed 2 or more alleles for some of the loci examined. The error rates for those samples that only had a single allele for every locus had an error rate of 0% for all loci (Table 4.1). The scoring errors associated with those samples that had 2 or more alleles for some of the loci consisted solely of whether or not the same number of alleles were scored each time. Determining

the presence of 2 or more alleles was highly contingent upon the signal strength of the alleles when scored, which tended to be lower when 2 or more alleles were present.

Discussion

Eight microsatellite loci were used to examine ploidy, multiple infections, and the distribution of alleles of *Hematodinium* sp. from infected blue crabs collected from six sites along the Delmarva Peninsula, Virginia. Given that 58% of all samples examined had only a single allele at all loci examined, it appears that all life history stages of *Hematodinium* sp. observed within blue crab hemolymph are most likely haploid. Multiple infections were common in this system as 42% of the samples showed 2 or more alleles for at least one of the microsatellite loci examined. Additionally, the distribution of the allele frequencies suggested mixing between the different coastal embayments, as the same alleles were generally observed at all sites and even rare alleles were observed in multiple and sometimes non-neighboring sites.

Evidence for haploidy and multiple infections in host hemolymph

The determination of ploidy is essential for understanding genome evolution, determination of sexual stage, and interpretation of population genetic data; however, prior to this study, the ploidy of *Hematodinium* sp. was unknown. The only evidence regarding ploidy for any species of *Hematodinium* was data indicating that the macrospore, microspore, and vegetative stages of the parasite all contained approximately the same amount of DNA, indicating that all of these stages were most likely the same ploidy (Eaton et al. 1991). Previous research used microsatellite data to infer that *Symbiodinium* spp. are haploid when residing within coral tissue (Santos and Coffroth 2003) and that *A. catenella* cysts are most likely diploid, while the planktonic stage is most likely haploid (Masseret et al. 2009). The microsatellite data in this study indicate that *Hematodinium* sp. is most likely haploid within the blue crab hemolymph as the majority of samples (58%) had a single allele at all microsatellite loci. There are other possible, but more unlikely explanations for this observation. First, this observation could occur with an extremely high scoring error. The error rates calculated for this study were generally low, but more importantly, the error rates for those samples with only one allele

per locus were zero. A second possible explanation is a high number of null alleles, which would result if mismatches occurred in the primer binding sites of one of the two alleles present, so that only one allele is scored when more than one allele is actually present. While null alleles may be present in this data set, the likelihood that null alleles existed at all eight loci for 58% of the samples is extremely low. Dakin and Avise (2004) found that when null alleles were reported, they were reported at relatively low frequencies (2-4%). Lastly, fixed homozygosity at these microsatellite loci could account for the observed data. However, this explanation is also unlikely because if *Hematodinium* sp. was diploid and primarily clonally reproducing, then it should show fixed heterozygosity. This is because the only genetic variation would be from mutations that would become fixed in a primarily clonal organism (Tibayrenc et al. 1990, Tibayrenc and Ayala 2002). Another possible means of acquiring a high level of fixed homozygosity is through inbreeding; however, inbreeding seems improbable given the high level of genotypic variation in this parasite (see Chapter 5). Thus, *Hematodinium* sp. is most likely haploid in the blue crab host. Additionally, these data support the assertion of Eaton et al. (1991), that all parasite stages observed in blue crab hemolymph are most likely haploid.

Multiple infections of different genotypes have been observed in *Symbiodinium* spp. (Coffroth and Santos 2003, Howells et al. 2009, Magalon et al. 2009) and in *Perkinsus marinus*, a parasitic protozoan of bivalves (Reece et al. 2001, Thompson et al. in press). Microsatellite markers have elucidated multiple infections of other protozoan parasites including *Plasmodium mexicanum* (Vardo-Zalik et al. 2009), *Plasmodium falciparum* (Babiker and Walliker 1997; Anderson et al. 2000), *Toxoplasma gondii* (Ajzenberg et al. 2002), *Trypanosoma cruzi* (Oliveira et al. 1998), and *Trypanosoma brucei* (Balmer et al. 2009). The present study indicated that multiple genotypes of *Hematodinium* sp. are capable of co-occurring within a single crab host; however, the effects of a single versus a multiple infection on the host are unknown. It is possible that infection with multiple parasite genotypes or strains could be more detrimental to the host as a result of intra-specific competition for host resources (van Baalen and Sabelis 1995, Mosquera and Adler 1998). However, studies have also shown that intra-specific competition between protozoan parasites can lessen the virulent impacts of the parasites,

causing a multiple infection to be equal to or more beneficial for the host than a single infection (Brown et al. 2002, Vardo-Zalik and Schall 2008, Balmer et al. 2009). While previous research has shown that being infected with *Hematodinium* sp. is detrimental and most likely deadly for a crab host (Shields and Squyars 2000), no laboratory experiments have included genotyping *Hematodinium* sp. to compare the potential impacts of multiple vs. single genotype infections on blue crab survival or disease progression.

Allele frequencies: mixing vs. isolation

Several pieces of evidence demonstrate mixing of *Hematodinium* sp. among the sites sampled along the Delmarva Peninsula. First, the same common alleles dominated in all sites examined, with little difference in the frequency of these common alleles among the sites. This suggests that sufficient mixing among the sites exists to maintain a relatively similar level of allele frequencies, which was also observed temporally for the three sites that were sampled over two years. Temporal stability of microsatellite loci has been observed for *A. minutum* over 14 years (McCauley et al. 2009) and *Symbiodinium* sp. over 3 years (Santos et al. 2003). In this study, some rare alleles were observed in multiple sites, including non-neighboring sites, another indication that genotype mixing is probably occurring among all locations along the Delmarva Peninsula. To maintain a panmictic population along the Delmarva Peninsula, *Hematodinium* sp. is either moving or being transported between the coastal embayments at a rate that results in genetic homogeneity throughout the coastal embayments. However, this movement does not necessarily have to involve a large number of parasite cells, as previous studies have shown that even a few migrants can substantially homogenize populations (Hartl and Clark 2007).

The evidence for population isolation was detection of private alleles in all but one site. While these may represent true private alleles, other evidence makes this unlikely. Specifically, the level of genetic variation observed was much greater than anticipated and additional analyses demonstrated that the sampling effort was insufficient for capturing the genetic variation in this parasite (see Chapter 5 for further explanation). Thus, while these private alleles may be due to a degree of genetic isolation, it is more

likely that these private alleles would be observed in other locations with additional sampling.

Guidelines for amplifying parasite microsatellites from infected host tissue

Amplifying parasite microsatellites from infected host tissue will probably continue to become a more commonly used procedure and it is already frequently used for assessing population structure in *Symbiodinium* spp. (Santos and Coffroth 2003, Magalon et al. 2006, Howells et al. 2009, Thornhill et al. 2009, Kirk et al. 2009) and in *Perkinsus* spp. (Thompson et al. in press, Vilas et al. in press). Based on the unexpected issues that arose during this project, I present an assessment of the pros and cons of this technique, along with a set of guidelines to follow if this technique is deemed appropriate for a given study.

There are two main alternatives to amplifying parasite microsatellites from infected host tissue. One alternative would be to micro-manipulate individual cells and remove them from infected host tissue, blood, or hemolymph, as has been described for *P. marinus* (Bushek et al. 2000). Microsatellites could be amplified from the individual cells, which was described for a free-living dinoflagellate, *Lingulodinium polyedrum* (Frommlet and Iglesias-Rodríguez 2008). However, for microsatellite loci used in population genetic studies there is generally only a single copy of the locus in a given cell, making the amount of starting material available for primer binding quite low. This technique also requires access to the equipment necessary to micro-manipulate cells and could be difficult depending on the size of the parasite cell in question. A second alternative would be to create multiple clonal cultures per infected host and then amplify microsatellites from the clonal cultures. This can be extremely time consuming, can create a space issue for the large number of clonal cultures that would need to be created, and it can be prohibitive if sufficient manpower is not available to assist in creating the clonal cultures at the time that infected hosts are being sampled.

Amplifying parasite microsatellites directly from infected host tissue can have certain benefits. It has the potential to be more cost effective and less time consuming than creating clonal cultures or micro-manipulating parasite cells. Additionally, depending on the purpose of the study, one could also process more samples of infected

hosts to assess the genetic variability of the parasite among host individuals vs. sampling more parasite cells from a single host individual to assess the genetic variability within a host. Additionally, this technique is essential for those parasites, such as *Hematodinium* spp. and *Symbiodinium* spp. that are not amenable to clonal culturing.

While the microsatellite data presented in this study has elucidated various important aspects of the life history of *Hematodinium* sp., this technique is certainly not without major drawbacks. First, the majority of samples in this study had to be amplified and scored multiple times (up to five times for one marker for one sample) because of low signal on the electropherogram after amplification. The low signal was most likely due to the fact that parasite microsatellites, presumably single copy sequences, were being amplified from samples with a substantial amount of host DNA. The use of Platinum Taq (Invitrogen) combined with an increased amount of DNA template was the most successful method of increasing the microsatellite signal; however, it was not successful in all cases. The varying intensity and number of different genotypes within a single host led to numerous problems with microsatellite amplification including low signal for all alleles amplified from samples with multiple infections. Additionally, the difficulty in determining the difference between a stutter band and a true allele was apparent in the comparison of the error rates calculated for the multiple infections. It was impossible to know if the low signal of a particular allele was due to a primer binding issue, lower intensity of a particular genotype, or if the allele in question was actually a stutter band and not a true allele. With an unknown number of alleles, scoring was quite daunting. Strict guidelines had to be developed to assess the difference between a stutter band and a true allele. These guidelines included carefully assessing the shape of each suspected allele on the electropherogram, considering the signal strength, and re-running samples where determinations could not made with confidence. Hence, all the alleles were scored multiple times, most samples were subjected to PCR amplification multiple times, and a final score was only determined after all electropherograms from all the microsatellite runs were compared.

The issues with microsatellite amplification from multiple infections have been addressed most thoroughly for studies that have used microsatellites to amplify *Plasmodium* spp. Laboratory simulations have been conducted where DNA from

previously genotyped clonal cultures were mixed together, at either similar or different (1:10) concentrations, and microsatellites were then amplified from these mixed samples to determine the efficiency of amplification (Havryliuk et al. 2008, Vardo-Zalik et al. 2009, Ford et al. 2010). Each study showed reproducibility, as the samples were run two or three times. All the studies demonstrated that the ability to amplify the known alleles depended on the microsatellite marker and the concentration of target DNA present. Most of the studies purposely chose clones with alleles that had very different sizes and the researchers knew what alleles were supposed to be present, which is generally not the case with field collected samples. All of these studies combined DNA from culture material, which dismisses the issue of having to amplify the parasite DNA in the presence of host DNA. Additionally, one study reported that similar amounts of DNA can cause different peak heights on the electropherogram, which means that peak intensity cannot be used to correlate with parasite intensity (Ford et al. 2010) nor should multi-locus genotypes be assembled from a sample that clearly has a multiple infection, as it is impossible to determine which alleles belong to the same parasite individual (Havryliuk et al. 2008). These studies demonstrate that the technique is possible, relatively reproducible, and informative if the appropriate precautions are taken when scoring the alleles.

