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# DETECTING AND VERIFYING MICROSATELLITE MARKERS ASSOCIATED WITH DISEASE RESISTANCE IN CULTURED POPULATIONS OF EASTERN OYSTERS, CRASSOSTREA VIRGINICA

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DETECTING AND VERIFYING MICROSATELLITE MARKERS ASSOCIATED

WITH DISEASE RESISTANCE IN CULTURED POPULATIONS OF EASTERN

OYSTERS, CRASSOSTREA VIRGINICA

BY

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**ABSTRACT**

The eastern oyster, *Crassostrea virginica*, is an economically important
aquaculture species in the USA, but several diseases, such as Dermo and MSX, have
an impact on production. Efforts have been made to develop disease-resistant oyster
lines using selective breeding techniques based on phenotype; however, achievement
of the desired trait is hindered by the inability to maintain consistent and intense
selection pressure in field trials. Marker-Assisted Selection (MAS) offers an effective
alternative to traditional breeding techniques by selecting based on genetic markers
associated with disease resistance, which can be done even in the absence of disease-
related selection pressure.  The greatest challenge in applying MAS is to identify
markers that are consistently associated with the disease-resistant phenotype.   In this
study, 20 previously published microsatellite markers, including 8 located in regions
previously associated with disease resistance were used to genotype and compare
oyster populations of the same stock (NEH-RI) deployed in 2012 at two sites (York
River, Virginia and Cape Shore, New Jersey) before and after a disease-caused
mortality period (March – November 2013). Two markers located in disease-resistant
QTLs (Cv02i23, Cvi1g3, and RUCV 97) exhibited significant post-mortality allelic
distribution shifts in one site but not the other. Significant differences in allelic
distribution before and after selection were detected in 3 markers with no prior
evidence of association with disease resistance at either both sites (RUCV270 and
RUCV 68) or one site (RUCV 27). These results strengthen the evidence associating
markers Cv02i23, Cvi1g3, and RUCV97 with the disease-resistant phenotype and

suggest that markers RUCV270, RUCV68 and RUCV 27 warrant further investigation. Additional genetic and functional genomic analyses are required to determine whether these markers are suited for MAS.

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# INTRODUCTION

## Disease management in oyster populations

Eastern oysters are a major part of the aquaculture industry in the United States of America (US).  In 2012, oyster production in the US totaled over 23 million pounds in production, with over 100 million dollars' worth of value (NOAA 2014). However, maximum production is impeded by mortalities caused by diseases such as Multinucleated Sphere Unknown (MSX), Seaside Organism disease (SSO), Dermo, and Roseovarius Oyster Disease (ROD, a.k.a. juvenile oyster disease). Among these diseases, MSX and Dermo have the widest geographical range and thus have the largest impact on oyster production. Each disease alone can cause up to 90% mortality in an oyster population (Yu and Guo 2006, Burreson et al. 2000). While a high parasite load of Dermo or MSX can lead to mass mortality, a lighter burden of the parasites has negative sub-lethal effects on the oysters, including lowered condition index and reduced reproductive output (Ford and Smolowitzv 2007, Dittman et al. 2001, Ford and Figueras 1988). Dermo and MSX are caused by the protozoan parasites *Perkinsus marinus* and *Haplosporidium nelsoni* respectively (Ewart and Ford 1993). They affect oyster populations on the Atlantic coast of the United States from Maine to Florida, but Delaware Bay and Chesapeake Bay areas are where they hit the hardest (Burreson et al. 2000, Ford and Haskin 1982). With the increase in sea surface temperature due to climate change, MSX and Dermo have been observed to extend their range to the northeastern US coast and to cause mortalities in this region (Cook et al. 1997, Ford and Smolowitz 2007).

Oyster farmers utilize several strategies to avoid Dermo/MSX-related mortality events. Neither Dermo nor MSX proliferate in low temperature (below 18 ℃ ) and low salinity (below 10 psu) environments (Ewart and Ford 1993, Cook et al. 1998). It has been found that altering the timing of seeding could lower mortality caused by MSX. Oysters deployed late in the season when temperature is falling generally has a lower level of disease intensity and mortality would not start until the following spring (Ewart and Ford 1993). Another approach is to take the advantage of MSX and Dermo pathogens' poor tolerance of low salinity and deploy oysters at sites of low salinity (Paynter and Burreson 1991). However, this approach sacrifices growth since oysters do not grow as well in low temperature or salinity sites (Ewart and Ford 1993, Paynter and Burreson 1991). Although MSX and Dermo prevalence and intensity of infection can be reduced with cold winters (both diseases) or low salinity (e.g. caused by freshwater influx after storms, mainly for MSX), disease prevalence and intensity increase as soon as environmental conditions become favorable to both parasite and oyster growth during summer and fall seasons (Ewart and Ford 1993). Farming practices alone are insufficient to prevent economic losses to disease.

