DERMO DISEASE ON MARTHA’S VINEYARD: INFECTIONS OF *PERKINSUS MARINUS*
AND ITS INFLUENCE ON OYSTER HOST POPULATION STRUCTURE

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A thesis submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements for the degree of

Master of Science

Graduate Program in Ecology and Evolution

written under the direction of

Dr. David Bushek

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New Brunswick, New Jersey

May 2011
ABSTRACT OF THE THESIS

Dermo Disease on Martha’s Vineyard: Infections of Perkinsus marinus and its Influence on Oyster Host Population Structure

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Dermo is a lethal disease of the eastern oyster Crassostrea virginica (Gmelin), and causes widespread mortality throughout most of its range. The pathogen is a protozoan parasite, Perkinsus marinus (Levine 1978). Despite its impact on oysters, there is little evidence demonstrating the development of resistance in wild oyster populations. On the island of Martha’s Vineyard off the coast of Massachusetts, four wild oyster populations inhabit separate coastal ponds with varying histories of P. marinus exposure. Two complementary studies were performed using these oyster populations to evaluate responses to Dermo disease: one study monitored disease levels to detect phenotypic response, and the other assessed genotypic differences that are the result of natural selection following disease exposure.

The first study tested the hypothesis that different patterns and levels of Dermo disease correlated to exposure history. Disease prevalence and intensity were monitored using Ray’s Fluid Thioglycollate Medium (RFTM) assay during the 2008 and 2009
summer seasons. One population remained uninfected. The infected populations experienced different patterns of Dermo that indicated the influence of factors such as salinity, water temperature, and the breaching of barrier beaches separating the ponds from the ocean, but did not correspond to exposure history.

The second study used putatively neutral and Dermo resistance-linked microsatellite markers to test the hypothesis that genetic divergence from the uninfected control population increases with duration of Dermo-mediated selection. Polymerase chain reaction (PCR) was used to genotype the oysters for both types of markers. Population analysis with resistance markers revealed a general increase in divergence of infected populations from the unselected population over time, whereas neutral markers showed little divergence and no correlation with Dermo exposure history. One pairwise differentiation with the resistance markers, however, showed no correlation to selection time, suggesting that the influence of other factors on selection dynamics can be important. This research indicates that, while wild oyster populations may develop genetics-based resistance to Dermo disease with time, there are many biological and physical factors that may alter the realization of disease resistance.
DEDICATION OF THE THESIS

I could not possibly dedicate this thesis to just one person. I must dedicate it to all the people in my life who have inspired me, pushed me, encouraged me, picked me up and settled me down. Thus, I dedicate this thesis to those who taught me lab techniques, field techniques and life techniques; to those that shared frustrations and experiences, or a cup of tea at just the right time; to those that have been by my side since before my journey through graduate school began and to those whom I met along the way that traveled with me as much as they could bare; and to those that taught me to love scientific exploration and discovery and to those that want to see results, progress and more shellfish!

I thank the Vineyard Vision Fellowship and Philip K. Evans Scholarship Foundation for financial support. The Fellowship brought to me many personal and spiritual connections to the Vineyard and its people that are invaluable. I am also so very grateful for the hard work and devotion of Marsha Morin to the Ecology and Evolution department and its students.

Most of all, I have to thank my mother who always believed in me, always forgave me for being absent, never doubted me and never ceased to nourish and support me. Everyone else – including my committee who never gave up on me, Uncle Roger and Aunt Liz, Corly, James, Rick and Amandine, Sammy, Cass, Alyssa, Iris, Emily, Jenn, Sue, Kathy, Susan and Josh – I hope you all know I could not have succeeded without your support, friendship and patience.
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CHAPTER ONE:
THE IMPACT OF DERM O DISEASE ON THE EASTERN OYSTER

Environmental and social importance of the eastern oyster

The eastern oyster, *Crassostrea virginica*, occurs naturally throughout the Atlantic and Gulf of Mexico coasts of North America (Galtsoff 1964). The complex matrix of an established oyster reef is often the sole hard substrate in an otherwise soft bottom environment and serves as substrate for other sessile animals and algae (Dame 1979). The interstitial spaces of a reef provide refuge from predators for juvenile oysters, other invertebrates and small fish, making reefs a foraging area for predators. By consuming seston from the water column, oysters can help prune phytoplankton biomass, cycle nutrients (Dame et al. 1984) and settle suspended solids, thus improving water clarity. The enhanced water clarity is beneficial to submerged aquatic vegetation as well as other estuarine organisms (Dame 1996, Cerco and Noel 2007).

Eastern oysters are also an important fisheries species that support a multi-million dollar fishing industry on the east and Gulf coasts of the US. Populations and landings have decreased, however, by more than 90% on average across the natural range since the 19th century (Rothschild et al. 1984). Much of this decline is due to fishing pressure and destructive techniques, yet it has been amplified by two protozoan parasites. *Perkinsus marinus* (=*Labyrinthomorpha marina* = *Dermocystidium marinum*), was first described in *C. virginica* in 1949 (Mackin et al. 1950) and MSX, caused by *Haplosporidium nelsoni*, was first discovered in dead oysters in 1957 in Delaware Bay (Wood and Andrews 1962). Extensive mortality in already over-fished populations resulted in the loss of ecosystem services provided by oysters, in
many locations. Water filtration, nutrient cycling, and provision of benthic habitat by oysters were diminished in much of the range of these diseases.

*Perkinsus marinus* was discovered in *C. virginica* after major mortalities occurred in the Mississippi Delta of Louisiana in the mid 1940s. Oystermen filed lawsuits against several major oil companies and the Freeport Sulfur Company, believing that petroleum operations were the cause of the deaths (Ray 1996). Mackin, Owen and Collier (1950) established that the mortalities were not petroleum related, but instead caused by a pathogen that inflicts the disease we now refer to as Dermo disease. Further epizootiological and experimental ecological studies by Andrews, Ray, Mackin and others yielded valuable information for oyster growers and managers. Among many other findings, they determined that direct transmission of infections is achieved through the water column and that the pathogen is most virulent at temperatures and salinities of 20-30°C and 20-30 ppt, respectively (Perkins 1996).

Dermo disease was a warm water pathogen, restricted to the Gulf of Mexico and Chesapeake Bay for most of the next 40 years. A series of droughts and notably warmer than usual winters in the late 1980s provided conditions that enabled the parasite to proliferate in previously uninfected populations and expand its impact to the north. Epizootics occurred from Delaware Bay to Cape Cod between 1990 and 1992, and the first infections were reported in Maine in 1995 (Kleinschuster and Parent 1995, Ford 1996).

*The parasite that causes Dermo disease*

*Perkinsus marinus* is the causative agent of Dermo disease, and is a member of a genus of protozoan parasites that infect marine molluscs on five continents, causing perkinsosis in a variety of molluscan shellfish (Villalba et al. 2004). Perkinsosis has likely caused more
economic losses than any other molluscan disease. Dermo is a type of Perkinsosis in oysters caused by *P. marinus*, which is found free in the water column and is ingested by the oyster during feeding activity (Mackin 1954). The parasite enters the epithelia of the gut, gill, mantle and labial palps (Chintala et al. 2002) where it multiplies and proceeds to colonize new organs. The mechanism by which it leaves the epithelia is not fully understood, but when it does, haemocytic circulation increases to infected tissue and haemocytes phagocytosize the parasite cells. Inside the haemocyte, parasite cells multiply and may rupture the host cell, setting free more parasites to infect additional organs (Mackin 1951, Perkins 1976, Bushek et al. 1997). The progression of infections through host tissues causes lesions, which lead to eventual death. Dermo-induced mortality can be extensive with considerable impact on a population. In addition to mortality, sublethal effects, such as depletion of energy reserves, decreases in fecundity and loss of resiliency against environmental stressors, can also have severe ecological and economic consequences (Villalba et al. 2004)

The seasonal infection pattern of *Perkinsus marinus* is largely water temperature-dependent (Ragone and Burreson 1993), and mortality is rarely observed at temperatures below 18°C (Chu and La Peyre 1993). Infections peak in prevalence and intensity in late summer to fall and it is then that the majority of the disease-induced mortality occurs. Infections regress with declining water temperature, and may be suppressed to undetectably low levels in the winter or spring, although the parasite may not be completely eliminated (Ragone and Burreson 1993). In the northeast and mid-Atlantic, infection rates are typically low at the end of spring, until water temperatures increase and the parasite multiplies rapidly within the host and low levels of mortality are often seen as the parasite becomes active again.

The influence of salinity on *P. marinus*, its virulence, and the ability of the oyster to
modulate its defense against the parasite, has been the subject of many studies. Several studies have found positive correlations between salinity and Dermo infections (Mackin 1956, Andrews and Hewatt 1957, Soniat 1985), showing that oysters grown in high salinity waters (15 – 30ppt) exhibit higher Dermo prevalence than those grown at lower salinities. In the laboratory, Ragone and Burreson (1993) found a significant effect of salinity on infection intensity and an even stronger effect of salinity on mortality. They found that infections in oysters held at 6 and 9 ppt did not change a great deal during the course of their 56 day study, while infections in oysters at 12 and 20 ppt progressed and caused mortality within the first few weeks. In vitro, Chu and Greene (1989) observed inhibition of zoosporulation by *P. marinus* at low salinities. This helps to explain the fact that historically, *P. marinus* is ineffective or absent in waters that maintain a low salinity. The areas of low salinity were considered Dermo disease-free, and were often located at the upper regions of the estuary. However, when periodic droughts increase salinity in these areas above 15 ppt, the parasite is able to invade. Once established, *P. marinus* can withstand salinities as low as 5 ppt for several months, making it virtually impossible to eliminate. Only the combination of freezing temperatures and very low salinity - such as can be found in parts of the New England and maritime Canada – appear to eliminate the parasite.

**Managing oyster resources**

Sensitivity of the parasite to low salinity may be utilized by oyster growers and resource managers to avoid or lessen mortality from Dermo disease (Andrews and Ray 1988). If possible, a grower may move their oysters from high to low salinity during the months of high Dermo intensity, or transplant oysters from low salinity seedbeds to high salinity before harvesting for
market. These strategies, however, are labor intensive and what is needed for aquaculture and restoration are disease-resistant oysters.