Given the above benefits and caveats of this technique, the following guidelines are proposed for amplifying microsatellite loci of parasites from host tissue. First, when amplifying parasite microsatellites from infected host tissue, one should always use more template than a traditional microsatellite PCR and a high-fidelity Taq polymerase. In this study, two times the amount of DNA template, compared to what has traditionally been used to amplify microsatellites from eastern oysters (Reece et al. 2004), and Platinum Taq (Invitrogen) provided increased signal strength in the microsatellite electropherograms. When scoring microsatellite alleles, allele scores must be visually confirmed, with strict attention paid to the signal strength and shape of the electropherogram. All samples should be run multiple times to assess the consistency of the microsatellite amplification, which is a commonly used method for assessing consistency when amplifying microsatellites from samples collected using noninvasive sampling methods (Taberlet et al. 1999, Pompanon et al. 2005). For example, in studies

where animal tracking is conducted for rare or difficult to capture animals, researchers will sample noninvasively by collecting samples of fecal piles, shed hair, or feathers. After DNA extraction, microsatellites designed for the organism of interest are used to amplify DNA from the samples. With this type of sampling, there is usually DNA from other organisms present in addition to the target DNA, as is the case in parasite amplification from host tissue. In some studies, multiple samples are taken initially and are run simultaneously to ensure that both samples yield the same result. Alternatively, the microsatellite loci can be amplified multiple times from the same sample until the exact same electopherogram is observed multiple times. This is in contrast to what was done in this study, as samples were re-amplified multiple times until the scorer had sufficient confidence in scoring a single electropherogram. Ideally, at least two electropherograms consisting of sufficient signal strength would have been generated, as is generally done in studies using non-invasive sampling techniques. Lastly, error rates (overall, single infection, and multiple infection) for allele scoring should always be calculated (Pompanon et al. 2005).

In conclusion, the microsatellite data for *Hematodinium* sp. demonstrate that the parasite is most likely haploid in the blue crab hemolymph and that infections from multiple genotypes of the parasite are fairly common. Additionally, there appears to be relatively high levels of genetic mixing among the sites that is sufficient to maintain similar allele frequencies along the Delmarva Peninsula. Lastly, amplifying parasite microsatellites from infected host tissue has both benefits and drawbacks. Following protocols developed for amplifying microsatellites from noninvasive samples may help mitigate problems in future studies.

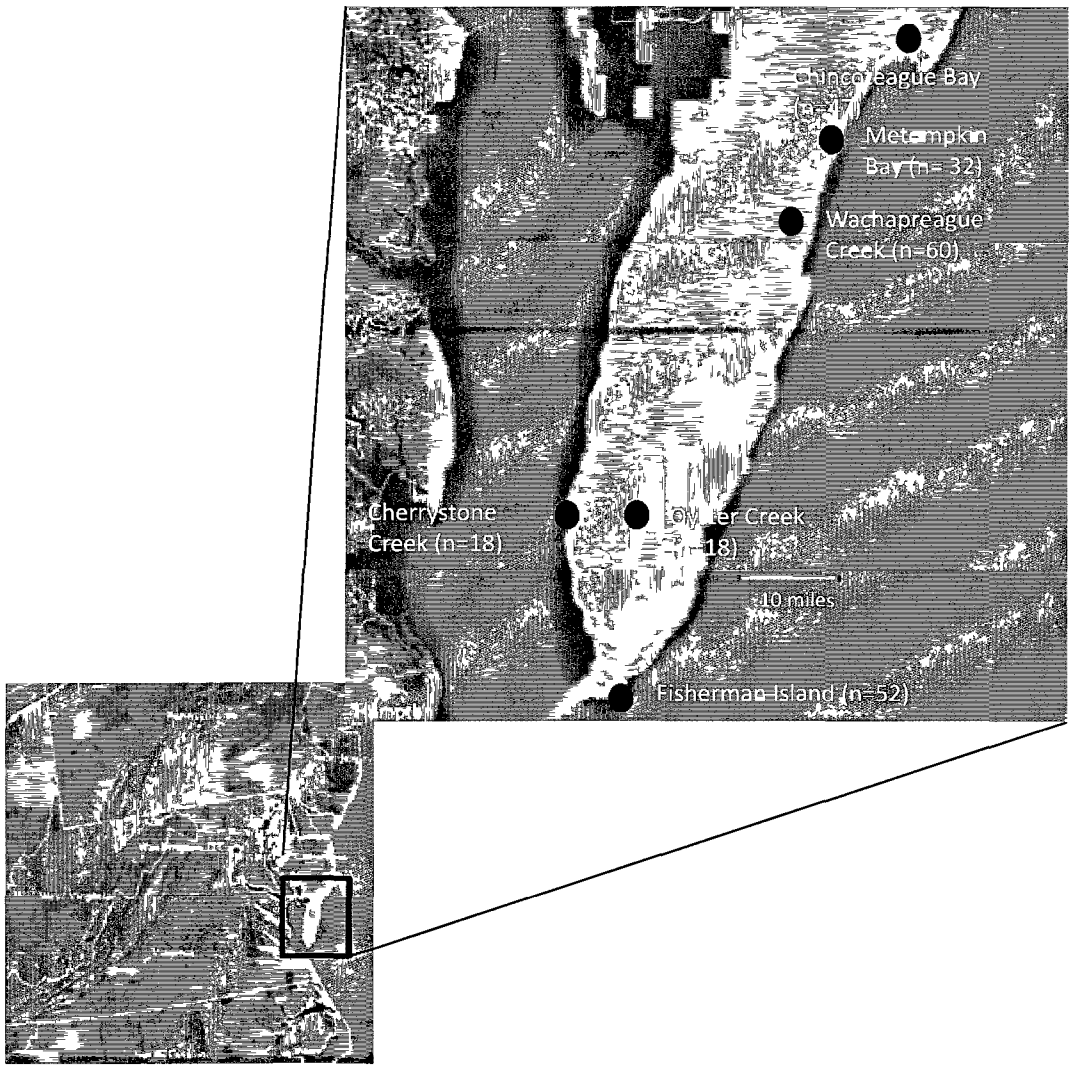


FIGURE 4.1. Sampling locations with the total number of infected blue crabs obtained from each site over the entire sampling period 2008-2009. Note that Metompkin Bay, Oyster Creek, and Cherrystone Creek were only sampled in 2008, while Chincoteague Bay, Wachapreague Creek, and Fisherman Island were sampled in 2008 and 2009.

FIGURE 4.2. Allele frequencies for the eight polymorphic microsatellite loci calculated by site. Note that the color scheme for the alleles is independently assigned for each locus.

FIGURE 4.2.

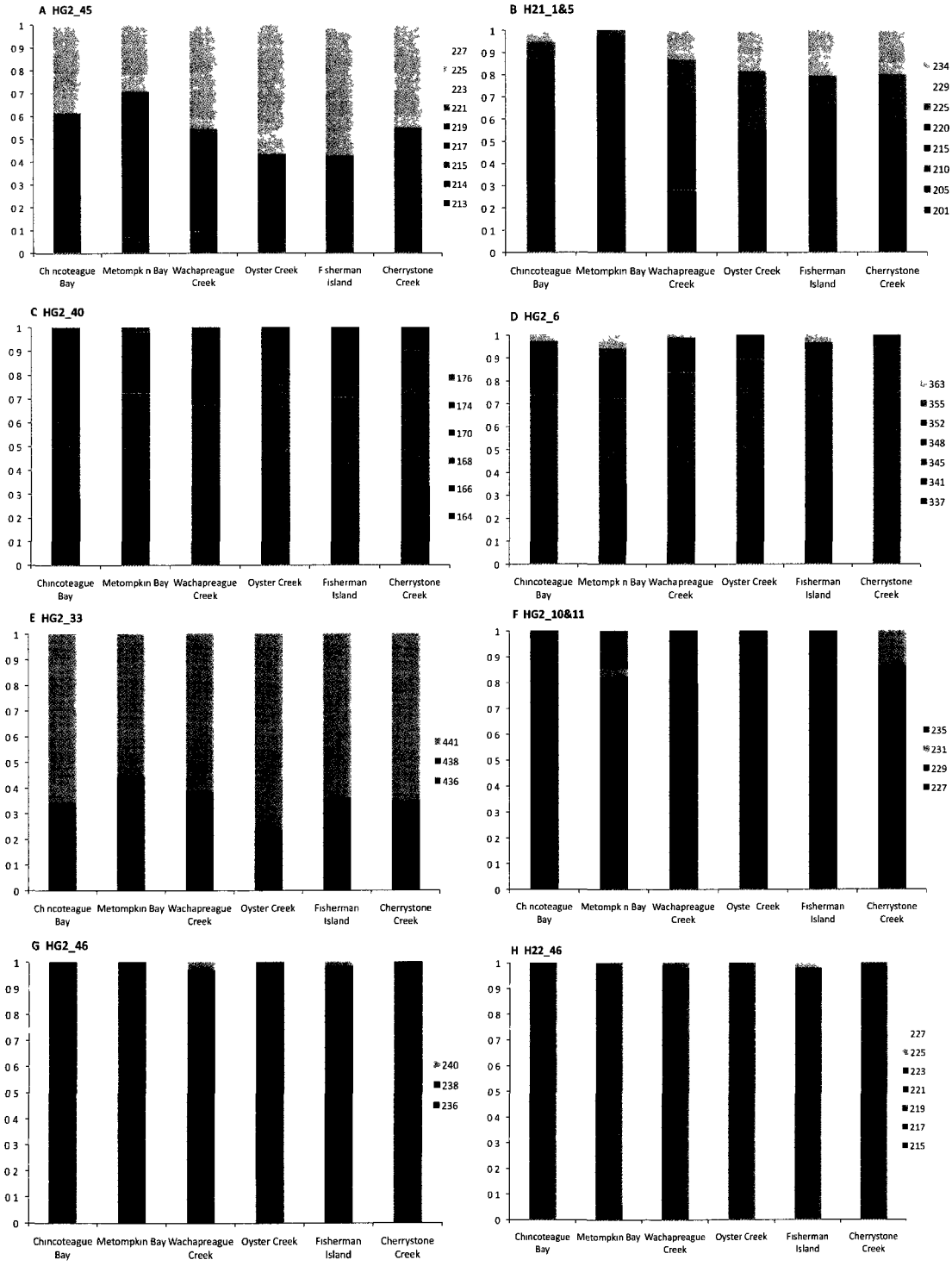
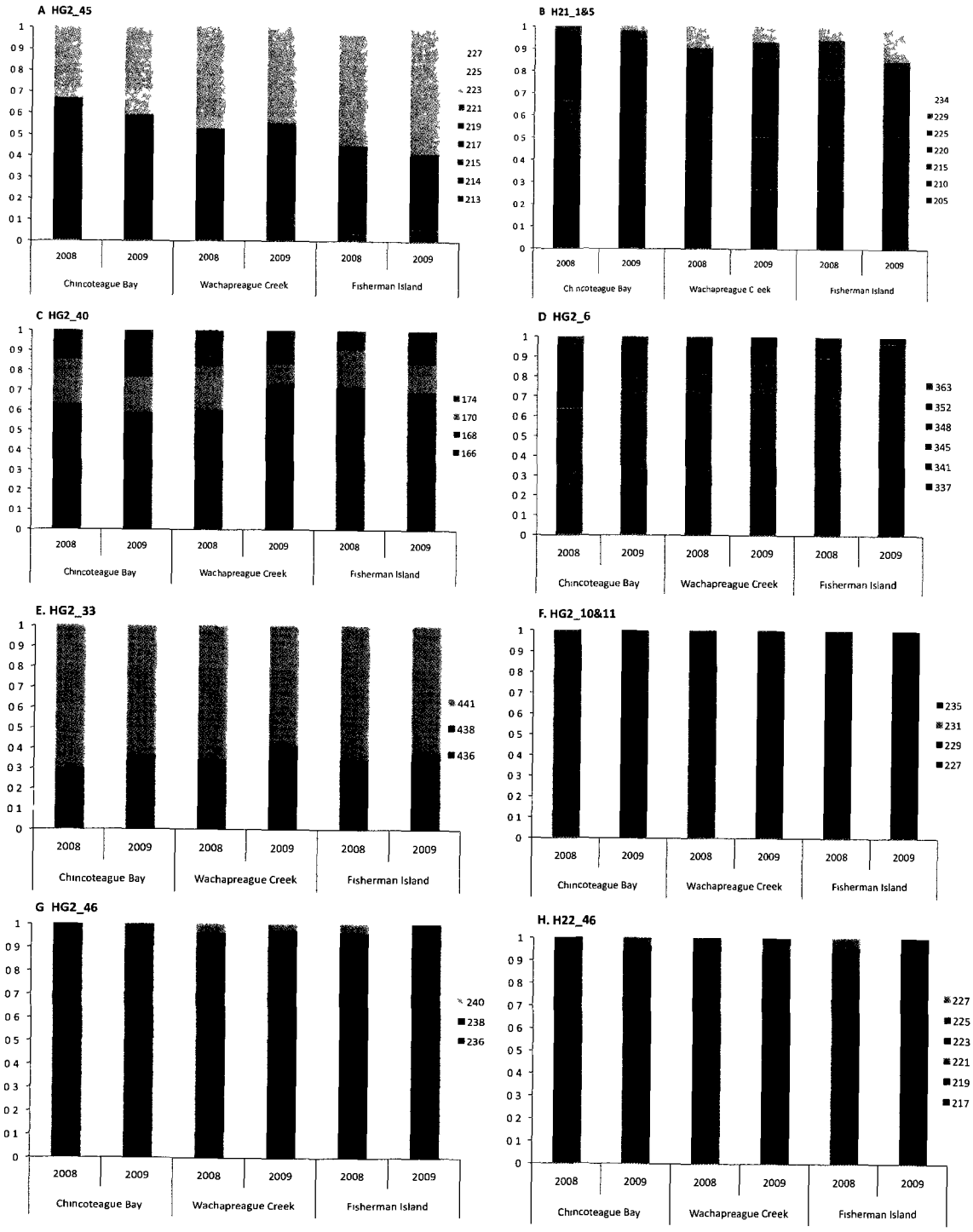