Artificial selection for disease resistance is another approach being employed to mediate the negative impacts of disease outbreaks on oyster production. Several lines of evidence indicate that disease resistance is a heritable trait, at least for MSX. For example, wild oyster populations growing in locations with a history of high MSX prevalence in the Chesapeake and Delaware Bays developed quantifiable levels of resistance to the disease in response to natural disease outbreaks (Haskin and Ford 1979). Selective breeding programs were established to build upon the naturally

acquired MSX resistance in oysters, and within a small number of successive

generations, strains with high resistance were established (Ford and Haskin 1987).

Selected, MSX-resistant lines demonstrate significantly higher survival compared to

oyster populations with a history of no or limited exposure to the disease when both

are deployed at locations with high disease pressure (reviewed in Carnegie and

Burreson 2011). Although oyster strains have been observed to differ in their response

to Dermo disease (Brown et al. 2005), their resistance level remain low compared to

resistance to MSX (Powell et al. 2011). Powell et al. (2011) speculated on why the

development of Dermo resistance had been slow. Oysters might have limited immune

responses to the pathogen or development of Dermo resistance might be hindered by a

rapidly changing virulence of its pathogen, *P. marinus*. Most importantly, Dermo

mainly affects mature oysters that have passed one spawning cycle and the infection

does not affect reproduction until it has reached lethal level. These factors make

selection for Dermo resistance hard. Nevertheless, oysters with dual resistance to both

Dermo and MSX are being developed (Calvo et al. 2003), since the parasites *H.*

*nelsoni* and *P. marinus* often co-occur in the Atlantic Coast of the United States

(Ewart and Ford 1993).   Currently, several oyster lines are available that have been

selected for fast growth and disease resistance to a variety of diseases in various

environmental conditions (Table 1).

**Table 1**. Examples of oyster lines/stocks developed through selective breeding for fast growth and resistance to a variety of diseases.

| Line | Developed at | Prevalent Environmental Conditions at sites in which the selection was performed* | Phenotype (disease resistance) | Reference |
|---|---|---|---|---|
| **UMFS (University of Maine Flowers Select)** | University of Maine | Cold, high salinity | Demonstrated high resistance to ROD; fast growth | Barber et al. 1999 Hawes et al. 1990 |
| **Clinton** | Clinton, CT | Warm, low-high salinity | Hypothesized resistance to MSX/Dermo/ROD based on disease levels at grow site and preliminary testing | Sunila (pers. Comm.) |
| **NEH-RI** | Narraganset t Bay, RI | Warm, high salinity | Developed using NEH oysters surviving an SSO outbreak in 2010-2011 | Gomez-Chiarri et al. (unpub.) |
| **NEH** | Cape Shore, NJ | Warm, medium salinity | Demonstrated high resistance to MSX and moderate to Dermo | Haskin and Ford, 1979, Guo et al. 2003 |
| **DEBY: (Delaware Bay)** | York River and Lynnhaven River, VA | Warm, low-high salinity | Demonstrated high resistance to MSX and moderate to Dermo | Calvo et al. 2003 |
| **hANA: high-salinity LouisiANA** | York River and Lynnhaven River, VA | Warm, med-high salinity | Demonstrated moderate resistance to MSX and high resistance to Dermo | ABC 2009 |

*Salinity range, low: 8-15 psu, medium: 17-25 psu, high: 28-35 psu; Temperature range, cold: average winter temperature < 3 ℃, warm: average winter temperature >3 ℃.

Despite the aforementioned genetic improvements gained via selection, traditional methods of selective breeding have some limitations. First, these methods select for phenotypes (e.g. survival to disease outbreaks) instead of genotypes. However, survival is affected by many factors besides disease, such as temperature, salinity, and predation. Thus, the phenotype selected for might not indicate the presence of disease resistance. Secondly, the occurrence of disease is sporadic over space and time. Since it is very important for selective breeding to keep a constant selection pressure on the oyster populations, years with no disease pressure will have a negative impact on the selection process (Guo et al. 2008). Although traditional selective breeding methods through either mass or family selection are a powerful method for producing lines for the industry with certain desired phenotypes, a faster and more accurate way of selecting for disease resistance in oysters is needed.