For well over a decade, researchers have been working to create and identify lines of oysters that can withstand Dermo and another eastern oyster parasite *Haplosporidian nelsoni* (MSX). In theory, every generation that survives Dermo disease to reproduce will, presumably, produce a generation of offspring that is more resistant than the last. Studies clearly show that improved growth and survival amidst *H. nelsoni* can be accomplished through selective breeding (Ford and Haskin 1987) and oyster seed that is resistant to MSX is available to oyster growers. Some studies suggest natural selection is providing some resistance to Dermo (Brown et al 2005, Roberts et al. 2008), yet selective breeding for resistance to Dermo disease has not been as easily achieved as it was for MSX. One explanation for this difference is the role of locations where salinity is low that allow the Dermo parasite to persist at low levels. These areas may be called ‘disease refugia’ because as most of the larger population is selected by Dermo, subpopulations in the refuge are not. Unselected oyster populations can live and reproduce in the refugia and contribute unselected genes to the overall gene pool, thus diluting the potential for resistance in the next generation. It has been suggested that the many low-salinity tributaries of Chesapeake Bay provide refugia to MSX, hindering the progress towards resistance to MSX in the wild populations, whereas the oyster population in Delaware Bay has largely become resistant (Hofmann et al. 2010).

Resistance can be defined as either the ability of an organism to avoid infection, or to tolerate the effects of disease (Ford and Tripp 1996). Ray’s fluid thioglycollate medium, or RFTM, allows researchers to easily assess the level of infection in an individual and a population (Ray 1952). However, no detection method thus far can undoubtedly determine which infections
will lead to imminent death of the oyster and which infections will be tolerated for possibly many years. Consequently, although we can quantify infection intensity and prevalence, resistance is difficult to measure. Nevertheless, Bushek and Allen (1996) found that in the laboratory, the rate infections developed generally decreased among populations with time from the first exposure to Dermo disease.

More recently, molecular tools have been developed to recognize the genetic basis for disease resistance (Roberts et al. 2008, Guo et al., 2008, Yu and Guo 2005, Wang and Guo, unpublished). Resistance to, or tolerance of *P. marinus* results when selective pressure favors particular genotypes and the genotypes that are less favorable, become less common. Yu and Gou (2005) showed that shifts in genotypic frequencies following epizootic mortality events are caused by preferential survival of individuals with specific genotypes. These genotypes increase the fitness of the host in a way that may affect metabolism and reduce susceptibility to infection, or affect cellular responses to the pathogen, thus reducing the effects of disease on the host and, in the case of Dermo in *C. virginica*, avoid death.

In 1994, Dermo disease was detected in one of four brackish ponds that contain self-recruiting oyster populations on the island of Martha’s Vineyard off the coast of Massachusetts, USA. In 1999 and 2004, the disease was detected in oysters from two of the other ponds, while oysters in the fourth pond appear to have remained free of the disease. The sequential exposure to *P. marinus* established a natural experiment to examine the response of oyster populations to Dermo disease. Specifically, this natural experiment tests the hypothesis that populations exposed to the disease should show different levels of disease as a function of their time since initial exposure, and that there would be genetic differentiation related to the time since initial exposure among all four populations at microsatellite loci that are structurally linked to disease
resistance genes. By comparison, unselected loci should show no pattern related to time of exposure to Dermo.
CHAPTER TWO:

PATTERNS OF DERO DISEASE ON MARTHA’S VINEYARD

INTRODUCTION

The oyster disease Dermo is caused by infestation of the protozoan parasite *Perkinsus marinus*. Scientists identified Dermo disease as the cause of oyster mortalities in *Crassostrea virginica* in the Gulf of Mexico in 1946 (Ray 1996) and Chesapeake Bay in 1949 (Mackin et al. 1950). The parasite was predominately detected south of the Chesapeake Bay until 1990, when, concurrent with a series of unusually warm winters, *P. marinus* infected oysters in Delaware Bay and populations as far north as the Damariscotta River in Maine, within a few years (Ford 1996). Dermo epizootics have devastated many oyster populations and altered associated estuarine communities because oysters were often the dominant species, an important provider of habitat, and a functionally important water filtration system. In turn, the economies of many oyster fishing communities throughout the U.S. mid-Atlantic and Northeast regions were drastically suppressed.

*Dermo in Martha’s Vineyard Oyster Populations*

Martha’s Vineyard is an island off the southeast coast of Massachusetts, on the northeastern coast of the United States. Four oyster populations exist on Martha’s Vineyard, occupying four different coastal salt ponds. From east to west across the island they are Edgartown Great Pond (EGP), Oyster Pond (OYP), Tisbury Great Pond (TGP) and Squibnocket Pond (SQP) (Figure 1). These populations are isolated from each other and the ocean by barrier
beaches that are mechanically breached several times a year. Perhaps due to this isolation, they were initially infected by \textit{P. marinus} at different times (Figure 2).

The first Dermo infection on Martha’s Vineyard was detected in EGP in 1994 (Figure 2). Prevalence the following summer was 85% (Ford 1996) and was followed by the first major Dermo-induced mass mortality. Shell stocking (the planting of shell to serve as hard substrate for setting bivalve larvae) by the town shellfish department for the past decade has helped the population persist. More recently, the planting of hatchery-reared spat on shell (larvae set on shell to reduce predation) by Martha’s Vineyard Shellfish Group, Inc. was resumed in 2008 with considerable success. Today, local oystermen report that the oysters of EGP are growing to market size and larger despite the disease, and little summer mortality is observed. The population still fails to thrive, however, due to combined pressures of heavy sedimentation, substrate limitations, predation by crabs and fishing pressure (P. Bagnall, pers. comm.).

Dermo infection was first detected in Tisbury Great Pond (TGP) in 1999 (Figure 2), with 8% prevalence (Smolowitz 1999). The next year, mortality was estimated to approach 75% (R. Karney, pers. comm.) Annual restoration efforts by MVSG help augment the population in TGP and support up to two dozen oyster fishermen. While oysters are growing to market size, mortality in the early fall is commonly noted by fishermen and constables (I. Scheffer, pers. comm.).

The first confirmation of Dermo in Oyster Pond (OYP) was in 2004 (Figure 2) with 100% infection prevalence and a 3.1 weighted prevalence (mean sample infection intensity) (Smolowitz 2004). The high levels indicate that \textit{P. marinus} was probably already present before 2004, but due to sporadic disease testing and no public oyster fishing, substantial mortality had not been reported before 2004 (Paul Bagnall, Richard Karney, pers. comm.).
Dermo disease has never been detected in SQP. There is no record of a natural oyster population in Squibnocket Pond (SQP), yet oysters from a wild, off-island population and from an off-island hatchery were planted in SQP in the mid to late 1970s (R. Karney, pers. comm.) and are now well established in the pond. There is no public access to the pond and bacterial levels often restrict shellfish harvesting. SQP may have remained free of detectable levels of *P. marinus* due to the lack of contact with infected populations resulting from the different drainage orientation and the fact that it is never breached to the ocean.

Pond openings are considered important to the health of the brackish ecosystems and they allow saltwater input to the system, migration of estuarine animals and larvae as well as flushing of excess nutrients brought to the pond from the land, to the sea. For a breach to be successful the water level in the pond must rise well above sea level so that the head pressure within the pond will force the cut in the barrier beach open and deep enough to sustain itself for at least several days. The result is a net loss of water from the pond, the volume of which depends on how high the water accrued before it was opened. The tidal range and exchange volume of every tidal cycle generally decreases with time after the breach as the opening fills in with sand (Wilcox 2009).

The sequential spread of the disease among the oyster populations on Martha’s Vineyard presents a natural experiment to observe the response of a broadly dispersed marine organism to selection pressure from a disease-causing pathogen in confined bodies of water. The findings of this study will help elucidate how oyster populations respond to Dermo disease and how exposure history affects seasonal infection patterns. This information will advance efforts to develop Dermo resistant oysters in selective breeding programs, as well as identify wild
populations that have already developed resistance through natural selection. This study commenced with the following hypotheses:

- $H_0$: Oyster populations on Martha’s Vineyard experience *similar* infection patterns of Dermo disease at *similar* levels.

- $H_1$: Oyster populations on Martha’s Vineyard experience *different* infection patterns and levels of Dermo disease that are *unrelated* to their history of exposure.

- $H_2$: Oyster population on Martha’s Vineyard experience *different* infection patterns and levels of Dermo disease that *correspond* to their history of exposure to Dermo disease.
METHODS

Study Sites

Martha’s Vineyard is an island located south of Cape Cod in Massachusetts, USA (Figure 1). The salt ponds harboring the oyster populations, Edgartown Great Pond (EGP), Oyster Pond (OYP), Tisbury Great Pond (TGP) and Squibnocket Pond (SQP), are separated from the Atlantic Ocean by a narrow barrier beach, three of which (EGP, OYP and TGP) are breached mechanically several times a year, primarily to allow for tidal flushing of land-derived nutrients. Once a beach is breached it may remain open for five days to two months, or until it closes naturally. Unlike the three ponds that are periodically breached, SQP is connected to the Vineyard Sound on the north side of the island via a small tidal creek that connects to Menemsha Pond, which is kept open to the ocean with jetties. The beach that separates SQP from the Atlantic Ocean is never breached mechanically or naturally and all water exchange occurs though the small tidal creek.

Monthly Oyster Collection

From May through September 2008, thirty oysters (50 in October; see Chapter 3) were collected monthly from the four coastal salt ponds. Squibnocket Pond was not sampled in June 2008. Oysters being 65 mm to 85 mm in shell height were targeted for collection from all of the ponds. The oysters were collected with a small dredge or a hand rake from three to five locations within each pond to ensure adequate representation of the population within each pond (Figure 3). Squibnocket Pond had three sites because the oysters exist predominantly in the main body of the pond. Oyster Pond also had three sites because it is narrow with a relatively simple
configuration. Tisbury Great Pond had five sites; two in semi-remote coves and three from different sides of the main body of the pond. Edgartown Great Pond initially had five sites; three in remote coves and in two different regions of the main body of the pond. However, no oysters were found on the site closest to the beach after the first sample was taken, so oysters were often collected from additional locations. The resulting samples were intended to represent an average of potential infection levels and genetic heritage of the population in each pond.