A HG2_45
B H21_1&5
C HG2_40
D HG2_6
E HG2_33
F HG2_10&11
G HG2_46
H H22_46
Chincoteague Bay
Metompkin Bay
Wachapreague Creek
Oyster Creek
Fisherman Island
Cherrystone Creek

FIGURE 4.3. Allele frequencies for the three sites that were sampled in 2008 and 2009. The number of infected samples collected in 2008 from Chincoteague Bay, Wachapreague Creek, and Fisherman Island were 18, 27, and 24, respectively. The number of infected samples collected in 2009 from Chincoteague Bay, Wachapreague Creek, and Fisherman Island were 29, 33, and 28, respectively. Note that the color scheme for the alleles is independently assigned for each locus.

FIGURE 4.3


A HG2_45
227
225
223
221
219
217
215
214
213
B H21_1&5
234
229
225
220
215
210
205
C HG2_40
174
170
168
166
D HG2_6
363
352
348
345
341
337
E. HG2_33
441
438
436
F. HG2_10&11
235
231
229
227
G HG2_46
240
238
236
H. H22_46
227
225
223
221
219
217
2008
2009
Chincoteague Bay
Wachapreague Creek
Fisherman Island

Marker	Number of alleles observed	Missing data	Error rate for single infections	Error rate for multiple infections
HG2_45	9	0.0%	0%	15.0-29.2%
H21_1&5	8	1.8%	0%	30.4-33.0%
HG2_40	6	0.0%	0%	16.0-16.0%
HG2_6	7	0.0%	0%	4.2-8.3%
HG2_33	3	3.5%	0%	8.0-12.5%
HG2_10&11	4	11.9%	0%	4.2-12.5%
HG2_46	3	0.4%	0%	0.0-8.3%
H22_46	7	0.4%	0%	0%
H21_1	1	2.2%	0%	0%
H22_5_2	1	2.2%	0%	0%
H21_9	1	0.9%	0%	0%

TABLE 4.1. Microsatellite loci that were used for this study with the total number of alleles observed and the missing data (%) and the calculated scoring error rate for each marker. The error rates for single infections and multiple infections are listed with the same comparison of the error rates for the final (first number listed) vs. initial (second number listed) allele scores. Note that the error rates for single infections were zero for all microsatellite loci.

	Samples collected	Samples with 1 allele per locus	Samples with 1-2 alleles per locus	Samples with 1-3 or more alleles per locus
Chincoteague Bay	47	24	9	14
Metompkin Bay	32	18	8	6
Wachapreague Creek	60	25	17	18
Oyster Creek	18	13	3	2
Fisherman Island	52	38	7	7
Cherrystone Creek	18	14	4	0
Total	**227**	**132**	**48**	**47**

TABLE 4.2. The number of infected hemolymph samples collected per site, the number of samples that had a single allele at every locus, 1-2 alleles per locus, or 1-3 or more alleles per locus.

	Parasite Life Stage			
	Filamentous trophont	**Ameboid trophont**	**Multiple Stages**	**Other**
1 allele only	69.6 (16)	50.0 (71)	66.7 (20)	78.1 (25)
1-2 alleles	13.0 (3)	26.1 (37)	16.7 (5)	9.4 (3)
1-3 or more alleles	17.4 (4)	24.0 (34)	16.7 (5)	12.5 (4)

TABLE 4.3. Number of alleles versus the percentage of samples with various life history stages of the parasite from microscopic examination of hemolymph smears. The numbers in parentheses are the total number of observations in each category. Due to low sample sizes, samples containing clump colonies, prespores, and samples where life history stage was not recorded were combined and listed as other. Multiple life stages refers to when a combination of filamentous trophonts, ameboid trophonts, clump colonies, or prespores were observed.

	Parasite Intensity		
	Light	**Moderate**	**Heavy**
1 allele only	73.6 (53)	54.4 (43)	47.2 (34)
1-2 alleles	16.7 (12)	19.0 (15)	27.8 (20)
1-3 or more alleles	9.7 (7)	26.6 (21)	25.0 (18)

TABLE 4.4. The number of alleles versus the percentage of samples with different parasite intensities from microscopic examination of hemolymph smears. The numbers in parentheses are the total number observed in each category. Parasite intensity was not listed for 4 samples, so these were excluded for this analysis.

Chapter 5. Genotypic variation of *Hematodinium* sp. along the Delmarva Peninsula, Virginia

Abstract

Infections of *Hematodinium* sp., a parasitic dinoflagellate, have been reported in blue crab populations in the USA from the eastern seaboard and the Gulf of Mexico. While this parasite has the potential to impact several important commercial fisheries, little is currently known about the genotypic variation within and among populations of this parasite. To assess genotypic variation and population structure, blue crabs were collected from six sites along the Delmarva Peninsula from 2008-2009 and eight microsatellite loci were used to genotype *Hematodinium* sp. from infected blue crabs. The composition of multi-locus genotypes indicated high genotypic variation, as 84% of the genotypes recovered were unique. The distribution of genotypes from *Hematodinium* sp. indicates that this parasite constitutes a single panmictic population along the Delmarva Peninsula as multiple analyses showed genotypes from all locations grouping together. However, there were also some indications of a low level of population differentiation among sites. Finally, the large number of unique genotypes, with most differing by only one or two alleles, combined with the lack of linkage disequilibrium at the loci examined, is indicative of recombination. Thus, this is the first report indicating that sexual reproduction occurs in the life cycle of this parasite. However, where sexual reproduction occurs in the life cycle of *Hematodinium* sp. remains unknown.

Introduction

Hematodinium sp. is a parasitic dinoflagellate that was first reported from American blue crabs, *Callinectes sapidus,* in North Carolina, Georgia, and Florida (Newman and Johnson 1975). Additional studies have shown that this parasite infects blue crabs from Delaware to Texas (Newman and Johnson 1975, Messick 1994, Messick

and Shields 2000, Gruebl et al. 2002, Chapter 2). Multiple studies have shown that the majority of blue crabs naturally or experimentally infected with this parasite do not survive more than 45 days (Messick and Shields 2000, Shields and Squyars 2000). Additionally, this species of *Hematodinium* is generally reported in coastal waters with salinities >19ppt (Messick and Shields 2000). Thus, a high prevalence of *Hematodinium* sp. infections, typically in high salinity areas, could cause significant crab mortality due to disease.

A general life cycle for the species of *Hematodinium* that infects the blue crab was described from the parasite stages that occur within the hemolymph of the blue crab host by Stentiford and Shields (2005). Only recently has the entire life cycle of this parasite in culture been described by Li et al. (in review), which includes seven additional life history stages of the parasite that were observed in culture, but not in the hemolymph of the blue crab. In both described life cycles, the first parasite stage is the filamentous trophont (e.g. vermiform plasmodia, Stentiford and Shields 2005), which can replicate via budding to create more plasmodia, or undergo merogony to create vegetative amoeboid trophonts. Both filamentous trophonts and ameboid trophonts are observed in the hemolymph. The life history stages described by Li et al. (in review) that occur between the observation of ameboid trophonts and sporonts in the hemolymph are believed to occur in other tissues of the host, most likely the hepatopancreas. The remaining life stages observed in the hemolymph include the rounded sporont, which undergoes what appears to be sporogony to create either macro- or microdinospores. These dinospores then exit the host, generally via the gills, and enter the water column. What becomes of dinospores once they enter the water column is unknown. Additionally, how *Hematodinium* sp. infects its various crustacean hosts and the transmissive stage of the parasite are currently unknown, though transmission via a dinospore stage seems likely (Stentiford and Shields 2005, Chapter 1). Life cycle observations of histological preparations, hemolymph smears, and parasite culture indicate that this parasite undergoes substantial asexual reproduction in the host. Whether or not sexual reproduction occurs in the life cycle of this parasite was unknown.

In studies of other parasites, specifically protozoan parasites of humans, microsatellite markers have been used to elucidate aspects of the life history of these

parasites, such as recombination due to sexual reproduction, which were difficult or impossible to decipher using other techniques. Microsatellites are simple sequence repeats that are generally found in the nuclear genome and are characterized by high levels of polymorphism, resulting from high mutation rates (for review, see Li et al. 2002, Ellegren 2004). They are generally believed to be selectively neutral and have been used to examine life history characteristics and population structure in a variety of organisms. Microsatellite markers have been used to assess the extent of clonal versus sexual reproduction in a number of important human parasites (Tibayrenc et al. 1990, Tibayrenc and Ayala 2002). For example, analyzing multi-locus genotypes (MLGs) obtained from microsatellite data revealed population structure consistent with recombination due to sexual reproduction in *Plasmodium falciparum*, though the degree of clonal population structure appears to depend on the prevalence of the parasite in the region (Anderson et al. 2000, Razakandrainibe et al. 2002).

Genotype data has also been used to examine population structure and life history traits for several species of free-living dinoflagellates, as well as the endosymbiotic *Symbiodinium* spp. Most studies conducted on free-living dinoflagellates have reported extremely high levels of genotypic variation, with the same genotype rarely being observed multiple times (Nagai et al. 2007a, 2009, Alpermann et al. 2009, McCauley et al. 2009, Lowe et al. 2010). The same studies report no linkage disequilibrium between microsatellite loci, an observation consistent with recombination. On the contrary, most studies of *Symbiodinium* spp. in the Caribbean have shown that a particular coral species is dominated by either one or a few genotypes, indicating a predominance of clonal reproduction (Santos et al. 2003, Thornhill et al. 2009, Kirk et al. 2009). Genotype data has also been used to differentiate populations of several dinoflagellates including: *Alexandrium catenella* (Masseret et al. 2009), *Cochlodinium polykrikoides* (Nagai et al. 2009), *Alexandrium tamarense* (Nagai et al. 2007a), *Alexandrium minutum* (McCauley et al. 2009), *Oxyrrhis marina* (Lowe et al. 2010), *Symbiodinium* clade B (Santos et al. 2003, Thornhill et al. 2009, Kirk et al. 2009), and *Symbiodinium* clade C (Howells et al. 2009).

Previous work has demonstrated that the same species of *Hematodinium* is capable of infecting multiple crustacean hosts within the Delmarva Peninsula and infects blue crabs from Virginia to Texas (Chapters 1, 2). *Hematodinium* sp. from blue crabs is

most likely haploid and multiple infections are common (Chapter 4). Allele frequencies generated from eight microsatellite loci showed no population differentiation among six sites along the Delmarva Peninsula, indicating that this geographic region probably consists of a single population of the parasite (Chapter 4); however, more refined population level structuring could not be assessed with the allele frequency data due to the high prevalence of multiple infections.