Marker-assisted selection, whereby selection is imposed on genotype rather than phenotype, could enhance and accelerate the development of disease-resistant oyster lines (Lande and Thompson 1990). It identifies oysters with disease resistance based on genotypes by detecting markers associated with disease resistance. This approach allows the accurate identification of disease resistance in a species in the absence of disease pressure (Collard et al. 2005). In order to perform marker-assisted selection, breeders need genetic markers that have been confirmed to be associated with disease resistance in that species.

**Association Studies for the Identification of Markers Associated with Particular**

**Traits**

Association studies are an effective approach to identify genetic markers

associated with a certain trait, such as growth, shape, or disease resistance. It is

particularly helpful for complex traits that are controlled by multiple genes, such as

disease resistance in oysters (Ford and Haskin 1987).  Association studies utilize

genetic markers and compare allele frequencies of these markers between two groups

of organisms, one group with the trait of interest (case group) and one control group

without the trait of interest (Cardon and Bell 2001).  If a significant difference in the

genotype is consistently observed at a certain marker between the two groups, it

indicates that the marker is closely located to a nearby genetic variant that is causing

the difference or, less likely, is the direct genetic cause of the observed difference in

phenotype (Hirschhorn et al. 2001).  Such studies have been commonly used to

identify causes of diseases in humans (Hirschhorn et al. 2005, Oka et al. 1999) and

economically important traits in agriculture, such as increased yield and disease

resistance (Bai et al. 1999, Zhou et al. 2002, Brondani et al. 2002, Bermingham et al.

2013, Zila et al. 2013). These studies usually are supported with genetic maps for the

targeted species, so that the relative positions of markers on the chromosomes are

known. It is worth noting that for the purpose of MAS, an available genetic map is not

mandatory. Genetic markers with unknown locations on the chromosome can still be

tested for association with the trait of interest, and once validated, can be used in MAS.

But the availability of the genetic map of the targeted organism will be helpful in

selecting genetic markers to make sure that they span the whole genome, ensuring

high coverage.  Furthermore, once a genetic marker detects a signal of difference between the control and case groups, a genetic map facilitates locating the genetic markers that have evidence of association on the chromosome and pin-pointing the functional genes through fine-scale mapping (Cardon and Bell 2001, Zhou et al. 2002). It has also been noted that the results of association studies often cannot be replicated by further studies, and that an association from a single report should not be trusted fully (Hirschhorn et al. 2005). Thus, verification of previous association study results in multiple populations is necessary.

**Marker Development in Eastern Oysters**

Association studies required a large number of polymorphic markers (Liu and Cordes 2004). Recently, an increasing number of various types of genetic markers have been developed for eastern oysters, such as Amplified Fragment Length Polymorphism (AFLP) markers, microsatellites (MS), and Single Nucleotide Polymorphisms (SNPs). Over 200 AFLP markers were used in construction of the first genetic map for eastern oysters (Yu and Guo 2006).  AFLP markers are dominant and cannot be transferred among populations, which makes them less favorable than other co-dominant markers like microsatellites and SNPs, but they are still valuable for saturating linkage maps and increasing coverage (Guo et al. 2008). Over 300 microsatellite markers have been generated in eastern oysters (Brown et al. 2000, Wang and Guo 2007, Wang et al. 2009, Reece et al. 2004). Many of the newly developed microsatellite markers were generated from ESTs (Expressed Sequence Tags), meaning that they are part of the coding sequence. More than 100 SNPs

markers have been generated from both ESTs and resequencing of functional genes with known or predicted functions. If association is detected using markers developed from coding sequences, there is a higher chance of detecting functional genes underlying the disease resistance trait, compared to an association detected at neutral loci (Liu and Cordes 2004).

Among these markers, microsatellite markers are particularly informative because they are highly polymorphic and are inherited in a co-dominant manner, meaning that heterozygotes can be identified (Wright and Bentzen, 1995). They have been used in association studies to identify markers associated with various traits in humans, plants and animals (Oka et al. 1999, McKnight et al. 2006, Zhou et al. 2002, Vigouroux et al. 2002).