Sampling methods were modified in 2009 to provide mortality data and to investigate the effects of breaching the barrier beach on Dermo infection levels. In June, 300-400 oysters were collected from throughout each of the three Dermo-infected ponds and combined into floating containers at a site within the respective pond that was easily accessed from the shore. Each month, 30 oysters were collected from the containers, then measured and dissected for Dermo diagnosis as described below. Temperature, salinity and mortality were recorded during each collection.

**Water Quality Monitoring**

Salinity and temperature were recorded during each collection within centimeters of the sediment, using a YSI85 water quality monitoring instrument (YSI Inc. Yellow Springs, OH, USA). In addition, Hobo® Water Temp Pro temperature loggers (Onset Computer Corporation, Falmouth, MA, USA) were deployed in each pond to record temperature at 3 hr intervals throughout the 2008 sampling period. One temperature logger per pond was deployed at a central location relative to the sample sites by fastening them close to the bottom of a buoy anchor line to record bottom temperatures. The temperature logger in OYP was lost, but data from the other three loggers were uploaded using Onset Computer Corp, Boxcar® software.
**Sampling Breach Events**

Oysters in EGP and OYP were sampled before, during and after breaches during 2009 to observe the impact of breaches on Dermo infection prevalence and intensity. Thirty oysters were collected within 7 days before the barrier beach was breached, 2 – 3 days after it was opened, and 14 days later. Oysters were processed for Dermo as described below.

**Oyster Processing**

Oysters were transported to the MVSG laboratory, where height was recorded with electronic calipers. Height was measured as the longest point from hinge to bill of the oyster, as it is the most commonly used measurement to describe oyster size. Each oyster was dissected using sterile technique to remove tissues for Ray’s fluid thioglycollate medium (RFTM) assay to detect Dermo infections (Ray 1954), and for genetic analysis. For Dermo analysis, rectal and mantle tissues were placed in 5 ml of sterile RFTM, fortified with 0.5 ml Penicillin/Streptomycin antibiotics, and incubated in the dark for five to seven days. The tissues were removed to a glass slide, stained with Lugol’s iodine, minced, and examined under a compound light microscope for parasitic infection. Infections were rated from 0.0 – 5.0, with 0.0 representing no detectable infection, and 5.0 representing a very heavy parasitic infection (Ray 1954). Mantle, gill and adductor tissues were preserved in 95% ethanol for genetic analysis (see Chapter 3).
Data Analysis

Dermo infection prevalence was calculated as the percent of infected oysters in the sample, while weighted prevalence was calculated as the sum of infection scores, divided by the number of oysters in the sample. Prevalence and weighted prevalence were plotted over time and inspected for seasonal patterns as well as differences among ponds. Squibnocket was excluded from this analysis because no infections were detected. Analysis of variance (ANOVA) using MyStat 12.02.00 (Systat Software, Inc., Chicago, IL) and Scheffé’s post hoc test were used to detect significant differences in both oyster shell height and weighted prevalence among sampling dates within populations. Water temperature, salinity, mortality and mean shell height were plotted over time and inspected for differences among ponds that could impact infection levels.
RESULTS

Dermo in Tisbury Great Pond

TGP showed the expected, seasonal infection pattern both years of the study, characterized by low levels of infections in the beginning of the summer, a sharp increase with increasing water temperature, and a gradual decrease as water temperatures wane in the fall (Figure 4). Weighted prevalence reached peaks in August 2008 (1.55) and September 2009 (2.2) then decreased in October of each year to 1.34 in 2008 and 1.77 in 2009 (Figure 5). Similarly, prevalence peaked at 100% in September both years and remained high in October. Oyster shell height did not vary significantly in 2008 \((p = 0.772)\), but did in 2009 \((p = 0.038)\) (Figure 6). However, Scheffe’s test indicated that the difference in height between the most different pair of data - August 26th and September 21st, 2009 - was not significant \((p = 0.065)\), and was not reflected by disease levels. Mortality (only measured in 2009) peaked in August and cumulative mortality exceeded 16% by the end of the season. (Figure 7). Salinity varied throughout the season, but in very different patterns between years. Overall, salinity was above 15 ppt for the majority of both summers (Figure 8).

Dermo in Edgartown Great Pond

Dermo patterns in Edgartown Great Pond (EGP) were neither typical nor similar to TGP. Prevalence and weighted prevalence were lower in EGP than in TGP during both years of the study (Figure 5). With the exception of prevalence and weighted prevalence on August 5th of 2009, Dermo infection levels in EGP were quite similar both years of the study. Weighted prevalence and infection prevalence varied throughout the summer each year in ways that were
not readily explained by water temperature or salinity. Weighted prevalence paralleled TGP in 2008, from May to late August. From August 1 to August 23, 2008, a notable decrease occurred in EGP (wp: 1.27 to 0.59); yet it was not significant (Scheffe’s test; $p = 1.000$). Late September weighted prevalence was nearly equal to August 1st then decreased in October (wp = 0.57) with falling water temperatures. In 2009, there was a dramatic increase in weighted prevalence between July 10th and August 5th (wp: 0.82 to 1.88) followed by a sharp decrease in weighted prevalence just two days later on August 7th (wp = 1.08). Weighted prevalence increased slightly in late August and September before falling to 0.62 in October 2009. Although oyster height did vary significantly ($p = 0.000$) between sample collections in both years of the study, fluctuations were independent of each other and do not reliably predict infection levels for either year (Figure 6). For example, while Dermo prevalence and weighted prevalence decreased from September to October of both years, and oysters in October of 2009 were significantly smaller than those collected in September ($p = 0.014$), in 2008 they were not ($p = 0.974$). Cumulative mortality did not exceed 2% in 2009 (Figure 7). Salinity was above 15 ppt from June through October 2008, yet below 15 ppt for all of 2009 (Figure 8).

**Dermo in Oyster Pond**

Like EGP, Dermo disease in Oyster Pond (OYP) did not progress in a similar manner to TGP. Weighted prevalence in OYP followed a trend very similar to EGP in 2008, although at a greater magnitude (Figure 5). Similar to EGP, there was a decrease in weighted prevalence from July 28 to August 19, 2008 (wp = 1.45 to 1.07) that was not statistically significant (Scheffe’s test; $p = 0.929$). Oyster shell height was not significantly different across sampling dates in 2008 despite the variability in prevalence and weighted prevalence (Figure 6). In 2009, height
varied significantly ($p = 0.000$), yet the increases in oyster height did not correspond well to infection levels. For example, there were significant differences in shell height, according to Scheffe’s test, from the September 5th and 7th samples, and the late October sample, which were not reflected in disease levels. In contrast to EGP, salinity was below 15 ppt in June and below 10 ppt from July through October of 2008 and comparably low in 2009 (Figure 8). OYP had the highest observed weighted prevalence for most of 2008 (maximum in September, wp = 1.63) and the lowest for most of 2009 (maximum on September 24th, wp = 1.45). Weighted prevalence decreased very little in October of either year; contrary to EGP and TGP, and infection prevalence was below 70% only once in 2008 (on August 19, corresponding to decreased weighted prevalence) and did not fall below 75% at any sampling date in 2009. Cumulative mortality reached nearly 11% by the end of the summer of 2009 (Figure 7), however, the initial mortality count after the oysters were collected and deployed into floats was suspiciously high. It is possible that some dead oysters were mistaken to be alive at the first collection and if they are removed from the calculation, cumulative mortality may have been closer to 6%, which is still lower than TGP and higher than EGP.

**Dermo in Squibnocket Pond**

There was no Dermo found in any oyster from Squibnocket Pond (SQP) at any sampling date in 2008, or in the single sample of 30 oysters collected in October 2009. Thus, SQP remained free of *Perkinsus marinus* throughout the study. Based on data from the temperature logger deployed in 2008, water temperature in SQP is very similar to that of TGP and EGP (OYP logger was not recovered). This is a reasonable result based on the close proximity of the coastal ponds and the approximate water depth, which rarely exceeds 3 meters in any pond. Unlike the
ponds that are breached to the ocean, SQP maintained a narrow salinity range (10.5 ppt to 13.0 ppt) from May through October of 2008 (although June 2008 sample was not collected). In October of 2009 salinity was 12.5 ppt and water temperature was 13.9°C, which was comparable to the other ponds.

**Water Quality and Oyster Shell Size**

Seasonal water temperature profiles were nearly identical for all ponds on Martha’s Vineyard both years (Figure 8), with the exception of Oyster Pond in September of 2009, when it spiked from 6.8°C to 12.6°C when the water level dropped after the pond was opened. Salinity varied between sampling dates within each pond, and across both sampling seasons (Figure 8). Unlike temperature, salinity varied independently in each pond and was not correlated to seasonal changes in water temperature.

Oyster height varied across sampling dates during both years of the study (Figure 6). The largest and most significant difference was between June 2008 and September 2008 in EGP (92 mm, 75 mm; \( p = 0.000 \)). It is hard to say if this difference affected the observed Dermo infection levels because weighted prevalence was not significantly different between these dates (0.97, 1.25; \( p = 0.949 \)). Overall, the differences in oyster shell height are not strong enough to predict Dermo infections.

**Dermo Levels Before and After Breaching Events, 2009**

Oysters were collected from Edgartown Great Pond just before the barrier beach was breached on August 5th, in order to observe what effect the breach may have on Dermo levels.
The subsequent sample taken on August 7\textsuperscript{th} showed a drop in prevalence from 100\% to 70\% and weighted prevalence from 1.88 to 1.08, which was not significant according to Scheffe’s post hoc test ($p = 0.205$). The reduction was not caused by mortality because no oysters were found dead on August 7\textsuperscript{th} and sampled oysters were the same size as the samples on August 5\textsuperscript{th} ($p = 0.658$) and on August 20\textsuperscript{th} ($p = 0.975$) (Figure 5) according to Scheffe’s test. Water temperature and salinity were not affected by the opening at the sample location, although the pond was open to the ocean through August 11\textsuperscript{th}. Considering the statistic insignificance of the decrease in infections observed, it is possible that the decrease was simply the result of sampling error.