In this study, eight microsatellite markers were used to assess genotypic variation and population structure of *Hematodinium* sp. from blue crabs collected along the Delmarva Peninsula, Virginia. More specifically, multi-locus genotypes were constructed and evaluated to determine the genotypic variation along the Delmarva Peninsula, as well as among the sampling locations to determine if population structure was present. Finally, a rarefaction analysis was conducted to determine if the amount of sampling was sufficient to assess genotypic variation in the region.

Materials and Methods

Sampling

Infected blue crabs were collected from six sites along the Delmarva Peninsula, Virginia as described in Chapter 1 (Figure 5.1). Infection status was ascertained as described in Chapter 1. Hemolymph samples from infected crabs were procured and handled as described in Chapters 1 and 2.

ITS1 and microsatellite PCR amplification

All DNA extractions were conducted as described in Chapter 1. Briefly, total DNA was extracted from 200μL of hemolymph in 95% ethanol suspension with a Qiagen Tissue and Blood kit (Qiagen, Valencia, California) following the manufacturer's instructions for animal tissue. All extractions completed within the same day included a blank column extraction, which, when subjected to PCR analysis alongside samples, serves as a control for extraction contamination. After extraction, separate aliquots (~20 μL) were made to avoid contamination of original DNA stock material. Stock DNA was stored at -20°C and aliquoted DNA was stored at 4°C. DNA extracts were quantified using a NanoDrop 2000 (Thermo Scientific, West Palm Beach, Florida).

All DNA samples were amplified as previously described in Chapter 1 with the nSSU PCR assay (modified from Medlin 1988) and the *Hematodinium* sp. ITS1 PCR assay (Small et al. 2007b) to ensure that the DNA was of high quality and that amplifiable *Hematodinium* sp. DNA was present. Only samples that were positive for both of these PCR assays were amplified with the microsatellite PCR assays.

Microsatellite PCR assays were performed as described in Chapter 3, with the exception that Platinum Taq (Invitrogen) was used if amplifications with Taq polymerase (Invitrogen) were unsuccessful or had an insufficient signal on the electropherogram.

Microsatellite data analyses

Allele sizes were resolved using a 3130xl Genetic Analyzer (Applied Biosystems, Norwalk, Connecticut). Allele size determinations were made using GeneMarker 1.75 (SoftGenetics LLC, State College, Pennsylvania). The minimum detectable peak height was set at 100 arbitrary fluorescence units (FU) and all alleles were verified by visual inspection. Only those samples that showed a single allele for all loci were used in this study. Due to small sample sizes, the samples collected from the same sites over multiple years were combined.

Multi-locus genotypes (MLGs) were sorted in Microsoft Excel and compared by eye. Missing data were ignored for genotype comparisons. To make the distribution of genotypes more conservative, an effort was made to first match genotypes that contained missing data to another genotype from the same location; however, if no matching genotype was found from that location, then the genotype with the missing data was paired with the first genotype it matched, regardless of location. The number of genotypes obtained and the total number of unique genotypes were calculated manually for the entire region as well as by location. A rarefaction curve was generated to determine if the number of samples collected was sufficient to assess the genotypic variation using these markers.

GenoDive (Meirsman and Van Tienderen 2004), a program for assessing genotypic diversity in clonally reproducing organisms, was used to calculate parameters of genotypic diversity. The clonal diversity suboption in GenoDive was used to calculate the effective genotypes per location, Nei's genetic distance with a correction for sample

size, and evenness. In GenoDive, the effective number of genotypes, which is an equivalent measurement to the effective number of alleles, is the number of equally frequent genotypes necessary to create the same level of genotypic variation observed in the population. The calculation for evenness assesses how evenly the various genotypes are divided over the locations sampled, using the effective number of genotypes. For this test, an evenness of 1 indicates that all genotypes have equal frequencies in a population.

Genodive was also used to assess whether the observed diversity was attributed to sexual or asexual reproduction. First, clones were assigned within Genodive using the infinite allele model, threshold=0, corrected Nei's diversity index, 10,000 permutations, and alleles were randomized over individuals within populations. The threshold was set at zero, as the scoring error was zero for all samples with single infections. Significance is based on $\alpha = 0.05$.

To assess the level of population differentiation, F_{ST}, R_{ST}, and AMOVA calculations were conducted using Arlequin 3.5.1.2 (Excoffier and Schneider 2005). The default settings were changed so that all loci were included in the analysis regardless of the amount of missing data. In order to conduct these tests, the haploid data had to be converted to diploid homozygotes. Both F_{ST} and R_{ST} values were calculated separately as each test assumes a different model of evolution: F_{ST} assumes an infinite allele model (IAM), while R_{ST} assumes a step-wise mutation model (SMM; for review see Balloux and Lugon-Moulin 2002). Pairwise comparisons to determine F_{ST} values were calculated using Slatkin's distance, 10,000 permutations, and the suboption number of different alleles (F_{ST}-like). The significant values indicated by the F_{ST} pairwise comparisons were used to determine the groupings in an AMOVA, which was conducted using the number of different alleles (F_{ST}-like) suboption and the following groups: Group 1 (Metompkin Bay), Group 2 (Oyster Creek), Group 3 (Fisherman Island), and Group 4 (Chincoteague Bay, Cherrystone Creek, and Wachapreague Creek). The significance of the covariance components and fixation indices were examined with 10,000 permutations. Pairwise comparisons to determine R_{ST} values were calculated using Slatkin's distance, 10,000 permutations, and the suboption sum of squares differences (R_{ST}- like). The significant values indicated by the R_{ST} pairwise comparisons were used to determine the groupings in a second AMOVA, which was conducted using the sum of squares (R_{ST}-like)

suboption and the following groups: Group 1 (Metompkin Bay), Group 2 (Oyster Creek), and Group 3 (Chincoteague Bay, Wachapreague Creek, Fisherman Island, and Cherrystone Creek). The significance of the covariance components and fixation indices were examined with 10,000 permutations. For both AMOVA analyses, the Global AMOVA values are presented as they are averaged over all eight loci, thus reducing the impact of the missing data on the analyses. For all F_{ST} and R_{ST} values, significant levels for multiple comparisons of loci were adjusted using a sequential Bonferroni correction (Rice 1989). Significance was based on an initial $\alpha = 0.05$. In order to determine if significant F_{ST} and R_{ST} values were driven by a particular locus in a single location, pairwise comparisons to obtain F_{ST} values and allele frequencies were calculated for individual loci per location in Genodive using 1,000 permutations and the AMOVA F_{IS} option.

Two factorial correspondence analyses (FCA) in 3D were performed with Genetix 4.05 (Belkhir et al. 1996-2004). The first FCA used only the contributions of individuals and alleles, while the second FCA assessed the contributions of the populations in addition to the contributions from the individuals and alleles. In both cases, only the first four factors were used in the analyses, as these are generally sufficient. Genetic distances using either the Cavalli-Sforza and Edward (1967) index or the Nei (1978) index were calculated in Genetix using the real data suboption (sur données réelles) including all data (totalité des données). Both models of genetic distance were calculated as each calculation assumes a different model of evolution: Nei (1978) genetic distance calculation assumes an IAM (for review see Balloux and Lugon-Moulin 2002) and includes a correction for small sample sizes, while Cavalli-Sforza and Edward (1967) incorporates only the effects of selection and genetic drift. The two genetic distance matrices were used to compute separate UPGMA dendograms in MEGA 5.0 (Kumar et al. 2004).

Linkage disequilibrium was assessed using GenePop on the web (http://genepop.curtin.edu.au/, Raymond and Rousset 1995) with 10,000 dememorizations, 1,000 batches, and 10,000 iterations per batch. In order to conduct this test the haploid data had to be converted to diploid homozygotes. Significance levels for

multiple comparisons of loci were adjusted using a sequential Bonferroni correction (Rice 1989). Significance was based on an initial $\alpha = 0.05$.

Results

In total, 132 samples showed a single allele at all eight loci and these were assumed to be infections of a single genotype of *Hematodinium* sp. For this data set, the total number of alleles detected was 8, 8, 5, 5, 2, 4, 2, and 6 for the eight polymorphic loci, HG2_45, H21_1&5, HG2_40, HG2_6, HG2_33, HG2_10&11, HG2_46, and H22_46, respectively. Among all locations, 110 total MLGs were observed, and 84% (n=92) of the MLGs observed were unique, having been observed only once (Table 5.1).

Eighteen MLGs of *Hematodinium* sp. were amplified from multiple crabs; however, no single MLG was observed more than three times (Figure 5.2). In addition, when MLGs were observed multiple times, they were not always observed in the same location or in neighboring locations. For example, MLG8 was observed once at Fisherman Island and once at Metompkin Bay, and MLG10 was observed once at Cherrystone Creek and once at Chincoteague Bay. While Oyster Creek had the highest proportion of unique MLGs (Table 5.1; Figure 5.3), unique MLGs were frequently observed at all sites, with 61-77% of the genotypes from the six locations being unique. Due to the fact that the majority of MLGs in each location were unique, the effective number of MLGs was highly similar to the total number of MLGs (Table 5.1). The corrected genotypic diversity values showed high levels of genotypic diversity at all locations. The evenness values indicated that the MLGs were essentially in equal frequencies in all locations.

The genotypic composition of parasites from Metompkin Bay was significantly different from Oyster Creek ($F_{ST} = 0.10296$, $p = 0.01079$) and Fisherman Island ($F_{ST} = 0.0796$, $p = 0.00475$; Table 5.2); however, these values were not significant after Bonferroni correction. Using the groupings based on the F_{ST} values that were significant before Bonferroni correction, the AMOVA showed the majority of the variation was attributed to individuals within the groups, but this variation was not significant (96.2%, FST=0.038, p=0.128; Table 5.3). While less variation was attributed to among the groups (2.56%), it was a significant portion of the variation (FCT= 0.0256, p=0.021).

After Bonferroni correction for 15 comparisons, one R_{ST} pairwise comparison was significant, indicating a significant difference in genetic diversity between the parasite populations in Metompkin Bay and Oyster Creek (R_{ST}=0.36427, p-value=0.00129; Table 5.4). The AMOVA based on the groupings yielded by the R_{ST} values revealed that there was a significant amount of variation both among individuals within populations (10.6%, FST=0.12882, p=0.02; Table 5.5) as well as among groups (87.1%, FCT=0.106, p=0.013).

Two loci appeared to be responsible for the significant F_{ST} values. The significant difference between Metompkin Bay and Fisherman Island could be attributed to a significant difference between these locations at HG2_45 (F_{ST}= 0.214, p=0.004). The significant difference between Metompkin Bay and Oyster Creek could be attributed to a significant difference between these locations at HG2_33 (F_{ST}= 0.337, p=0.007). However, examination of the allele frequencies did not reveal any apparent differences between these sites compared to the allele frequencies observed at the other sites.

The majority of the MLGs were grouped together in a single cluster with few genotypes dispersed as outliers and no structuring by location was apparent (FCA without locations, Figure 5.4). When location was included, MLGs from all locations overlapped (FCA with locations, Figure 5.5). Though the MLGs were not randomly dispersed and might be clustered by location, this grouping was difficult to partition due to the extensive overlapping of the MLGs from different locations.

Both UPGMA dendograms placed the genotypes from Chincoteague Bay and Wachapreague Creek into a single group (Figure 5.6, 5.7); however, the remaining topologies of the two dendograms differed. One placed the MLGs from Cherrystone Creek and Oyster Creek into one group, which was sister to a group of MLGs from Chincoteague Bay and Wachapreague Creek (UPGMA using Cavalli-Sforza and Edward (1967) genetic distance; Figure 5.6). These two groups were sister to a third group, which included MLGs from Fisherman Island and Metompkin Bay. The second dendogram placed MLGs from Cherrystone Creek and Metompkin Bay into one group, which was sister to the group of MLGs from Chincoteague Bay and Wachapreague Creek (UPGMA using Nei (1978) genetic distance; Figure 5.7). These two groups were sister to a third group containing the MLGs from Fisherman Island and Oyster Creek.