**Previous Association Studies on Disease Resistance in Eastern Oysters**

Successful association studies have been done to map disease resistance QTLs and to identify markers associated with disease resistance in eastern oysters (Yu and Guo 2006; Guo et al. 2008). Several challenges to association studies for disease resistance in oysters exist. First, it is hard to quantify the phenotype of disease resistance. The easiest evaluation of disease resistance in the field is survival. However, survival can be affected by factors other than disease, including environmental stress and predation. Disease prevalence and intensity in the presence of disease pressure is a more specific indicator of disease resistance, but, it is expensive and, in some cases, oysters may be able to tolerate a high parasite load without obvious negative effects on survival or performance (Guo et al. 2008).

Secondly, there is a lack of well-characterized highly-inbred susceptible and resistant oyster lines. As a result, the usual control-case association study design cannot be directly implemented, since there are no obvious candidates for the control groups and case groups. To overcome this challenge, another approach has been used in oysters, in which control and case oysters are taken from a single population but at different times regarding the disease outbreaks. The control group consists of oysters randomly sampled from the population before a mortality event caused by diseases, usually at the beginning of the summer. The case group consists of oysters randomly sampled from the population right after a mortality event, as these survivors are likely to be resistant to diseases. The two groups of oysters are then sacrificed and genotypes are then compared. Markers that show significant allele distribution shifts between the two groups are thought to be linked to disease response. If affected markers are clustered close on the genetic map and have shifts in the same direction, the region containing these markers is identified as a QTL (quantitative trait loci) for disease resistance. In order to facilitate mapping these QTLs, oyster families (instead of oyster populations) were used in these association studies (reviewed in Guo et al. 2008).

The results from these family-based association studies in oysters are summarized in Figure 1. The first map (Figure 1a) summarizes the results from several association studies for resistance to Dermo disease, as evaluated through allele frequency shifts after a Dermo disease-related mortality event in the field (reviewed in Guo et al. 2008). This map includes 313 markers (249 AFLPs, 47 MS, and 17 SNPs), resulting in a total genetic length of 729.9 cM. The average interval between markers is 2.3 cM. The map describes the location of 47 functional genes or expressed sequences. Based on results

from previous association studies, 26 AFLP markers, 1 SNP, and 6 microsatellite markers had significant after-mortality frequency shifts. After mapping these markers on a linkage map, regions containing 2 or more markers with frequency shifts in the same direction were used to identify 8 QTL regions potentially associated with disease resistance (Figure 1a; Guo et al. 2008). The 6 microsatellite markers showing significant post-mortality shifts and clustered in QTL regions were RUCV 97, RUCV 58, Cv02i23, Cvi2j24, Cvi2i4, and Cvi1g4.

In an unpublished study performed in 2010, Zhang, Guo, and Gomez-Chiarri used the same association study strategy to identify genetic markers associated with ROD-resistance. Two oyster families with differences in susceptibility to ROD were used. In order to minimize the impact of field environmental conditions on survival, the oysters were challenged with cultured *Roseovarius crassostreae,* the causative agent of ROD, in laboratory conditions. The study tested 257 genetic markers (90 MS, 2 SNP, and 155 AFLP markers). Twenty-eight markers (11 MS, 1 SNP and 16 AFLP markers) were identified to experience significant post-mortality frequency shifts and 22 of them were mapped (Zhang, Guo, and Gomez-Chiarri unpublished, Figure 1b). Two microsatellite markers that had significant post-mortality shifts and are publicly available are RUCV 66 and Cvi2m10. We also decided to include it in this study RUCV 270, a marker in linkage group 10 located in relative proximity to another marker (Cvi12) for which a significant shift was detected after ROD mortality.

**Figure 1.** Two genetic maps used to select microsatellite markers for this study. (a) A modified version of the genetic map by Guo et al. (2008). In red are microsatellite or SNP markers, in black are AFLP markers. Markers in **bold** showed significant shifts in frequency after disease-related mortalities. Lines encompassing regions within selected linkage groups indicate disease resistance QTLs. In black boxes are the candidate markers selected for this study. (b) Genetic map with genetic markers showing shifts in frequency after an ROD experimental challenge (Zhang, Guo, and Gomez-Chiarri, unpublished). Markers in red were genetic markers showing association with resistance to ROD. Markers in black boxes were candidate markers investigated in this study.