Oyster Pond was sampled on September 21\textsuperscript{st} and was breached the following day. On September 24\textsuperscript{th}, temperature, salinity and Dermo infections had all increased considerably. The floating cage was deployed on a steep, submerged slope, where the depth decreased from over 1.5 m before the opening, to less than 0.30 m on the 24\textsuperscript{th}. The temperature increase was likely related to the change in depth and may not reflect the conditions in the majority of the pond, and the observed increase in infections may not either. Salinity increased from 6.8 ppt on September 21\textsuperscript{st}, to 12.6 ppt on October 7\textsuperscript{th}. Prevalence increased from 77\% to 93\% and weighted prevalence from 0.77 to 1.45 during the first three days after the breach, yet no mortality was seen on September 24\textsuperscript{th}. Oyster Pond was open until September 28\textsuperscript{th}, and sampled again on October 7\textsuperscript{th}. Infections dropped only slightly on the 7\textsuperscript{th}; one oyster was found dead.
DISCUSSION

Differences in Dermo Disease Patterns

The typical seasonal Dermo pattern is characterized by a rapid increase of infections as the water temperature nears and surpasses 20°C, continued rise in infections throughout the summer, and often mortality in late summer or fall (Chu and La Peyre 1993, Villalba et al. 2004). In some locations, where salinity may be low or water temperature is high for a short length of time, mortality may not be observed. Infections typically recess after water temperature begins to fall (Ragone and Burreson 1994).

Of the four oyster populations studied on Martha’s Vineyard, Massachusetts in 2008 and 2009 only TGP showed infection progression and regression patterns that are characteristic of the disease and are considered to be strongly guided by seasonal changes in water temperature. Squibnocket remained free of detectable infections during this period. Meanwhile, infection patterns of EGP and OYP populations were more intricate and were not clearly predicted by water temperature, salinity or oyster size. The three populations were clearly subject to different salinity regimes during both seasons of monitoring for this study, but salinity did not explain disease levels, nor did changes in salinity explain sudden changes in Dermo disease. Based on the occurrence of otherwise unexplainable perturbations in the seasonal progression-regression cycle, it seems apparent that the barrier beaches have strong influences over Dermo disease in these populations. While it was possible to observe the differences in infection rates, it is unfeasible to assess the causes of these differences or their relative resistance to Dermo without controlling for either of these physical factors, or performing a transplant experiment. What can
be said, is that all three populations, in their respective coastal pond, experience different patterns and levels of Dermo disease.

The initial foundation of this study was partly based on proposed Dermo resistance of EGP over TGP by Roberts et al. (2008). Due to unforeseen complexities of infection dynamics in these systems on Martha’s Vineyard, the question of resistance was not adequately addressed in this study. Disease pressure may vary significantly among the ponds, and, without controlling disease pressure, one cannot say which stock has performed better. If all factors were equal, mortality data could provide a measure of resistance or tolerance among the populations. The moderate infections and very low mortality experienced in moderate salinity Edgartown Great Pond in 2009 could be interpreted as tolerance to Dermo disease in comparison to OYP, which experienced low salinity and low infections, but still incurred more mortality than EGP. While these data may not warrant such conclusions, they surely warrant further investigation.

The Plausible Impacts of Pond Opening on Dermo Levels

As stated earlier, previous work has indicated that a decrease in infection level can be caused by lowered temperature or salinity, or by the death of heavily infected individuals, which will lower the mean infection level. Because no oysters died during the reduction in weighted prevalence in August 2009 in EGP, it is not likely that the depressions in August 2008 in OYP and EGP were caused by mortality. At that time, neither salinity nor temperature changed, so the decrease may be more closely related to the breaching of the pond than to the other typical factors of temperature and salinity.

Several authors have suggested that Dermo prevalence is often less in low salinity waters because of the dilution effect of the freshwater input at the head of an estuary (Mackin 1956, Ray
1954, Andrews and Hewatt 1957). While salinity was not a strong governing factor during this study, the concept of parasite dilution is worth visiting. When the pond is opened to the ocean, water level decreases substantially, nutrients are flushed from the system (W. Wilcox, pers. comm.) and parasites are likely flushed as well. A reduced density of *P. marinus* cells in the water column could be expected to lower incidence of exposure and new infections of Dermo in the oysters, thereby allowing host defense mechanisms to successfully expel parasites before new ones are acquired.

The similarities between prevalence and weighted prevalence trends (not their amplitude) in OYP and EGP in 2008 were reflections of their management. Both ponds were opened to the ocean within days of each other, which seemed to have caused the unexpected depression in infections that make OYP and EGP so different from TGP. EGP and OYP are both managed by the town of Edgartown, while TGP is managed cooperatively by the towns of Chilmark and West Tisbury. With the openings being controlled by different town groups, timing, duration, extent and frequency of openings are often different.

Breaches to TGP often stay open longer than those to OYP or EGP, for physical and geological reasons such as placement of the breach and pressure supplied by headwater streams. For a breach to be successful, the water level in the pond must rise above sea level so that pressure within the pond will force the cut in the barrier beach open and deep enough to sustain itself for several days. The result is a net loss of water from the pond. For example, water in TGP attained an elevation of 5.57 NGVD (4.97 feet above MSL) before it was opened to the ocean on April 9th, 2009 and reached a low of 0.7 NGVD during a low tide, which equates to a discharge of 133 million cubic feet of water, or 48% of the initial volume (Wilcox 2009). During the first week after being opened, the tidal range was about one foot, moving 24.7 million
cubic feet of water, or about 17.4% of the pond volume while tidal. In general, the tidal range decreased with time, down to 0.2 feet in mid July 2009. From its closure on September 9th, TGP rose three feet in 30 days, representing 113.8 million cubic feet, or 3.8 million cubic feet per day (Wilcox 2009). The longevity of the opening in TGP during the course of this study seemed to create conditions that caused infections to progress in a manner similar to those in other Dermo-infected estuaries that are in permanent contact with the ocean.

The effect of tidal flushing on weighted prevalence had varying impacts in this study. After the pond loses the tidal exchange with the ocean, infection levels returned to a level comparable to that before the breach. This could mean that a breach in mid-summer may not prevent mortality in late summer, but a breach at the right time – just before mortalities occur – could prevent death.

In the search for stable food sources, economic stability as well as remediation of negative impacts human activity has on our natural environment, oyster enhancement and restoration has several goals. If the goal is for those market sized oysters to survive until winter for commercial harvest season, the perfectly timed breach may aid in that effort. However, further investigation into this concept is necessary. Alternatively, avoiding disease-induced mortality may not be the appropriate strategy if selection for resistance to Dermo disease is the ultimate goal of the resource manager. Only via natural selection will the population achieve greater self-sustaining genetic tools.
Figure 1:
Map of Martha’s Vineyard, showing coastal ponds with wild oyster populations

Squibnocket Pond (SQP) is connected to a harbor through a small creek, while Tisbury Great Pond (TGP), Oyster Pond (OYP) and Edgartown Great Pond (EGP) are separated from the Atlantic ocean by a barrier beach which is mechanically breached several times a year.
Figure 2:
A timeline of initial infections of *Perkinsus marinus*, the parasite that causes Dermo disease

The wild oyster populations on Martha’s Vineyard have experienced different histories of exposure to Dermo disease, including Squibnocket Pond (SQP), which has remained free of detectable levels of *P. marinus* through October 2009. All Dermo diagnoses used Ray’s Fluid Thioglycollate Medium (RFTM) assay.
In 2008, oysters were collected monthly from three to five sites in each coastal pond. Water temperature and salinity were also monitored at these sites. Hobo® Water Temp Pro loggers were deployed at the first sampling in late May of 2008 and collected in October of 2008. In 2009, oysters were collected from three ponds (excluding SQP) and kept in floating cages in their respective pond. The three infected populations were sampled every month, while Squibnocket Pond (SQP) was sampled only in October.
The typical Dermo infection curve is exemplified here by the >10 year mean weighted prevalence (mean sample infection intensity) in Delaware Bay (Bushek 2011).
While TGP shows infection patterns that are typical of the disease, EGP and OYP do not; they show that there are interacting forces that affect Dermo disease patterns in these coastal ponds. These data were analyzed via one-way Analysis of Variance (ANOVA) and Scheffé’s post hoc test, using MyStat 12 and Systat 8, respectively.
One-way ANOVA ($P = 0.05$, MyStat 12) were used to detect significant differences in shell height. Scheffe’s test (Systat 8) was applied to identify significant post hoc variation in shell size as a result of sampling error that could effect Dermo infection levels. Error bars represent the standard error of the mean for each sampling event.
Mortality was highest in Tisbury Great Pond (TGP) and accumulated to approximately 16% by October. Second highest mortality rate was in Oyster Pond (OYP) and totalled almost 11% by late October, while Edgartown Great Pond (EGP) experienced less than 2% mortality throughout the whole study season.
Salinity and temperature were recorded with every sample collection. Salinity was highly variable between years within each pond, and between ponds. Conversely, water temperature profiles for all three ponds were very similar both years of the study.
CHAPTER THREE:

THE EFFECTS OF Dermo DISEASE ON OYSTER POPULATION STRUCTURE ON MARTHA’S VINEYARD

INTRODUCTION

Population structure in marine invertebrates

In contrast to terrestrial and freshwater species, marine invertebrates tend to show little genetic population structure over small or even large geographic scales. This is largely attributed to life history characteristics such as broadcast spawning, high fecundity and planktonic larval stages (Buroker 1983, Palumbi 1992, Waples 1998). About 70% of marine invertebrates produce pelagic larvae (Mileikovsky 1971), which may compensate for limited post-settlement mobility. Schooling and other behavioral associations may be responsible for distinct genetic assemblages in pelagic fish. Yet despite the apparent tendency towards panmixia, a few studies have found genetic differentiation in marine bivalves using nuclear genes (Reeb and Avise 1990, Hare and Avise 1996, Hare and Avise 1998, Hirschfeld et al. 1999, Hoover and Gaffney 2005, Murray and Hare 2006). Historical vicariance events, internal fertilization, brooding, larval behavior, habitat discontinuity, currents, and gyres may cause such structure (Bohanok 1999, Ridgway 2001, Barber and Mann 2003, Luttikhuizen et al. 2003, Costantini et al. 2007, Galindo-Sanchez et al. 2008, Zhan et al. 2009).