The rarefaction curve did not reach an asymptote (Figure 5.8), but instead showed a linear relationship between the number of samples collected and the MLGs observed. This relationship indicates the number of samples collected was insufficient for assessing the level of genotypic variation present in this geographic region.

One pair of loci, H21-1&5 and HG2_10&11 (p= 0.0013) appeared to be in linkage disequilibrium when compared across all populations, after Bonferroni correction for 28 comparisons. However, given that none of the loci pairs within the populations were significantly linked after Bonferroni correction, it seems unlikely that the above pair is truly linked, but more likely an artifact of both small sample size and the high frequency of a single allele at the HG2_10&11 locus.

Using Genodive, 108 MLGs were identified. The discrepancy between the number of MLGs from Genodive and the number manually calculated is probably due to how the missing data effects the genetic distance calculations used to assign genotypes in Genodive (Meirsman and Van Tienderen 2004). Regardless, the test for random mating based on the observed genetic diversity indicated that random mating was occurring within each location (Cherrystone Creek, p=0.3; Chincoteague Bay, p=1.0; Fisherman Island, p=0.5; Metompkin Bay, p=1.0, Oyster Creek, p=1.0; Wachapreague Creek, p=0.7) as well as over all the locations (p=0.9).

Discussion

This study used eight microsatellite markers to assess genotypic variation, composition, and distribution of *Hematodinium* sp. along the Delmarva Peninsula, Virginia. Though there was not an abundance of alleles at any particular locus, there was still an extremely high level of genotypic variation observed both among and within all locations. This high level of genotypic variation, combined with almost no evidence of linkage disequilibrium, is indicative of recombination occurring during sexual reproduction. This finding is further supported by the indication of random mating based on the genetic diversity within and among the locations. This is the first evidence that *Hematodinium* sp. has a sexual reproductive stage; however, the life history stage where sex occurs has yet to be determined. Additionally, the majority of the data indicated that *Hematodinium* sp. is most likely panmictic along the Delmarva Peninsula. However, as

demonstrated by the rarefaction curve, a larger sample size is necessary to truly assess the level of genotypic variation and population structure of this parasite in this region.

Evidence of high genotypic variation and panmixia

The high level of genotypic variation observed in *Hematodinium* sp. along the Delmarva Peninsula was unexpected, but is comparable to what has been observed in genotypic variation using microsatellite data in free-living dinoflagellates. Many studies using microsatellites to examine population structure in free-living dinoflagellates have reported high levels of genotypic variation, even in small geographic regions, and report only rarely if ever sampling the same genotype multiple times (Alpermann et al. 2009, 2010, McCauley et al. 2009, Nagai et al. 2007a, 2009, Lowe et al. 2010). This is in contrast with what has been reported on studies of *Symbiodinium* spp., where the same genotype is frequently recovered multiple times, resulting in strong population structuring either by reef or by depth (Santos et al. 2003, Kirk et al. 2009, Thornhill et al. 2009).

The maintenance of high genotypic variation is generally attributed to an increased ability to adapt to changing environments and hosts (Frankham 2005, Barrett and Schluter 2008). For *Hematodinium* sp., which must survive in two very different environments, a host and the water column, the high genotypic variation may facilitate adaptation in both environments. Previous work has shown that this species of *Hematodinium* is a host generalist (Chapter 1), so a high level of genotypic variation may facilitate the use of more host species. Similarly high levels of genotypic variation have been observed in another host generalist, the trematode *Maritrema novaezealandensis*, in New Zealand (Keeney et al. 2007, 2008). It is also possible that different strains of *Hematodinium* sp., which vary in virulence or environmental tolerance, are present and account for the high genotypic variation; however, there is currently no evidence that the observed genotypic variation correlates to differences in virulence or environmental tolerance. On the contrary, studies have been conducted examining mortality in blue crabs due to *Hematodinium* sp. infection using different *Hematodinium* sp. isolates from the Delmarva Peninsula, and these studies have reported crab death due to disease occurring between 13-42 days post-inoculation (Messick and Shields 2000, Shields and

Squyars 2000). Given the high level of genotypic variation in this parasite, it is highly likely that both of these studies used a different genotype of *Hematodinium* sp., so the similarity in time to death reported among the studies is not consistent with different strains from Virginia waters varying in virulence. On the other hand, results of studies conducted with *Hematodinium* sp. obtained in coastal Georgia suggested that those strains had higher virulence, with crab death due to disease occurring within 2-4 days post-inoculation (Lee and Frischer 2004, Frischer et al. 2006, Walker et al. 2009). Of course, these hypotheses for the genotypic variation observed in *Hematodinium* sp. are not mutually exclusive and genotypic variation may be driven by some combination of these factors.

In Chapter 4, the allele distributions among the sites demonstrated that *Hematodinium* sp. comprises a panmictic population along the Delmarva Peninsula, as the same alleles were generally dominant at all sites and rare alleles were observed in multiple and sometimes non-neighboring sites. In this chapter, focusing on results based on MLGs, the data also suggests a panmictic population, though the results are confounded by the insufficient sample sizes obtained for each location. The MLGs observed multiple times in different locations, both FCA analyses, the two UPGMA dendograms demonstrated that gene flow is occurring between locations.

There is some, though minimal, evidence for definitive population structure of *Hematodinium* sp. within the coastal embayments of the Delmarva Peninsula. First, as discussed in Chapter 4, private alleles were observed in five of the six sites sampled, although this could be a result of small sample sizes such that rare alleles were not detected at other sites. Second, one R_{ST} value was significant after Bonferroni correction and indicated that populations in Oyster Creek and Metompkin Bay were genetically distinct; however, it is important to note that R_{ST} significance can be biased by insufficient and unequal sample sizes (Ruzzante 1998). Third, the AMOVA analyses based on both the R_{ST} and F_{ST} values attributed a significant level of the genetic variation to that observed among the groups. Therefore, it is possible that this genetic differentiation is real and that long residence times at Oyster Creek and Metompkin Bay prevent gene flow between these populations and from those sites to other coastal embayments along the Delmarva Peninsula. No hydrological information specific to

these locations could be obtained from the published literature to assess the validity of this hypothesis, though long residence times are known for other coastal embayments along the Delmarva Peninsula (Pritchard 1960, Krantz 2000, 2001). However, given that Metompkin Bay and Oyster Creek represent two of the smallest sample sizes used in this study, this differentiation may be an artifact of sample size and additional sampling is needed to more confidently determine whether or not these populations are actually genetically distinct.

In studies of free-living dinoflagellates, genetic differentiation among populations was usually detected over large spatial scales, while genetic differentiation among studies of *Symbiodinium* spp. have generally shown genetic differentiation on much smaller spatial scales. Studies have shown population structure for *C. polykroides* over 800km (Nagai et al. 2009), for *A. tamarense* over 1600km (Nagai et al. 2007a), for *A. minutum* on a global scale (McCauley et al. 2009), and for *O. marina* over hundreds of kilometers (Lowe et al. 2010). One study of *A. tamarense* showed subpopulation structure within a single water sample (Alpermann et al. 2010), while a study of *O. marina* demonstrated local population structure at 2km (Lowe et al. 2010). For *Symbiodinium* spp., population structure has been detected at tens of kilometers (Santos et al. 2003), 5-200km (Kirk et al. 2009), 16-1,360 km (Howells et al. 2009), and ~12.5 km (Thornhill et al. 2009) with the largest spatial scale being >200km (Magalon et al. 2006). In this current study, genetic population structure was undetectable in *Hematodinium* sp. over ~ 150 km, from Chincoteague Bay, Virginia to Cherrystone Creek, Virginia.

Evidence for sexual reproduction

The presence or absence of multi-locus linkage disequilibrium is a common test for determining the level of recombination occurring in bacterial and protozoan populations (Maynard Smith et al. 1993, Anderson et al. 2000, Tibayrenc et al. 1990, Tibayrenc and Ayala 2002). Linkage disequilibrium, the nonrandom association of alleles, can be attributed to unknowingly analyzing a mixture of multiple populations, "true" clonal reproduction, natural selection, genetic drift, physically linked loci, inbreeding, or "epidemic" population structure (a single genotype that propagates clonally and subsequently dominates a population) (Hartl and Clark 2007, Maynard

Smith et al. 1993). Linkage equilibrium, or the random association of alleles, is generally attributed to recombination occurring as a result of sexual reproduction (Hartl and Clark 2007). It is important to note that clonal reproduction, as inferred from molecular data, does not imply a lack of sexual reproduction, only that the level of sexual reproduction is insufficient to mask the clonal population structure (Tibayrenc and Ayala 2002). Other indicators for clonal population structure for haploid organisms include multiple observations of the same widespread genotype, no recombinant genotypes, and similar findings with two independent sets of genetic markers (Tibayrenc et al. 1990, Tibayrenc and Ayala 2002).

Microsatellite data has frequently been used to assess the level of sexual reproduction occurring in a variety of organisms, particularly to assess the level of clonal or asexual propagation in human parasites (Tibayrenc et al. 1990, Tibayrenc and Ayala 2002) and *Perkinsus* spp. (Vilas et al. in press, Thompson et al. in press). Genotypic data has also been used to infer or confirm sexual reproduction in two free-living dinoflagellates, *A. tamarense* (Nagai et al. 2007a) and *O. marina* (Lowe et al. 2010).

The genotypic data obtained in this study strongly suggest that *Hematodinium* sp. undergoes recombination via sexual reproduction at some point during its life cycle. The high genotypic variation observed in all locations, with the majority being observed a single time, is consistent with recombination. Second, the large number of genotypes observed is not attributable to mutation alone, as the highest number of alleles observed at a single locus was only eight. In comparison, other studies have reported as many as 42 alleles at a single locus (Nagai et al. 2007a). In fact, the genotypic variation was not attributed to the number of alleles observed, but instead to the combination of those alleles. A number of the genotypes differed by only one or two alleles, which indicates variation due to recombination. Third, linkage disequilibrium was not observed consistently in pairs of loci across populations or within populations, indicating that the alleles are most likely randomly associated. Fourth, the test in Genodive for random mating was not significant, indicating that there was no difference between the observed genetic diversity and what would be expected if random mating were occurring.

There are other possible, but more unlikely, explanations for observing high genotypic variation. One is that these data suffer from extremely high error rates, so that

many alleles were called incorrectly and altered the multi-locus genotypes observed. However, error rates were calculated and determined to be zero for all samples with single genotype infections (Chapter 4). Another potential explanation was previously proposed to explain the high genotypic variation observed in *A. tamarense* (Nagai et al. 2007a, Alpermann et al. 2009). In this species, cysts are known to exist in the sediments for many years (up to 13 years, Nagai et al. 2007a), thus it has been proposed that high genotypic variation could be attributed to cysts from multiple cohorts simultaneously excysting and returning to the planktonic phase, so that what is observed is a temporal mixture of individuals from previous blooms (Alpermann et al. 2009). While no environmental reservoir of *Hematodinium* sp. is currently known, *Hematodinium* sp. has been detected via PCR in the sediments (Li et al. unpubl. data, Hanif et al. 2011), but the life stage detected is currently unknown. Thus, while an environmental reservoir may contribute to the observed genotypic variation, the existence of a sexually reproducing stage seems likely.