(a)

(b)

**1**

0.0 RUCV395**
7.9 RUCV424s
25.3 RUCV405B****
30.8 F1f358
33.6 C6f58
38.4 RUCV399
39.1 F3f367
41.3 C7f199
43.2 F2f431
43.5 RUCV876
44.1 H3f148
45.6 RUCV409
49.0 F2f268**
50.1 F2f301s
51.5 F2f435
52.0 F1f125**
54.0 F2f233
54.3 F2f367
55.8 H3f204
56.7 C7f183
57.1 F2f436*
59.4 F1f263*
62.9 RUCV046
65.9 F3f254
67.8 C7f361
70.4 RUCV113
74.8 RUCV061
77.8 RUCV374
84.0 RUCV287
89.2 RUCV119
93.6 RUCV051
99.6 RUCV265

**2**

0.0 RUCV073
1.3 F2f505
3.0 C6f113
8.8 RUCV075
11.1 H3H321
12.9 C6f198
14.4 cvi2k14
16.1 C6f301
17.3 Cvi2i23
18.0 H3f131
22.6 F2f293
23.6 F2f82
25.1 Cv01-03
30.2 C7f275
30.7 RUCV230
33.9 RUCV753
34.4 F1f179s
35.2 F2f197s
38.5 C6f245s
43.7 F2f276**
46.5 C7f119
48.9 H3f179
49.9 F2f181
50.3 H3f130
57.8 H3f154
63.5 BD-90
65.2 C6f169
66.7 F2f152
85.5 Cvi2j24

**3**

0.0 RUCV297
20.7 F3f224
23.7 F2f147
25.0 RUCV003
25.1 C7f372 RUCV114
27.8 F2f355
28.4 H3f195
29.2 RUCV851
42.1 RUCV769s
46.3 RUCV405C3**
48.1 C6f101 RUCV156
49.2 C7f68

**4**

0.0 H3H311
4.5 H3f112
6.6 F3f137
17.5 H3f111
26.3 F2f235
28.9 Cvi8
33.0 Cvi13
33.6 F3f372
34.4 F3f223
35.9 F2f236
37.0 H3f226
39.1 C7f278
39.9 RUCV013
40.2 F3f386
43.5 F2f178
44.7 RUCV772
46.1 RUCV045
47.8 F3f134
50.3 H3f276
51.8 F1f337
53.3 H3f274
53.8 RUCV183
54.4 RUCV027
54.6 F1f215
55.5 F1f226
57.7 H3f275

**5**

0.0 RUCV717
0.2 H3f267
17.9 RUCV108
26.5 H3H377
34.3 C7f352
38.2 C7f452
39.1 RUCV373
47.1 H3f130
49.9 F2f170
67.6 C7f175 C7f336
81.6 cvi9
81.9 C7f97

**6**

0.0 cvi2i20
10.4 C6f161**
10.7 F2f274**
26.7 H3f65*
37.6 RUCV131*
49.8 C6f445
50.8 RUCV109
51.5 cvi6
53.5 F1f64
55.1 RUCV066*
55.8 C6f315
61.0 Cvi2m10*
61.2 F3f91
67.4 RUCV279
67.5 Cvo1-c11
70.5 C7f334
73.5 F3f431
101.9 RUCV834C

**7**

0.0 F1f609
3.6 C7f329
7.7 RUCV274
9.3 C6f124
10.2 F2f270
11.3 Cvi1248
12.4 C6f339
13.8 RUCV025
14.8 RUCV008
19.7 F2f239
27.5 RUCV190
42.6 C6f411
62.4 RUCV834***
81.0 spi-224***
100.8 RUCV256
105.8 RUCV018

**8**

0.0 Cv01-d18
1.9 C6f99
11.0 C7f59
21.4 C7f263
42.7 F3f232

**9**

0.0 RUCV788
12.0 RUCV164
21.3 RUCV112 RUCV388
26.9 RUCV026
29.0 H3f233*
44.1 C6f192
54.0 C7f116

**10**

0.0 RUCV370**
5.3 RUCV423**
15.4 RUCV397*
24.1 C7f249
26.3 F3f142
42.7 F2f138
55.3 RUCV748
56.0 C7f246
56.8 C7f122
60.3 C6f105
62.3 RUCV270 Cvi12***
63.9 C6f111
64.7 H3f448
66.0 F2f205
67.7 C6f112
69.5 F3f273
72.9 Cvi2m14

12

**Goal of this Project**

        Previous family-based association studies have identified several markers (including 8 publically available microsatellites) associated with disease resistance in oysters (reviewed in Guo et al. 2008). However, due to families' low genetic diversity, a family-based approach might not truthfully reflect wild population situations. Thus, results obtained from family-based studies should be further confirmed by subsequent studies using multiple oyster lines (mixed families) or cultures stocks and ultimately in wild oyster populations.