The eastern oyster, *Crassostrea virginica* (Gmelin 1791) is a euryhaline, sedentary bivalve that produces planktonic larvae. Oyster larvae are planktonic for 2 – 3 weeks (Kennedy 1996), during which time they may travel on currents and tides for long distances, settling among
distant populations or returning to settle among neighboring oysters near their birthplace. Over evolutionary time, very little gene flow is required to create and support genetic homogenization, so only strong and persistent evolutionary barriers to gene flow will result in differentiation between populations (Palumbi 2003). In the presence of a barrier, Dermo may have the ability to create population structure based on differential selection. In order to detect differentiation caused by Dermo, genetic markers that are capable of detecting differential selection must be developed and used.

**Use of genetic markers in oyster population studies**

Allozymes (proteins that vary in amino acid sequence) were popular genetic markers for population studies (Buroker 1983) until DNA-based alternatives were discovered. Now, microsatellite markers are recognized to be better suited for detecting fine-scale population structure, due to their abundance, neutrality and highly polymorphic signature, offering high statistical power, by virtue of their high heterozygosity levels (Hedrick 1999). Dozens of microsatellite markers have been developed for the eastern oyster (Brown et al. 2000, Reece et al. 2004, Wang and Guo 2007, Wang and Guo 2009), and are now routinely used for oyster population studies (Rose et al. 2006, Galindo-Sanchez et al. 2008, Milbury and Guo, unpublished). Such population studies typically address the presence of structure-producing mechanisms such as vicariance, genetic drift, Wahlund effect and isolation by distance, using genes that are assumed to be neutral (or not under selection) but are often of uncertain biological function. This standard use of neutral or anonymous genes for population studies inhibits the detection of structure due to factors that affect reproductive fitness, specifically predation and disease (Addison and Hart 2004, Rose et al. 2006), and few studies have been undertaken that
specifically seek population structure caused by adaptive forces such as those for resistance to Dermo (Milbury and Guo, unpublished data). In order to detect genetic differences among populations that are the result of adaptive forces, markers linked to adaptive genes need to be employed (Johannesson et al. 2004, Karl and Avise 1992). This could be especially interesting in the case of Dermo disease; markers that point to genes that confer resistance to Dermo could clarify the relationship between genotypic and phenotypic responses to Dermo disease, as well as aid in the marker-assisted development of resistant biotypes.

For these reasons, research has been conducted to identify disease-resistance genes or markers (Yu and Guo 2006, Guo et al. 2008). In these studies, genetic markers were developed from genes that were mostly related to host-defense (Tanguy et al. 2004, Wang and Guo 2007, Wang et al. 2009). Field trials were used to identify which genes experience major shifts in genotypic frequencies, following widespread mortality from diseases, and those shifted markers, when verified as showing clustering on the genetic map, were considered as linked to disease resistance genes (Guo et al. 2008, Wang and Guo, unpublished). Those microsatellites that did not experience a change after disease-induced mortality were considered “neutral”. The term “neutral” here is not meant to say that these genes have no function, but simply that they are not associated with host-defense activity or Dermo resistance. In this study, I chose four putatively neutral markers and four markers that have shown linkage to disease resistance genes (referred to as resistance markers, here on out), to address the difference between unselected population structure and that caused by Dermo-based selection.
Selective pressure by Dermo disease on Martha’s Vineyard

Dermo is a lethal disease of the eastern oyster *Crassostrea virginica*, caused by the intracellular protozoan parasite *Perkinsus marinus*. The pathogen transmits itself directly through the water column and gains access to the oyster as the oyster filters water for phytoplankton food. The ability of *P. marinus* to infiltrate effectively and degrade host tissues is largely dependent on increasing water temperature and salinity (Mackin 1956, Andrews and Hewatt 1957, Soniat 1985), and there is an especially strong effect of salinity on disease-related oyster mortality (Ragone and Burreson 1993). Improved survival against Dermo occurs after significant mortality induced by repeated epizootics removes genotypically susceptible individuals from the gene pool. Some support for this process has been shown using selectively bred hatchery strains and some wild populations of oysters in locations where Dermo is enzootic (Ragone Calvo et al. 2003, Brown et al. 2005, Roberts et al. 2008). While these small improvements rarely provide enough relief from the disease for the population to regain the numbers lost to Dermo or for aquaculture to be viable, after epizootics in oyster populations on Martha’s Vineyard, Massachusetts, some recovery was recognized and some degree of resistance was inferred (Roberts et al. 2008).

The first positive Dermo diagnosis on Martha’s Vineyard was in Edgartown Great Pond (EGP) in 1994, the second in Tisbury Great Pond (TGP) in 2000, and the third in Oyster Pond (OYP) in 2004. There is a fourth wild oyster population in Squibnocket Pond (SQP) where Dermo has not been detected yet. Each coastal pond is separated from the ocean by a barrier beach, and SQP is also connected to an open bay by a small creek (for greater detail see Chapter 2). The barrier beach of the other three are breached mechanically several times a year, providing the only salt water input and the only opportunity to flush land-derived nutrients. The
barrier beaches isolate the oyster populations from each other, as well as from other populations in the region. Possibly because of this isolation, the four populations have different histories of exposure to Dermo (Figure 1). This situation created a naturally occurring experiment to study the response of populations to Dermo disease over time. I used putatively neutral microsatellite markers to assess population structure among the four oyster populations from Martha’s Vineyard, a population from lower Delaware Bay in New Jersey, and a population from Great Bay in New Hampshire. In addition, microsatellite markers linked to genes that are involved in host-defense mechanisms (henceforth *resistance markers*) were used to detect population structure resulting from disease pressure.

**Selection of Outgroups**

For this study, two outgroups were utilized as reference populations against which comparisons could be made; one from Delaware Bay, New Jersey (NJ), and one from Great Bay, New Hampshire (NH) (Figure 2). The NJ oysters were collected from a portion of the Bay where salinity and selection for Dermo resistance are high. The first Dermo epizootic in Delaware Bay occurred 1990 so the Delaware Bay population has presumably had a longer time to respond to selection from Dermo than any population on Martha’s Vineyard. The NJ population was therefore used to gauge the response among the infected oyster populations OYP, TGP and EGP. In contrast, Great Bay (NH) was used as a minimally selected population, not in close proximity to either New Jersey or Martha’s Vineyard (Figure 2). NH was use to gauge the divergence for the resistance-associated loci on a more regional scale, as well as to assess the effect of distance on the divergence with neutral markers.
Dermo is endemic to Great Bay, but large-scale mortality has not been reported, and it is assumed that little selection has taken place. Although available Dermo records are not inclusive of the entire Great Bay system, they do date back to the mid 1990’s, when prevalence of Dermo was very low. Dermo was not found consistently in Great Bay until 2002, with prevalences of 15%, 8% at two of the three sites tested. In 2003, only one site was tested, and found to have Dermo at 20% prevalence. In 2004, all three sites that were tested were infected with prevalences of 65%, 59% and 16%. Overall, Dermo seems to be increasing in Great Bay, and prevalence ranged from 30% to 95% in October of 2008, with a maximum weighted prevalence of 2.1, the highest to date at the time (Haskin Shellfish Research Laboratory, Unpublished pathology report).

OYP and NH are shown in Figure 1 as both having “advanced” infections in 2004, which likely indicates that 2004 was not the first discovery of the parasite, nor the occurrence of major mortality. Infection levels were very different for OYP and NH in 2004. While NH showed a maximum of 65% prevalence and a weighted prevalence of 1.1 at one site, OYP had 100% prevalence and weighted prevalence of 3.1. Based on this difference, NH is expected to be less selected than OYP.

Based on the premise that resistance to Dermo will increase with persistent disease pressure over time (Bushek and Allen 1996), I examined the following hypotheses (Figure 3) by comparing allelic frequencies at microsatellite loci linked to genes for Dermo resistance:

- H₀ (null hypothesis): disease-linked microsatellite loci show no genotypic difference among populations,
• H$_1$ (first alternative hypothesis): disease-linked microsatellite loci show genotypic differentiation among populations that is not related to the time since Dermo was first detected (i.e., there is no evidence of selection by Dermo), and/or

• H$_2$ (second alternative hypothesis): disease-linked microsatellite loci show genotypic differentiation among populations, corresponding to the time since Dermo was first detected indicating that selection has occurred.

If H$_0$ is true then pairwise F$_{st}$ values should be roughly equivalent among the populations. For example, if considering comparisons with SQP then F$_{st}$ (SQP vs. NH) = F$_{st}$ (SQP vs. OYP) = F$_{st}$ (SQP vs. TGP) = F$_{st}$ (SQP vs. EGP) = F$_{st}$ (SQP vs. NJ). Such a case is not expected if the populations are isolated as genetic drift should lead to differences among populations over time. If H$_1$ is true, then F$_{st}$ values will differ among populations, but not in any way that reflects time since detection of *P. marinus* and Dermo disease. This is essentially the expectation for neutral alleles as a result of genetic drift. Finally, if H$_2$ is true, then F$_{st}$ value will not only be different, but will reflect the time since detection of *P. marinus* and Dermo disease. In other words, those populations that have been exposed to *P. marinus* and experienced Dermo disease for longer periods of time will have higher frequencies of disease-resistant alleles at disease-linked loci when compared with populations that have a shorter disease exposure history. Here, using comparisons with SQP as above, the expectation is that F$_{st}$ (SQP vs. NH) < F$_{st}$ (SQP vs. OYP) < F$_{st}$ (SQP vs. TGP) < F$_{st}$ (SQP vs. EGP) < F$_{st}$ (SQP vs. NJ).
METHODS

Sample Collection, DNA Extraction and Gel Electrophoresis

In October of 2008, fifty oysters were collected from each of the six populations, ranging from a mean of 75 mm to 82 mm in shell height. Oysters were collected from several locations around each of the four coastal ponds on Martha’s Vineyard to supply a representative sample of each population. From Delaware Bay, I chose an area referred to as New Beds (39°14’54”N, 75°15’12W) for its long disease history and high disease pressure. Oysters were received from three locations in Great Bay, New Hampshire and all three sites were used in the population study (Figure 2).