Previous research has shown that *Hematodinium* sp. is most likely haploid at all life stages that have been reported from blue crab hemolymph (Chapter 4), but in order for recombination to occur, there must be a point in the life cycle when two haploid *Hematodinium* sp. cells merge to create a diploid cell. At this time, it is unknown where and when in the life cycle the sexually reproducing life history stage of this parasite occurs. Additionally, there is some circumstantial evidence that supports that sexual reproduction may occur in the host and additional circumstantial evidence that it may occur in the environment. If sexual reproduction occurs in the host, it may occur in a stage that has been observed in *in vitro* cultures that is currently referred to as an arachnoid sporont, which consists of extremely high densities of *Hematodinium* sp. cells that are probably sporonts (Li et al., unpubl. data); however, this life history stage has not been observed histologically in host tissues, which makes distinguishing it as a life history stage, rather than a culturing artifact, difficult. Additionally, if the prevalence values reported in Chapter 4 were accurate and 58% of infections consist of only a single genotype, then sexual reproduction in the host would result in self-mating for the majority of infections. Self-mating has been inferred from genetic data in other groups closely related to the dinoflagellates including the Apicomplexa (Anderson et al. 2000,

Razakandrainibe et al. 2002) and the Perkinsozoa (Vilas et al. in press, Thompson et al. in press). However, mating within the host may only occur in multiple infections when compatible mating types are present, which has been reported for some species of parasitic fungi (for review, see Giraud et al. 2008a, 2008b) and at least one ciliate, *Tetrahymena thermophila* (Doerder et al. 1996). For dinoflagellate species, mate recognition has also been examined for two free-living species of dinoflagellates. Research examining mate recognition and sexual reproduction in *Gymnodinium catenatum* demonstrated that some strains are self-compatible while other strains must be out-crossed (Figueroa et al. 2009). For *A. tamarense*, a free-living dinoflagellate, both isogamous and anisogamous mating has been observed (Turpin et al. 1978, Anderson 1980). Additional research has demonstrated that some *A. tamarense* strains require out-crossing while others are capable of self-mating, though the majority of self-fertilized hypnozygotes were not viable (Destombe and Cembella 1990).

Another option is that the parasite undergoes sexual reproduction at some stage in the environment. While sexual reproduction has only been explicitly demonstrated in a few species, it is believed to occur in the majority of "true" dinoflagellates (Taylor 1990). In a generalized life cycle of dinoflagellates, two gametes, which can be isogamous or anisogamous, fuse to create a planozygote, which then becomes a hypnocyst or resting cyst (Taylor 1990). Thus, sexual reproduction in *Hematodinium* sp. resulting in cysts in water or sediments would be consistent with the life cycle of the other dinoflagellates. Second, the genotypic data reported in this study are equivalent to genotypic variation reported for free-living dinoflagellates (Nagai et al. 2007a, 2009, Masseret et al. 2009, McCauley et al. 2009). Third, all genotypes would be present in the water column, which would presumably decrease the likelihood of inbreeding, and should generate higher levels of genotypic variation. There is PCR-based evidence indicating that *Hematodinium* sp. occurs in the water column (Frischer et al. 2006, Li et al. 2010) and may occur in the sediments (Li et al. unpubl. data, Hanif et al. 2011). However, the life stage of *Hematodinium* sp. that occurs in the water column is unknown, though it is presumed to be the dinospore stage (for review see Stentiford and Shields 2005, Frischer et al. 2006, Li et al. 2010) and it is possible that positive sediment samples were actually contaminated by *Hematodinium* sp. present in the water column (Li et al. unpubl. data).

In conclusion, the use of microsatellite loci to examine single genotype infections of *Hematodinium* sp. in blue crabs revealed high genotypic diversity in all locations sampled along the Delmarva Peninsula. The majority of analyses conducted suggest that *Hematodinium* sp. consists of a single panmictic population along the Delmarva Peninsula; however, the sample sizes obtained were insufficient for accurately assessing the total genotypic variation observed. Lastly, the level of genotypic variation coupled with the lack of linkage disequilibrium strongly suggests that *Hematodinium* sp. has a sexually reproductive stage; however, future work is needed to demonstrate when and where sexual reproduction occurs in the life cycle of this parasite.

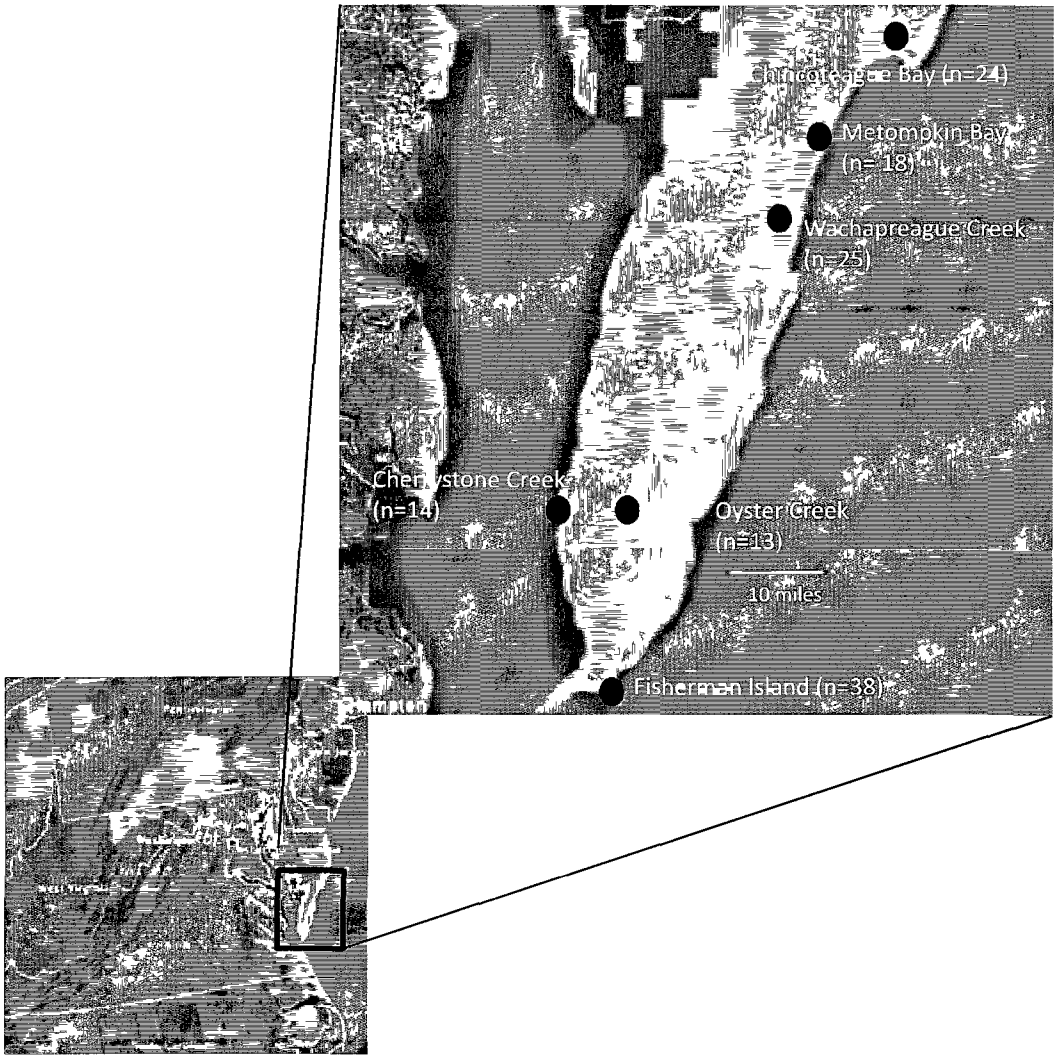


FIGURE 5.1. Sampling locations with the total number of infected blue crabs collected for this study in 2008-2009. Note that Metompkin Bay, Oyster Creek, and Cherrystone Creek were only sampled in 2008, while Chincoteague Bay, Wachapreague Creek, and Fisherman Island were sampled in 2008 and 2009.

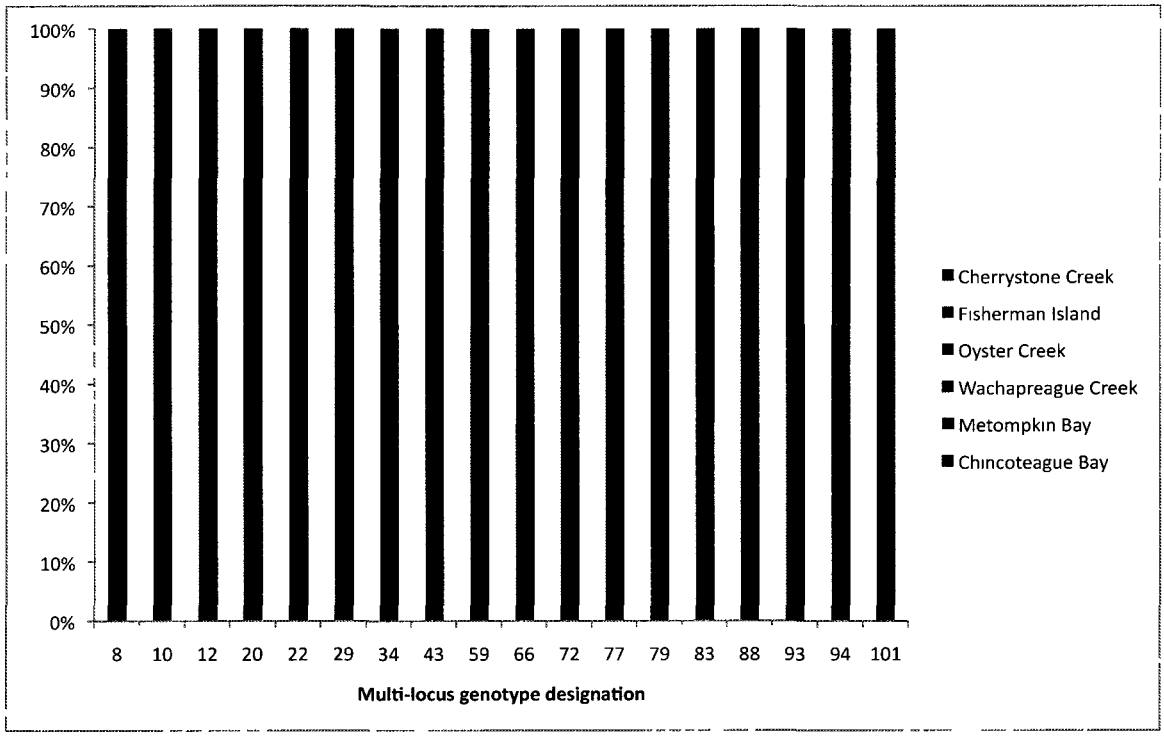


FIGURE 5.2. The distribution of the 18 genotypes that were observed more than once either in a single location or in more than one location. The numbers for the genotype designations were arbitrarily assigned.

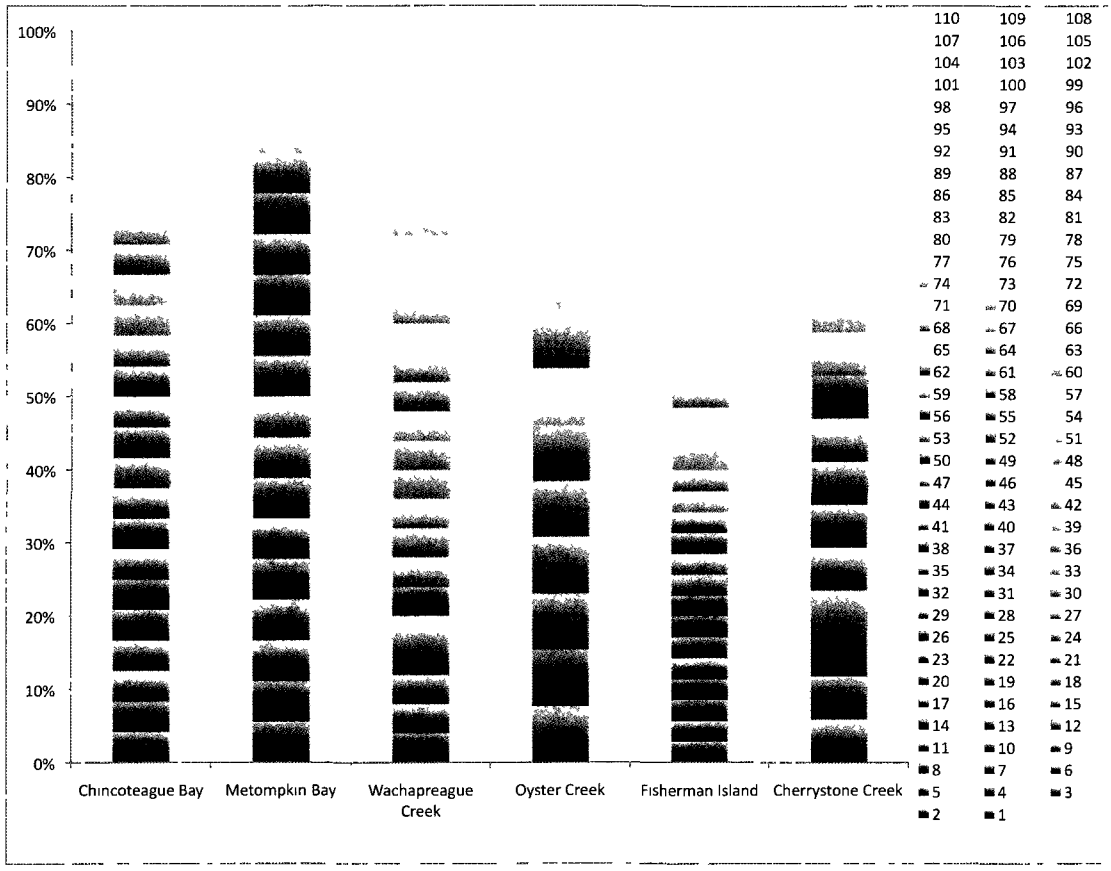


FIGURE 5.3. The distribution of all 110 genotypes among all of the sampling locations.