        This project aimed to verify 8 several previously identified microsatellite markers that had evidence of association to disease resistance in oysters, and to possibly identify new associated markers by testing 12 additional microsatellite markers without previous evidence of association using samples from an oyster stock deployed in two separate field locations that experienced mortality due to Dermo and MSX collected by a previous field performance study (Proestou et al. in preparation). Oysters before and after the disease-caused mortality event were genotyped at the 20 genetic marker loci. Significant post-selection allele distribution shifts, supported fully or in part by other lines of evidence (consistent shifts in allele frequency in the same direction at the 2 different sites and/or mapping of these markers to disease resistant QTLs in other studies), were considered as evidence for potential association with disease resistance.

## METHODS

### Oyster Samples

Oysters samples were available from a previous study that evaluated the performance of 6 oyster lines or stocks in 5 locations in the East Coast of the US (Proestou et al. in preparation). Samples of oysters (between 30 and 60) from each of the lines/stocks at each of the sites were collected at deployment (August 2012) and in Spring (March or April, depending on the site), Summer (August/September) and Fall (October/November) of 2013.  NEH-RI oysters (derived from brood stock oysters from the NEH line deployed in Rhode Island that survived an SSO outbreak) deployed in August 2012 at two locations (Cape Shore, NJ and York River, VA) were used for this study, because of the high disease-related mortality (more than 30%) that these oysters experienced 1 year after deployment at both sites during the period spanning the summer and early fall (July – September 2013, Supplementary Figures 1 and 2). Mortality in this period was attributed to MSX and/or Dermo diseases based on high prevalence and intensity of the parasites *P. marinus* and *H. nelsoni* in oysters collected in September 2013 (as determined by quantitative real time PCR; qPCR; Supplementary Table 1; Proestou et al. unpublished).


### DNA Extraction and Evaluation

Oysters were shucked, and tissue samples from mantles and gills were stored in 70% ethanol at -20 degrees Celsius until extraction. Genomic DNA was extracted from mantle and gill tissues using either a Chelex method (Aranishi and Okimoto 2006) or Autogen's Quickgene Mini80 system (Autogen Inc., Holliston, MA; Pereira

et al. 2011). The Chelex method has the advantage of being fast and inexpensive, but the DNA extracted was fragmented to ~500bp fragments, which reduces the success of PCR amplification for genetic markers of longer fragment. Therefore, the Autogen method was used later in the study, for it yielded higher quality DNA. The change of extraction method should not affect the integrity of the data since the same genotypes were obtained from successful PCR amplification of DNA from tissues of selected individual oysters that was extracted using both methods (data not shown). DNA quantity and quality were evaluated using a NanoDrop 8000 instrument (Thermo Fisher Scientific Inc., Waltham, MA). DNA was diluted to 7.5-10 ng/ul for PCR amplification. DNA was stored at -20˚C until use.

**Microsatellite Marker Selection**

Twenty previously published microsatellite markers were used to genotype the control and selected populations (Reece et al. 2004, Wang and Guo 2007, Wang et al. 2009). These markers were chosen based on allele size range and ability to multiplex, level of polymorphism, and reported frequency of null alleles and are therefore appropriate for detecting genetic differentiation among oyster populations. Moreover, 9 of the 20 loci included in this study were located within previously defined disease resistance QTL or near (RUCV 270) a marker showing a significant shift in allele frequency after ROD mortality (Table 2, Guo et al. 2008, Guo, Zhang, and Gomez-Chiarri, unpublished). The 5'end of the forward primer from each locus was labeled with one of four fluorescent dyes (PET, NED, 6-FAM and VIC) and markers were assigned to one of five plexes, based on allele size range and dye color (Table 2).