Each oyster was measured with digital calipers and dissected using sterile procedures. Adductor muscle tissue was dissected and preserved in 95% ethanol until DNA was extracted using an E.Z.N.A™ Mollusc DNA Kit from Omega Bio-Tek, according to the manufacturer’s protocol. DNA stock solutions were stored at -20°C and working solutions were aliquotted and diluted 40 times with sterile dionized water, and stored at 4°C while they were in use.

DNA quality was evaluated by gel electrophoresis (Figure 4). Gels were produced using a solution of 0.5x Tris/Borate/EDTA (TBE) buffer (C$_4$H$_{11}$NO$_3$) and 0.9% agarose, which was heated in the microwave for 60 – 120 seconds. I then fortified the hot solution with 5.0 µl ethidium bromide per 100 ml agarose solution, as a fluorescent stain of DNA for visualization under ultraviolet light. The solution was poured into gel trays at approximately 60°C and combs inserted to produce wells. After the gel cooled to room temperature, the combs were removed, and the gel was removed from the gel plate and placed into the electrophoresis box containing 0.5x TBE buffer. I pipetted 3.0 µl DNA stock solution, mixed with 1.0 µl PCR loading buffer,
into each well with sterile pipette tips. Gels were electrophoresed until loading dye appeared to be two-thirds of the way down the gel and the gel was evaluated under ultraviolet light (Figure 4).

**Amplification and Genotyping of Microsatellites**

I performed polymerase chain reactions (PCR) in 10 µl reactions and amplified PCR products on an iCycler thermocycler (Bio-Rad, Hercules, CA) or a GeneAmp 9700 thermocycler (Perkin Elmer, Weiterstadt, CA). PCR amplification was optimized using several mixes and thermocycler schedules until the results were stable and satisfactory (Figure 5) and those protocols were used for the remainder of the study (Table 1).

PCR for three markers (Cvi13, Cvi2k14 and RUCV45, with a forward primer directly labeled at 5’ with NED, FAM and PET, respectively), used the following mix: 1X PCR buffer, 1.5 - 2.5mM MgCl$_2$, 0.6mg/ml or 6% bovine serum albumin (BSA), 0.2mM deoxynucleoside triphosphate mix, 0.2µl each of forward and reverse 5.0 µM primers, 0.05 U of Taq DNA polymerase, and 3 – 10 ng of oyster genomic DNA template. Amplification was achieved with the following schedule: initial denaturation at 94°C for 5 minutes, followed by 35 – 40 cycles of: 94°C for 30 seconds, 55 – 60°C for 30 seconds and 72°C for 30 seconds; a final extension at 72°C for 10 minutes, then held at 4°C.

PCR mix for markers Cvi2i23 and RUCV27 were the same as above, with the addition of 0.2 µl labeled M13-primer (5’-TGTAACGACGGCCAGT-3’) (Schuelke 2000). Amplification was achieved by using an initial denaturation for 5 minutes at 94°C, followed by 35 – 40 cycles of: 94°C for 30 seconds, 55 – 60°C for 30 seconds, and 72°C for 30 seconds; 19
cycles of: 94°C for 30 seconds, 53°C for 30 seconds, and 72°C for 30 seconds; a final extension at 72°C for 10 minutes, then held at 4°C.

Three markers (RUCV46, RUCV63 and Cvi1g8, which contained a directly labeled forward primer), were blended for multiplex PCR, using: 2X PCR buffer, 2.5mM MgCl2, 1.0mg/ml BSA, 0.4mM deoxynucleoside triphosphate mix, 0.03 - 0.12µl combined forward and reverse 10.0 µM primers, 0.09 U of Taq DNA polymerase, and 3 – 10ng of oyster genomic DNA template. Amplification of these markers was achieved using an initial denaturation for 5 minutes at 94°C, followed by 40 cycles of: 94°C for 45 seconds, 50°C for 45 seconds, and 68°C for 2:30 minutes; a final extension at 68°C for 7 minutes, and final holding at 4°C (adapted from Henegariu et al. 1997).

I combined all size compatible PCR amplicons in a single well for genotyping on an Applied Biosystems Inc. (ABI) 3130xl genetic analyzer. PCR products were diluted according to amplification intensity to prevent oversaturation and to allow for proper peak identification. 0.5 – 1.0µl of each diluted PCR product was added to 9.8 µl deionized formamide and 0.2 µl GeneScan™ 600-LIZ® size standard by ABI in a 96 – well ABI MicroAmp® Optical Reaction plate and run on the genetic analyzer. RUCV27, RUCV45, Cvi2k14, Cvi2i23 and Cvi13 were typed together, while RUCV46, RUCV63 and Cvi1g8 were amplified in a multiplex PCR reaction and genotyped at the same time. Scoring of allele size was performed manually, using GeneMapper 4.0 (ABI) software (Figure 6). Two peaks were recognized as being a heterozygote genotype if the smaller was at least 10% of the larger in height. If a score was questionable the individual was either rerun or PCR was repeated.
**Marker Selection**

Eight polymorphic microsatellite markers were considered for population genetic analysis; four that had been linked to disease resistance and four that were not linked to disease resistance. Each microsatellite marker was used to genotype 288 individuals from six populations. Forty-eight individuals were used from each population to fill 288 wells between three 96-well PCR plates. Analysis of these data in Micro-Checker 2.2.3 (Van Oosterhout et al. 2004) revealed a significant shortage of heterozygotes and apparent single-nucleotide repeat-unit genotypes at loci RUCV46 and Cvi1g8 in population SQP, possibly resulting from stuttering (slipped-strand mispairings during PCR), which caused scoring errors at these loci. No evidence of stuttering, scoring error or large allele drop out were detected at any other locus.

Across all loci, all six populations deviated significantly from Hardy-Weinberg equilibrium. Across all populations, six out of eight loci showed significant departures from Hardy-Weinberg equilibrium \((p < 0.01)\). Three of those loci – Cvi1g8, Cvi13 and RUCV27 – exhibited very large excesses of homozygosity, measured by \(F_{IS}\), likely due to the particularly high frequencies of null alleles (Table 2). These three markers were also relatively difficult to score. Therefore, only two neutral (RUCV46 and RUCV63) and three resistance markers (RUCV45, Cvi2i23 and Cvi2k14) were used for population genetic analysis.

**Statistical Analysis**

With the remaining five microsatellite loci, expected (\(H_E\)) and observed (\(H_O\)) heterozygosity and linkage disequilibrium were calculated using GENEPOP version 4.0.10 [(Raymond and Rousset 1995); http://genepop.curtin.edu.au/index.html] with MCMC chain parameters of 10,000 dememorization steps, 1,000 batches, and 10,000 iterations (Fisher’s
method) where appropriate. Departure from Hardy-Weinberg equilibrium was measured using Wright’s $F_{IS}$, which measures the inbreeding of an individual relative to that of the subpopulation (or pond, in this case). To prepare the remaining data for further analysis, individuals with a null homozygote at one or more of the five remaining loci were removed. There were 16 null homozygotes, which reduced the overall dataset from 288 to 272 individuals, with a minimum of 41 (NJ) and a maximum of 48 (NH) individuals per populations. Two unbalanced analyses of molecular variance (AMOVA) were performed in GenAlEx 6.4 (Peakall and Smouse 2006) using 999 permutations to obtain Wright’s $F$ coefficients of population differentiation. Neutral (RUCV46 and RUCV63) and resistance (RUCV45, Cvi2i23 and Cvi2k14 markers were analyzed and interpreted separately. $F_{ST}$ is a comparison/ratio of the variation that exists within a subpopulation with the variation of the total population. $F_{ST}$ can be measured for a pair of populations to relay how divergent they are from each other, compared to the total variation in the two-population sample. $F_{IS}$ and $F_{IT}$ are measures of deviation from Hardy-Weinberg proportions within a subpopulation and the total population, respectively. $F_{IS}$ is also called the *inbreeding coefficient*, because it measures the relationship between two alleles of one individual to the alleles of the subpopulation. Large positive $F_{IS}$ values are commonly seen in marine bivalves and are caused by inbreeding, selection, null alleles and Wahlund effect (McGoldrick et al. 2000, Reece et al. 2004).
RESULTS

Across all populations, all five informative loci were significantly out of Hardy-Weinberg equilibrium ($P < 0.05$), demonstrating heterozygote deficiencies, and four out of five deviations were highly significant ($P = 0.001$), excluding Cvi2i23 ($F_{IS} = 0.053, p = 0.003$) (Table 3). Neutral locus specific $F_{IS}$ across all populations showed higher positive values ($F_{IS} = 0.117$ and 0.073 for RUCV46 and RUCV63, respectively) than those at resistance loci ($F_{IS} = 0.067, 0.006$ and 0.053 for RUCV45, Cvi2k14 and Cvi2i23, respectively) (Table 3). It is likely that high null allele frequencies in the neutral marker RUCV46 caused some heterozygotes to appear as a homozygote.