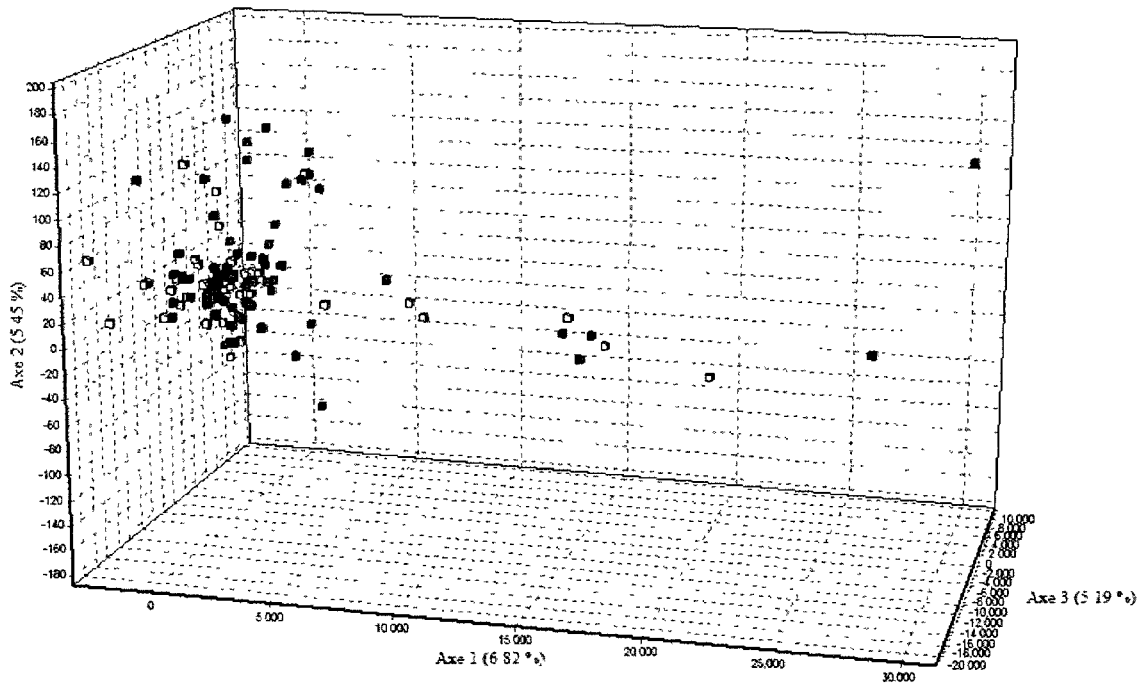


FIGURE 5.4. FCA analysis conducted in Genetix with only the contributions of individuals and alleles. Only the first four factors are shown, as these factors are generally sufficient for this analysis. The colors correspond to genotypes obtained from the following locations: Oyster Creek= pink, Fisherman Island= clear, Wachapreague Creek= teal, Cherrystone Creek= yellow, Chincoteague Bay= blue, and Metompkin Bay= gray.

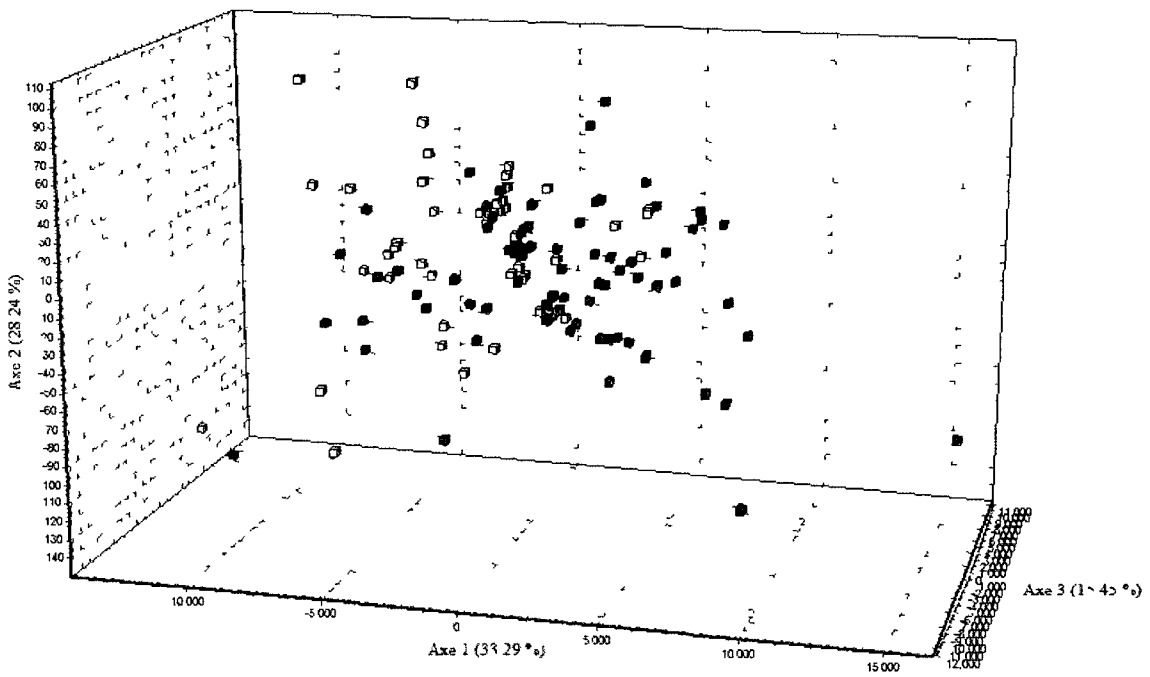


FIGURE 5 5 FCA analysis conducted in Genetix including contributions of individuals, alleles, and populations. Only the first four factors are shown, as these factors are generally sufficient for this analysis. The colors correspond to genotypes obtained from the following locations. Oyster Creek= pink, Fisherman Island= clear, Wachapreague Creek= teal, Cherrystone Creek= yellow, Chincoteague Bay= blue, and Metompkin Bay= gray.

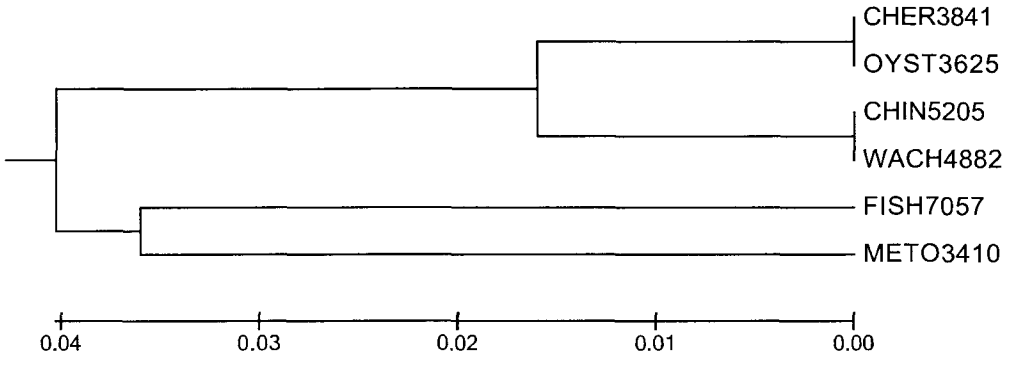


FIGURE 5.6. UPGMA dendogram generated in MEGA 5.0 using Cavalli-Sforza and Edward (1967) genetic distance calculated in Genetix. The abbreviations are as follows: CHER=Cherrystone Creek, OYST= Oyster Creek, CHIN= Chincoteague Bay, WACH= Wachapreague Creek, FISH=Fisherman Island, and METO=Metompkin Bay.

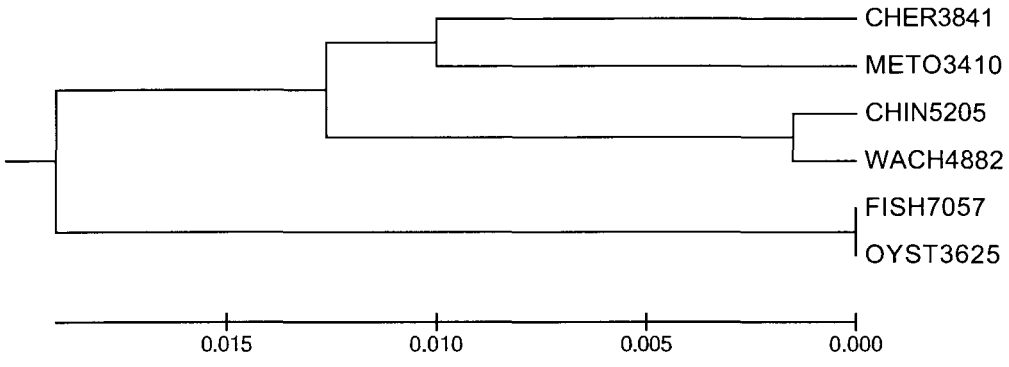


FIGURE 5.7. UPGMA dendogram generated in MEGA 5.0 using Nei (1978) genetic distance, which assumes an infinite allele model (IAM) and includes a correction for small sample sizes, calculated in Genetix. The abbreviations are as follows: CHER=Cherrystone Creek, OYST= Oyster Creek, CHIN= Chincoteague Bay, WACH= Wachapreague Creek, FISH=Fisherman Island, and METO=Metompkin Bay.

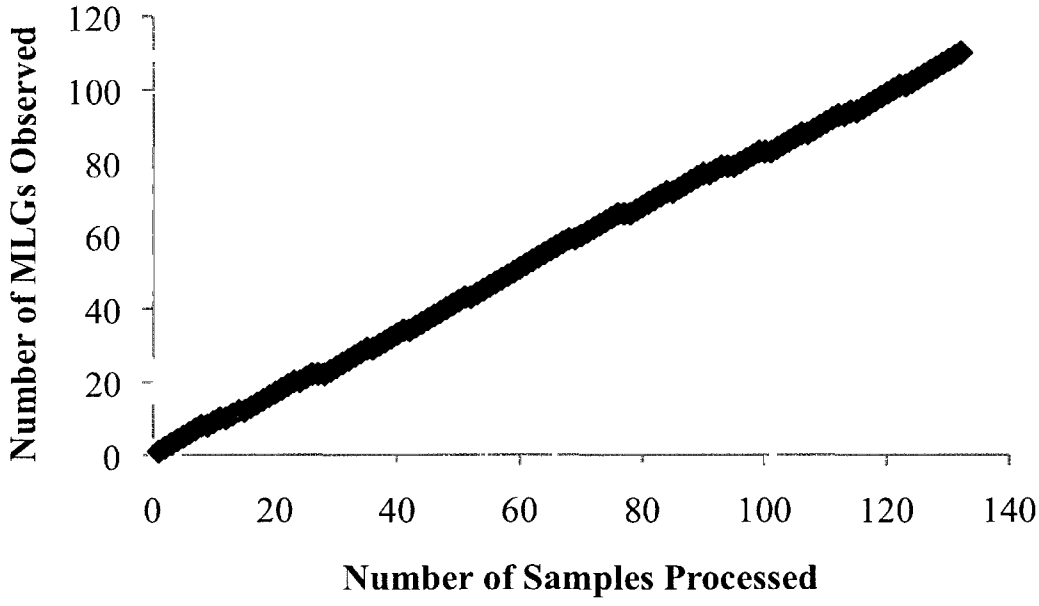


FIGURE 5.8. A rarefaction analysis comparing the number of genotypes compared to the number of samples analyzed.