**Table 2**. Microsatellite markers used in this project. Markers shaded grey represent those located within disease-resistance QTLs. *Marker located near a marker showing a significant shift in allele frequency after a mortality event (Guo et al. 2008, Guo, Zhang, and Gomez-Chiarri, unpublished).

| Plex | Name | Ta | MgCl$_2$ | Direction | Primer Sequence | Reference |
|------|------|----|----------|-----------|-----------------|-----------|
| A | RUCV 1 | 60 | 1.5 | fwd | AGTCAAGAACTATACAAATTTACGCT | Wang and Guo 2007 |
|   |        |    |     | rev | CTCACAGACCATGAAAATGGGCTGTT |  |
|   | RUCV 3 | 60 | 1.5 | fwd | AGTTATCCATTCTGTTGTGGAAGTGA | Wang and Guo 2007 |
|   |        |    |     | rev | GTTTGTCCCGACAACATACCGCCATT |  |
|   | RUCV 46 | 60 | 1.5 | fwd | GTCGTGCAAGTTGACATTCC | Wang and Guo 2007 |
|   |        |    |     | rev | TCCACCTCTATTTCATGTTGTCC |  |
|   | RUCY 28 | 55 | 1.5 | fwd | GGAGGCCCAAGAACTGCGAGGGGACC | Wang and Guo 2007 |
|   |        |    |     | rev | TTGAAAACATGCACGTCCGGCAACAT |  |
| D | RUCV 11 | 60 | 1.5 | fwd | TGCCGGTCGTTCTTTCAGGTATGTTC | Wang and Guo 2007 |
|   |        |    |     | rev | TTTCTGAAGGGACACTGATAGTGAGT |  |
|   | RUCV 27 | 60 | 1.5 | fwd | GCTGATCGGGATGGCGAGAGAGTGAC | Wang and Guo 2007 |
|   |        |    |     | rev | TGAAAACATGCACGTCCGGACAACAT |  |
|   | Cv02i23 | 51.5 | 1.5 | fwd | TAACACAAAGCCAACATCGCC | Reece et al. 2004 |
|   |        |    |     | rev | AAGTAAAAGACGGTCAAAGGGTCC |  |
|   | RUCV 66 | 60 | 1.5 | fwd | ACCATCAGCAACACAGAACG | Wang and Guo 2007 |
|   |        |    |     | rev | GGGTCCCAAGTGTTGTCG |  |
| E | RUCV 24 | 55 | 1.5 | fwd | AAAAGGGAATTTTGTTACACAATCCA | Wang and Guo 2007 |
|   |        |    |     | rev | AAAAACAAAATAATGAATACATTGGC |  |
|   | RUCV 97 | 55 | 1.5 | fwd | AGCCATGATTGAGGAATTGG | Wang and Guo 2007 |
|   |        |    |     | rev | ATCCCCTAAAGTGCGACTGG |  |
|   | RUCV 68 | 60 | 1.5 | fwd | TCTTGGAATGACAAGCAAGC | Wang and Guo 2007 |
|   |        |    |     | rev | CCAGGGGTCAACAGTTTCC |  |
|   | Cvi2j24 | 53 | 1.5 | fwd | CGTAGCCAGAAGGGGGGTTTC | Reece et al. 2004 |
|   |        |    |     | rev | GCAGTGAGACACGATAAAAGAAGCC |  |
| F | RUCV 23 | 55 | 1.5 | fwd | GCAAGATGGGGATGATCAACCTGCAT | Wang and Guo 2007 |
|   |        |    |     | rev | GGACATCGGATCCCAGTGTCGGTTGA |  |
|   | Cvi2i4 | 46 | 1.5 | fwd | AATAATACAAAATCCAGTAGC | Reece et al. 2004 |
|   |        |    |     | rev | CCAATCAAATCTCACTAAAG |  |
|   | RUCV 18 | 60 | 1.5 | fwd | TACTTTAATTGCATGCATGTGGTTGT | Wang and Guo 2007 |
|   |        |    |     | rev | GTCGGTCTGCTTGATCTGTGAAGGTT |  |
|   | RUCV 270* | 60 | 1.5 | fwd | GGACCAAATATTCCACATCACAC | Wang et al. 2009 |
|   |        |    |     | rev | AAGCTGAATGCCCAAACATC |  |
| G | RUCV58 | 60 | 2.5 | fwd | TTGGAATTTTAATAAATGTCAGAAAGG | Wang and Guo 2007 |
|   |        |    |     | rev | AACTTAGTTAACATTTTGGAATTGG |  |
|   | Cvi1g4 | 50 | 1.5 | fwd | TCATAAACAATCAGTGACACAG | Reece et al. 2004 |
|   |        |    |     | rev | GCAAAGTAAGGGGTAAGATG |  |
|   | Cvi1g3 | 51 | 1.5 | fwd | CATAAAGTTAATGCTTC | Reece et al. 2004 |
|   |        |    |     | rev | ATAGCGAGTTGAGGAACC |  |
|   | Cvi2m10 | 50 | 2 | fwd | CGAATCTGTGTTGATGAAAGG | Reece et al. 2004 |
|   |        |    |     | rev | CCATGTCTCTCATCGTCAGTGCC |  |