Linkage disequilibrium (Fisher’s method) using GenePop 4.0.10 (Raymond and Rousset 1995) was found between RUCV46 and RUCV63 in NH ($p = 0.013$), RUCV46 and RUCV45 in NH ($p = 0.006$) and between Cvi2k14 and Cvi2i23 in EGP ($p = 0.012$). Only Cvi2i23 and Cvi2k14 showed consistently significant linkage disequilibrium across all populations ($p = 0.033$). None of these relationships remained significant after Bonferroni correction for multiple tests, although Cvi2k14 and Cvi2i23 both exist within linkage group 2 and are 21.1 centimorgans (cM) apart on the female *C. virginica* genetic map (X. Guo, unpublished data). Thus, the general level of disequilibrium was consistent with the level of sampling, particularly in view of the fact that consistent inbreeding ($F_{IS} > 0$) within any of these populations would result in at least a bit of sampling disequilibrium.
Summary of population structure

Divergence for neutral markers RUCV46 and RUCV63 varied widely among population pairs and was not correlated to history of exposure to Dermo disease (Figure 7). Squibnocket Pond (SQP) was used as an unselected reference for the three Dermo-selected populations on Martha’s Vineyard, which, under the second alternative hypothesis $H_2$, should diverge progressively from SQP in reverse order of their first exposure to the disease (Figure 1). $H_2$ was generally supported, and divergence of the selected populations on Martha’s Vineyard from the unselected population SQP roughly corresponds to history of initial exposure. In terms of Wright’s $F_{ST}$ for the resistance markers RUCV45, Cvi2k14 and Cvi2i23: $F_{ST}$ (SQP vs. OYP, 2004) $< F_{ST}$ (SQP vs. TGP, 1999) $< F_{ST}$ (SQP vs. EGP, 1994). As predicted under $H_2$ (Figure 3), NH was less resistant than OYP, or $F_{ST}$ (SQP vs. NH) $< F_{ST}$ (SQP vs. OYP). What was not predicted under $H_2$ or any other hypothesis, was that NJ was not the most divergent from the control population, despite its long history of exposure to Dermo.

Genetic divergence for neutral loci

A total of 544 genotypes were described for the two putatively neutral marker loci, RUCV46 and RUCV63 (272 individuals, 2 markers). Divergence was highly significant between NH and all four Martha’s Vineyard populations (Table 4, below diagonal): NH & SQP ($F_{ST} = 0.015, p = 0.001$), NH & OYP ($F_{ST} = 0.019, p = 0.001$), NH & TGP ($F_{ST} = 0.013, p = 0.001$), NH & EGP ($F_{ST} = 0.016, p = 0.001$). NH & NJ ($F_{ST} = 0.010, p = 0.004$) showed little more divergence than OYP & SQP ($F_{ST} = 0.009, p = 0.007$) or TGP & SQP ($F_{ST} = 0.008, p = 0.007$). EGP & SQP, EGP & TGP, NJ & TGP and NJ & EGP were the only population pairs to show no
divergence ($F_{ST} < 0.001, 0.479 > p > 0.446$) for these two ‘neutral’ loci, an important finding to be discussed later.

**Genetic divergence for disease-associated loci**

A total of 816 genotypes were described for the three Dermo resistance marker loci (272 individuals, 3 markers). There was no divergence among the Dermo-selected populations on Martha’s Vineyard (EGP, TGP and OYP), as $F_{ST} = 0.000$ for all three pairs, but they were all divergent from the unselected population, SQP (Table 4, above diagonal), and the relative level of divergence corresponded to the order in which they had been exposed to *P. marinus*. Specifically, EGP & SQP showed the largest difference ($F_{ST} = 0.022, p = 0.001$) followed by TGP & SQP ($F_{ST} = 0.019, p = 0.001$) and then OYP & SQP ($F_{ST} = 0.014, p = 0.004$).

Comparing the outgroups with populations on Martha’s Vineyard showed that NJ & SQP ($F_{ST} = 0.019, p = 0.002$) were as divergent as TGP & SQP, but less than EGP & SQP ($F_{ST} = 0.022, p = 0.001$). In contrast, NH & SQP ($F_{ST} = 0.008, p = 0.043$) were less divergent with the resistance markers than were OYP & SQP ($F_{ST} = 0.014$), despite the greater distance between them and the questionable date of first infections in OYP and NH. Interestingly, the most divergent population pair was NH & NJ ($F_{ST} = 0.025, p = 0.001$), which was not the case using the neutral markers. Four population pairs showed no divergence with the neutral markers ($F_{ST} < 0.001$), but one of these one of these was highly divergent with the resistance marker set: EGP & SQP ($F_{ST} = 0.022, p = 0.001$). NJ & TGP ($F_{ST} = 0.004, p = 0.103$) and NJ & EGP ($F_{ST} = 0.006, p = 0.064$) showed low divergence and TGP & EGP remained undifferentiated ($F_{ST} = 0.000, p = 0.000$).
DISCUSSION

Hardy-Weinberg and Inbreeding Coefficients

Significant heterozygote deficiencies were detected at all five markers used for population analysis, which could be caused by inbreeding, Wahlund effect, null alleles or selection. Although hermaphroditism in oysters is very rare (<1%) and they are a functionally protandric dioecious species, inbreeding cannot be ruled out as a possible source of heterozygote deficits, because five of the six populations (excluding Delaware Bay) are quite limited in size and the chances of inbreeding may be high. The Wahlund effect is the reduced frequency of heterozygotes that occurs when multiple populations with different allele frequencies are pooled together and analyzed as one population. There is no evidence of internal subdivision within any of these populations that would indicate a Wahlund effect in this study.

Selection also has the ability to increase $F_{IS}$ values. If it is assumed that resistance alleles are rare, and therefore homozygous resistance genotypes are very rare, heterozygotes containing a resistance allele would be favored over a susceptible homozygote. Selection favoring heterozygotes would make $F_{IS}$ more negative, or less positive, such as those $F_{IS}$ values for the resistance markers compared to the neutral markers. This may be, however, what happens during the initial resistance selection and as selection continues, purifying selection for resistant homozygotes would likely become the dominant process, thus increasing $F_{IS}$.

Null alleles are a possible cause for the apparent excess of homozygotes, especially with marker RUCV46, where 13 of the 16 null homozygotes appeared in the original data set of five markers. One null homozygote was found in each of RUCV63, Cvi2k14 and RUCV45, all of which were pruned out of the data set prior to performing AMOVA, and none were found in
Considering the high frequency of null homozygotes in the neutral marker RUCV46, there is a high chance that null alleles masked many genotypes as homozygotes when in fact they were not, and this is likely the greatest cause of homozygote excess.

**Comparison of Population Structure Using Neutral and Resistance Markers**

This study was designed to look closely at the impact of Dermo disease on the genotypic divergence of several wild populations of eastern oysters and to see if history of exposure to the pathogen determined resistance, as inferred by divergence from a control. Specifically, I set out to test the null hypothesis that there is no genotypic difference between selected and unselected populations. Alternatively, I allowed for the possibilities that there is population structure between selected and unselected populations in no particular pattern, or that divergence from the control population is related to the length of exposure and selection from *P. marinus* (Figure 3).

After comparing the population structure presented by the two neutral markers to that of the three Dermo-resistance markers, it is evident that Dermo disease pressure has impacted the genetic population structure of the oyster populations on Martha’s Vineyard. Across both neutral loci employed in this study (RUCV46 and RUCV63), very little population structure was found among the oyster populations on Martha’s Vineyard, although the small levels of divergence in three out of six pairs were significant. It was not surprising that a small level of divergence was detected ($F_{ST} < 0.009$), considering the separation from each other provided by the physical barriers of the ponds. Aside from oyster restoration activities, of which there are some, the exchange of genes between populations requires that one or more beaches be breached when gametes or larvae are present in the water column so that the gametes or larvae can migrate between ponds. For Squibnocket, gametes or larvae must travel around the landmass on the east
or west end of the island to get between Squibnocket Pond on the northwest shore and the other three ponds on the south side of the island (Figure 2).

NH & NJ showed less divergence with the neutral markers ($F_{ST} = 0.009$) than NH & MV as a whole, suggesting that distance is not the primary factor in divergence of these populations. NJ was less divergent from all four MV populations ($F_{ST} < 0.006$) than three out of six MV population pairs: SQP & TGP ($F_{ST} = 0.008$), SQP & OYP ($F_{ST} = 0.009$) and EGP & OYP ($F_{ST} = 0.007$). This suggests that the gene flow between NJ and MV is comparable to that between the populations on MV and that distance is not a strong determinant of divergence. Most importantly for this study, none of the genetic population structure detected using RUCV46 and RUCV63 was related to the history of exposure to Dermo disease.

Across all three Dermo-resistance linked loci (RUCV45, Cvi2k14 and Cvi2i23), there was a greater degree of population structure than across neutral markers. Because SQP was a Dermo-naive reference point, divergence (measured by $F_{ST}$) from it across resistance-linked markers was interpreted as resistance to Dermo disease, and the greater the divergence from SQP, the greater the Dermo resistance of that population. The three selected populations on Martha’s Vineyard diverged from SQP according to their length of exposure to P. marinus (Figure 1). From SQP, divergence of EGP ($F_{ST} = 0.022$) is greater than that of TGP ($F_{ST} = 0.019$), which is in turn greater than that of OYP ($F_{ST} = 0.014$), as predicted by $H_2$ (Figure 3). It is especially important to note that while EGP & SQP are highly divergent at resistance linked loci, they were statistically identical ($F_{ST} = 0.000$) for the two neutral loci. While there were small measures of divergence among the populations on Martha’s Vineyard with neutral markers, EGP, TGP and OYP were not genetically different for the resistance markers ($F_{ST} = 0.000$, all three pairs); they are more like each other than they are like SQP (Figure 7). This
supports the hypothesis that genetic divergence increases by Dermo-mediated selection rather than by drift or distance, and has a tendency to increase (relative to unselected SQP) as time since exposure to Dermo increases.

The finding that NJ & SQP ($F_{ST} = 0.019$) were less divergent than EGP & SQP was unexpected because NJ has had Dermo disease longer. There are at least three possible explanations for this. First, the resolution provided by the markers used for this study was not strong enough to accurately describe differences between EGP and NJ. Second, EGP may have developed slightly more resistance to Dermo disease than NJ. Third, there are likely many possible genotypic responses to selection by Dermo, and EGP and NJ may not have been on the same selective trajectory.