	Sample size	Number of genotypes	Number of unique MLG	% Unique MLG	Effective number of genotypes	Corrected Genotypic diversity	Eveness
Chincoteague Bay	24	24	18	75.0	24	1	1
Metompkin Bay	18	18	13	72.2	18	1	1
Wachapreague Creek	25	24	18	75.0	23. 15	0.997	0.965
Oyster Creek	13	13	10	76.9	13	1	1
Fisherman Island	38	34	25	73.5	30.08	0.993	0.885
Cherrystone Creek	14	13	8	61.5	12.25	0.989	0.942

TABLE 5.1. Sample size, number of multi-locus genotypes, number of unique multi-locus genotypes per site, the percentage of unique genotypes per site, effective number of genotypes, corrected genotypic diversity (Nei's genetic diversity), and evenness for each location sampled.

	Cherrystone Creek	Chincoteague Bay	Fisherman Island	Metompkin Bay	Oyster Creek	Wachapreague Creek
Cherrystone Creek	---	0.02604	0.04967	0.03115	0.03167	0.01606
Chincoteague Bay	0.50203	---	0.03002	0.02444	0.05484	-0.00016
Fisherman Island	0.1088	0.16523	---	0.0796	0.05179	0.00489
Metompkin Bay	0.475504	0.46164	0.00475*	---	0.10296	0.03724
Oyster Creek	0.54104	0.12949	0.10336	0.01079*	---	0.03672
Wachapreague Creek	0.6833	0.9407	0.8216	0.21226	0.33719	---

TABLE 5.2. Pairwise F_{ST} values calculated by Arlequin 3.5.1.2 using Slatkin's distance and 10,000 permutations. F_{ST} values are above the diagonal and the corresponding p-values are below the diagonal. Note the * indicates those p-values that were significant before Bonferroni correction and used to determine groupings for the AMOVA calculations. These p-values were not significant after Bonferroni correction for 15 comparisons (α=0.003).

	Sum of squares	Variance components	Percent variation
Among groups	16.071	0.04611	2.55959
Among populations within groups	5.21	0.02244	1.24574
Among individuals within populations	438.674	1.73307	96.19467

Among individuals:	0.03805
P=	0.12842
Among populations within groups:	0.01278
P=	0.88089
Among groups:	0.0256
P=	0.02139

TABLE 5.3. AMOVA values with F_{ST} calculations using Arlequin 3.5.1.2 with 10,000 permutations and the following groupings: Group 1 (Metompkin Bay), Group 2 (Oyster Creek), Group 3 (Fisherman Island), and Group 4 (Chincoteague Bay, Cherrystone Creek, and Wachapreague Creek). Significance is based on α=0.05.

	Cherrystone Creek	Chincoteague Bay	Fisherman Island	Metompkin Bay	Oyster Creek	Wachapreague Creek
Cherrystone Creek	---	0.07635	0.02064	0.21474	0.0009	-0.01211
Chincoteague Bay	0.13553	---	0.04587	0.05747	0.18593	0.02145
Fisherman Island	0.37838	0.13801	---	0.16213	0.06528	-0.00678
Metompkin Bay	0.01881	0.14771	0.0096	---	0.36427	0.14688
Oyster Creek	0.57965	0.02069	0.16236	**0.00129**	---	0.06335
Wachapreague Creek	0.7727	0.34779	0.77903	0.02109	0.19226	---

TABLE 5.4. Pairwise R_{ST} values calculated by Arlequin 3.5.1.2 using Slatkin's distance and 10,000 permutations. R_{ST} values are above the diagonal and the corresponding p-values are below the diagonal. Note the * indicates those p-values that were significant after Bonferroni correction for 15 comparisons (α=0.003) and used to determine the groupings for the AMOVA calculations.

	Sum of squares	Variance components	Percent variation
Among groups	1781.742	13.48041	10.62897
Among populations within groups	742.516	2.85702	2.25269
Among individuals within populations	27913.072	110.4897	87.11834

Among individuals:	0.12882
P=	0.02
Among populations within groups:	0.02521
P=	0.38109
Among groups:	0.10629
P=	0.01307

TABLE 5.5. AMOVA values with R_{ST} calculations using Arlequin 3.5.1.2 with 10,000 permutations and the following groupings: Group 1 (Metompkin Bay), Group 2 (Oyster Creek), Group 3 (Fisherman Island, Chincoteague Bay, Cherrystone Creek, and Wachapreague Creek). Significance is based on α=0.05.

CONCLUSION

In 2005, a review paper reported that *Hematodinium* species had been reported from 11 regions, 7 countries, and 37 host species (Stentiford and Shields 2005). These parasites have had major impacts on multiple crustacean fisheries, including those targeting *Chionoecetes opilio* (Taylor and Khan 1995, Pestal et al. 2003, Shields et al. 2005), *Callinectes sapidus* (Newman and Johnson 1975, Messick 1994, Messick and Shields 2000), and *Nephrops norvegicus* (Field et al. 1992, 1998). Since 2005, *Hematodinium* species have been discovered in multiple new hosts, new locations, and have impacted additional fisheries. Ryazanova (2008) reported infections of a *Hematodinium* species in red and blue king crabs, *Paralithodes camtschaticus* and *Paralithodes platypus*, respectively, from the Sea of Okhotsk, Russia. Tully et al. (2006) reported a *Hematodinium* species infecting *C. pagurus* in Irish waters in 2004. Subsequent research indicated that disease from this parasite detrimentally impacted this fishery in Ireland (Chualáin et al. 2009). Hamilton et al. (2009) reported infections of a *Hematodinium* species in two novel hosts, *Pagurus prideaux* and *Munida rugosa*, as well as infections in *L. depurator*, *N. puber*, and *C. pagurus* for the first time in the Clyde Sea. Eigemann et al. (2010) reported infections of a *Hematodinium* species in three crustaceans (*N. norvegicus*, *P. bernhardus*, and *L. depurator*) in Danish waters and two crustaceans (*C. opilio* and *Hyas araneus*) in Greenlandic waters for the first time. Li et al. (2008) reported infection of a *Hematodinium* species in *Scylla serrata*, a portunid crab that is cultured along the southeastern coast of China for the first time. Xu et al. (2007) reported infection of a species of *Hematodinium* in *Portunus trituberculatus* in China. Xu et al. (2010) reported infection of a species of *Hematodinium* in *Exopalaemon carinicauda*, the ridgetail white prawn, which is cultured in eastern China. Thus, not only are members of this genus found worldwide, they may also constitute an emerging disease in a number of commercially important fisheries.

All research conducted thus far demonstrates that *Hematodinium* species are host generalists. Molecular evidence indicates that a species of *Hematodinium* parasite infects multiple crustacean species in the waters of the UK (Small 2004, Small et al. 2007a, Hamilton et al. 2007, 2009). Additional molecular evidence supports that a *Hematodinium* species is capable of infecting boreal crustaceans worldwide (Jensen et al. 2010). This study demonstrated that the species of *Hematodinium* that infects blue crabs also appears to be a host generalist that is capable of infecting a number of different crustaceans, potentially from different Orders (Chapter 1). However, the ecological roles of each of these hosts has not been demonstrated, though this determination is critical for assessing how these additional hosts could impact disease transmission and cycling between commercially important hosts and alternate hosts. If the alternate hosts represent true reservoirs with respect to the commercially important hosts, then if the populations of the commercially fished host declined, the parasite would be maintained in the system through using other crustaceans as hosts. On the other hand, if these alternate hosts represent incidental or spillover hosts or the parasite is being cycled independently within these hosts, with little transmission back to the commercially important hosts, then the parasite might be eradicated from the system once the populations of the commercially important hosts fell below the critical threshold value necessary for transmission (Begon 2008). Speculations have been put forth as to the ecological roles of spider crabs, hermit crabs, and mud crabs with respect to transmission between those populations and blue crab populations in the Delmarva Peninsula (Chapter 1). However, no evidence exists regarding the potential roles of alternate hosts for other *Hematodinium* species, though the impacts of those roles could be important in maintaining the parasite in other regions of the world where populations of crustaceans targeted by fisheries have declined.

Previous studies using sequence data from the rRNA gene complex indicated that a species of *Hematodinium* parasite occurs throughout the waters of the UK (Small 2004, Small et al. 2007a). Similarly, ITS1 region sequence data from this study demonstrated that a single species of *Hematodinium* occurs from Virginia to Texas (Chapter 2). This finding is particularly interesting given than multiple studies have shown population differentiation between the eastern seaboard of the USA and the Gulf of Mexico in other marine species (for review, see Avise 1992) and for another marine parasite (Reece et al.

2001). Additionally, while the same *Hematodinium* species occurs throughout the UK, there are differences in the sequence data that suggest genetic differentiation may be present (Small et al. 2007a, Hamilton et al. 2010); however, this was not the case for the *Hematodinium* sp. in blue crabs, as there was very little variation in the ITS1 region sequence data over the entire geographic range examined. Determining the mechanisms that have maintained this low level of variation could yield some interesting information about the life history and spread of this parasite.

Due to the low level of variation examined in the ITS1 region sequence data, it was necessary to develop new molecular markers for examining the intra-specific variation of this parasite. Thus, using enriched microsatellite library techniques, eleven microsatellites markers were developed for the species of *Hematodinium* that infects blue crabs (Chapter 3). When primers for eight polymorphic markers were used to amplify DNA extracted from the hemolymph of infected blue crabs. These findings from the microsatellite data include that this parasite is haploid in the host hemolymph, multiple infections are common along the Delmarva Peninsula, and that this parasite has high genotypic variation strongly indicative of sexual reproduction in the life cycle (Chapter 4, 5).

The microsatellite markers developed in this study have the potential to elucidate further aspects of the ecology and life history of this parasite, and possibly other members of this genus. These markers could be used to genotype cultures used for laboratory experiments and, through these experiments, could provide insight into potential strain variation and composition. These markers could also be used to examine differences in the genotypic composition of this parasite between and among blue crab populations from various geographic locations. More specifically, these markers could be used to compare genotypes of *Hematodinium* sp. from Virginia and Georgia. Differences in the genotypic composition of *Hematodinium* sp. between these locations may explain the differences in virulence reported from *Hematodinium* sp. from Virginia (Messick 1994, Shields and Squyars 2000, Messick and Shields 2000) versus *Hematodinium* sp. from Georgia (Lee and Frischer 2004, Frischer et al. 2006, Walker et al. 2009). Ongoing research with these microsatellite markers is examining other species of *Hematodinium* that are closely related to the species that infect blue crabs, including *Hematodinium*

perezi from the type host from France and the *Hematodinium* species that infects *Portunus trituberculatus* in China (Small et al., in review). Preliminary data has shown that different alleles are present in *H. perezi* compared to those that have been observed to date in the *Hematodinium* sp. in blue crabs. Additional microsatellite data may be useful for elucidating the relationships between these parasites and could give insight into the historical origin and spread of this parasite to these locations.

Prior to this study, little molecular work had been conducted to examine host specificity, population structure, and life history traits of *Hematodinium* sp. in blue crabs. This study clearly demonstrates the utility of microsatellite markers for examining aspects of the ecology and evolution of parasites. The tools described in this work have the potential to contribute to a variety of additional studies and potentially can be applied to other species of *Hematodinium*. Overall, the techniques described in this study could be employed to examine other host-parasite systems, particularly those where the parasite is difficult to culture.

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