If EGP is, in reality, more resistant to Dermo than NJ, it could be the result of disease refugia that are present in Delaware Bay, but not in Edgartown Great Pond. Refugia exist in the upper reaches of an estuary such as Delaware Bay (Hofmann et al. 2009), where salinity is low and the parasite is not typically able to inflict advanced infections or mortality, thus no selection for resistance occurs there. When adult oysters in refuge areas spawn, their genes may travel to lower portions of the bay as their larvae disperse, essentially weakening the effect of selection on the local gene pool. The coastal ponds on Martha’s Vineyard, on the other hand, do not have such an area, mainly because they are shallow, wind-mixed and without a salinity gradient. The absence of a disease refuge in the ponds of Martha’s Vineyard should accelerate the development of resistance. This refugia principle could also hold true for NH, where, in spite of the presence of Dermo for several years, no epizootics have been reported, advanced infections are not common and divergence from the control population is minimal.
In conclusion, the null hypothesis of this study was rejected, and the alternative hypotheses were supported. There is genotypic differentiation between selected and unselected oyster populations on Martha’s Vineyard, and divergence from the unselected population increased with the length of exposure to Dermo disease, caused by *Perkinsus marinus*. Like many studies, this one may conclude by offering more questions than answers. It does, however, give substantial evidence that the Dermo parasite *P. marinus* can impact genotypic frequencies in a manner that is traceable with current genetic technology. And, by utilizing a natural experiment of differential temporal exposure to the disease, this study suggests that increased resistance may be achievable in wild oyster populations.
This study took advantage of the different histories of exposure to Dermo that exist on Martha’s Vineyard. Squibnocket Pond (SQP) has not been infected by the Dermo and was used as a control population from which to gauge divergence of the infected populations. An oyster population from lower Delaware Bay (NJ) was used as a highly-selected comparison, and Great Bay (NH) was used as a minimally-selected population for comparison.
Figure 2:
Maps of six oyster populations in three regions

Divergence from SQP, the uninfected control population, by the three infected oyster populations on Martha’s Vineyard (A) was gauged against that of Great Bay, NH (B) and lower Delaware Bay, NJ (C). General sample locations in Great Bay and Delaware Bay are indicated by a star.
Figure 3: Hypotheses of Dermo-induced population differentiation

(A) The null hypothesis (H₀): there is no genetic divergence among populations at disease resistance loci. (B) The first alternative hypothesis: there is genetic differentiation between selected and unselected populations, with resistance markers. C. The second alternative hypothesis: the population structure presented by the resistance markers corresponds to the length of exposure to Dermo disease.
Figure 4:
Gel electrophoresis of genomic DNA

DNA was extracted using E.Z.N.A™ Mollusc DNA Kit from Omega Bio-Tek and electrophoresed on gel of 0.9% agarose and 0.5x TBE buffer tested for quality.
Figure 5:
Gel electrophoresis of successful and unsuccessful PCR products

* PCR product containing excessive primer-dimer. These markers were not used in the study. Three well amplified markers (Cvi2i23, RUCV45 and Cvi2k14) were used in the study. Electrophoresis on 0.9% agarose gels were used to confirm stability of PCR protocol.
Figure 6:
Sample plots of eight microsatellite markers showing relative allele size

(A) Sample plot of five markers that were analyzed together on the Applied Biosystems (ABI) 3130xl genetic analyzer. (B) Sample plot of three markers that were analyzed together. Distance along the x-axis shows allele size in number of basepairs. All scoring of allele size was done manually, using GeneMapper 4.0 software (ABI).
Neutral markers showed no divergence pattern related to history of exposure to Dermo. Five of the populations (excluding NH) showed very low divergence from each other (in the shaded oval) and NH diverged from all of the five to a greater extent.
Dermo resistance-linked markers showed population divergence that largely corresponded to the history of exposure to the disease among these populations.
Table 1:
Primer properties and PCR amplification conditions for eight microsatellite markers

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<th>Primer</th>
<th>Primer Sequence (5'→3')</th>
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<th>MgCl₂</th>
<th>Tₘ (°C)</th>
<th>Allele size</th>
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</table>
| Cvi1g8*1 | F  TCATAAAACAATCAGTGACACAG  
               R  GCCAAAGTAAGGGGTAAGATG  | CT           | 2.5   | 50     | 226-300     |
| Cvi223*1 | F  TGAAAACGACGGCCAGTGCTACACACH  
                    AAAAATGGG  
               R  TCAAATGAAGGACACCTCC  | GTTT         | 1.5   | 57     | 284-544     |
| Cvi214*1 | F  CCAAAAGAAGGTTGGATGATGCC  
               R  GGATGATGAAATGGATGACGG  | CAG          | 2.5   | 50     | 194-215     |
| Cvi13*1  | F  ACCGGAGATGTTGATATTTC  
                R  GTGTTGCAAGAAGACTTACAGAGAAGAAC  | CAAA         | 2.5   | 55     | 112-312     |
| RU2*3    | F  TGAAAAACGACGGCCAGTGCTGATCGGGGAT  
                        GGCAGGCGGCGTGA  
               R  TGAAAAACATOCACGTCGGGACACAT  | GA           | 2.5   | 58     | 200-258     |
| RUCV45*3 | F  TGTITAGTCATGGCAAGTGTGC  
                R  GTGACTTCTAATTGAGCCTTTACC  | GA           | 2.5   | 60     | 186-288     |
| RUCV46*3 | F  GTGACGTGACGTTGTGACATCC  
                R  TCCACCTCTATTTCAAGTGTTGCC  | GA           | 2.5   | 50     | 90-188      |
| RU6*3    | F  TGATACCTTTCTGTATTGCTTG  
                R  GATGTTCAATTTATTTGAACATT  | (GA)₄,(AG)₄  | 2.5   | 50     | 214-388     |

* used multiplex PCR (Henehan et al. 1997); * used forward primers with M13-linker sequence (in bold) (Schuelke 2000). Forward and reverse primer sequence, SSR repeat motif, MgCl₂ concentration (mM), annealing temperature (Tₘ) measured in °C and observed allele size (base pairs) for eight microsatellite loci. Primer and PCR conditions adapted from *Reece et al. (2004), *Brown et al. (2000), and *Wang and Guo (2007).
Table 2:
Genetic variability of six eastern oyster populations for eight microsatellite loci

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Populations analyzed: Squibnocket Pond (SQP), Tisbury Great Pond (TGP), Oyster Pond (OYP), Edgartown Great Pond (EGP), Great Bay, New Hampshire (NH) and Delaware Bay, New Jersey (NJ). Number of positively genotyped individuals per population (N). Expected (H_e) and observed (H_o) heterozygosity. F_is, inbreeding coefficient of each sample using ANOVA procedure as in Weir and Cockerham (1984). Null allele frequences (r) calculated according to Brookfield's method (1996). Neutral markers are shown shaded, resistance markers are not.
Table 3: Inbreeding coefficient $F_{IS}$ of each population, locus and sample

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<td>0.007</td>
</tr>
<tr>
<td>RUCV45</td>
<td>0.066</td>
<td>0.150</td>
<td>0.093</td>
<td>0.075</td>
<td>-0.016</td>
<td>0.025</td>
<td>0.067</td>
<td>0.079</td>
<td>0.007</td>
</tr>
<tr>
<td>Cvi2k14</td>
<td>-0.004</td>
<td>-0.004</td>
<td>-0.119</td>
<td>0.070</td>
<td>0.048</td>
<td>0.049</td>
<td>0.006</td>
<td>0.403</td>
<td>0.005*</td>
</tr>
<tr>
<td>Cvi2i23</td>
<td>0.054</td>
<td>0.066</td>
<td>0.070</td>
<td>0.016</td>
<td>0.074</td>
<td>0.035</td>
<td>0.053*</td>
<td>0.066</td>
<td>0.015</td>
</tr>
<tr>
<td>$F_{IS\ All}$</td>
<td>0.113</td>
<td>0.067</td>
<td>0.081</td>
<td>0.087</td>
<td>0.050</td>
<td>0.023</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>45</td>
<td>47</td>
<td>46</td>
<td>45</td>
<td>48</td>
<td>41</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P > 0.05; *P < 0.05; all other locus specific $F$-statistics were highly significant. No $P$-values were calculated for population specific $F_{IS}$.

$F_{IS}$, departure from Hardy–Weinberg proportions for each population at each locus and across all loci, using ANOVA procedure (as in Weir and Cockerham 1984) in GenePop 4.0 (Raymond and Rousset 1995). Locus specific Wright's $F$-statistics from unbalanced AMOVAs in GenAlEx 6.4 (Peakall and Smouse 2006), $p = 0.001$ unless noted otherwise. Neutral markers are shown shaded; resistance-linked markers are not.
Table 4:

**Wright’s pairwise $F_{ST}$ using neutral and disease resistance markers.**

$F_{ST}$ for resistance markers above diagonal

<table>
<thead>
<tr>
<th>Pop. Pair</th>
<th>SQP</th>
<th>TGP</th>
<th>OYP</th>
<th>EGP</th>
<th>NH</th>
<th>NJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>SQP</td>
<td>---</td>
<td>0.019**</td>
<td>0.014*</td>
<td>0.022**</td>
<td>0.008*</td>
<td>0.019*</td>
</tr>
<tr>
<td>TGP 0.008*</td>
<td>---</td>
<td>0.000</td>
<td>0.000</td>
<td>0.011*</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>OYP 0.009*</td>
<td>0.003</td>
<td>---</td>
<td>0.000</td>
<td>0.008</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>EGP 0.000</td>
<td>0.000</td>
<td>0.007*</td>
<td>---</td>
<td>0.010*</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>NH 0.015**</td>
<td>0.013**</td>
<td>0.019**</td>
<td>0.016**</td>
<td>---</td>
<td>0.025**</td>
<td></td>
</tr>
<tr>
<td>NJ 0.006*</td>
<td>0.000</td>
<td>0.005</td>
<td>0.000</td>
<td>0.010*</td>
<td>---</td>
<td></td>
</tr>
</tbody>
</table>

$F_{ST}$ for neutral markers below diagonal

• $P < 0.05$; **$P < 0.001$

Pairwise $F_{ST}$ calculated using analysis of molecular variance (AMOVA) in GenAlEx 6.0 (Peakall and Smouse 2006). Values above diagonal for resistance markers, values below diagonal for neutral markers (shaded).
REFERENCES


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eastern oyster, *Crassostrea virginica* (Gmelin) and identification of microsatellite and SNP markers, *BMC Genomics*, 8, 157.