**PCR Amplification and Genotyping**

DNA from each oyster was amplified using PCR for each microsatellite marker individually and then the amplification products for 4 microsatellites were multiplexed for genotyping. PCR reactions were run in a 10-µl solution containing 1X PCR Buffer with 1.5-2.5mM $MgCl_2$, 0.2mM dNTPs, 0.5 U of Qiagen's Toptaq DNA polymerase, 0.3 µM of each primer and 10-15 ng of oyster genomic DNA (Qiagen's TopTaq Polymerase Kit; Qiagen, Hilden, Germany). PCR for all but three markers (RUCV 58, Cvi2m10, Cvi2i4) was carried out using a touchdown PCR protocol (used to increase specificity and sensitivity of amplification; Korbie and Mattick 2008) as follows: 94℃ for 3 minutes, 10 cycles of 94℃ for 30s, 5℃ above annealing temperature (Ta) of the marker for 30s (decrease 0.5℃ every cycle), 72℃ for 30s, followed by 25 cycles of 94℃ for 30s, annealing temperature (Ta) for 30s and 72℃ for 30s, and a final extension at 72℃ for 30 min (Jaris 2014). Cvi2i4, RUCV 58, and Cvi2m10 were amplified as previously described (Reece et al. 2004, Wang and Guo 2007). RUCV 58 and Cvi2m10 required 2.5mM and 2mM $MgCl_2$ respectively for successful amplification. Gel electrophoresis was used to confirm successful amplification using 2% agarose gel. For each individual oyster sample, PCR products from the four loci belonging to a particular plex were pooled in equimolar amounts and purified with the Agencourt AMPure XP system (Beckman Coulter Inc., Brea, CA) according to the manufacturer's protocol. Purified pooled PCR products were sent to Yale DNA Analysis Facility for fragment analysis. Genotypes were called with GeneMarker software (SoftGenetics LLC., State College, PA), and subsequent manual editing.

Allele binning was conducted using Allelogram software (Morin et al. 2009), where

the length of the repeat motif for each marker provided a guideline for bin size.

**Data Analysis**

   Population multi-locus pairwise $F_{ST}$ (an index between $0 - 1$ that estimates the

degree of genetic differentiation between oyster populations at each of sampling time

points, with 0 indicating no differentiation; Wright 1978) was calculated to determine

population structure within the NEH-RI stock in each site between each of the

sampling time points. Arlequin software ver 3.5 was used to conduct data analysis

(Excoffier and Lischer 2010). Data from the allele binning needed to be formatted for

Arlequin software to recognize and process. The format conversions, along with the

generation of allele frequency data was done by the software CONVERT (Glaubitz).

The $F_{ST}$ calculation was done through the "Population comparisons" function in

Arlequin. Number of permutations was set to 3000 with a significance level ($p$) of

0.05. Arlequin was set to tolerate 15% of missing data. Locus-by-locus AMOVA was

also conducted to calculate the $F_{ST}$ value for each locus, in order to determine which

loci may be under selection during the mortality event.

   The statistical analysis package R (R Development Core Team 2008) was used to

perform Fishers' exact test on allele count data to determine the probability of whether

allele distributions of pre- and post-selection samples within each of the sites were

different.  Fisher's exact test was chosen over the chi-square test because of the small

sample size (McDonald 2014). The $p$-value  returned from Fisher's exact test was

corrected (*Pc*) by multiplying by the number of alleles for each marker, in order to compensate for multiple comparisons (Oka et al. 1999). Markers that showed significant shifts in both sites and markers that had significant shift in one site but were previously known to be in disease resistance QTLs were considered to be markers of interest.

In order to find out whether affected markers were related to known functional genes related to host-defense, translated protein (tblastx) and nucleotide (blastn) blast (Stephen et al. 1997) were used to conduct homology searches in the NCBI server.

**Amplification and Genotyping**

All 20 microsatellite markers were successfully amplified in most samples, with

some samples that failed to amplify for one or more marker loci due to low DNA

quality or errors in carrying out PCR. In order to reduce the amount of missing data,

samples with failed amplifications for five or more (≥25% of total markers) markers

were discarded (Table 3).

**Table 3.** Number of samples used in statistical analyses after discarding samples with five or more unsuccessful amplifications.

| Time point | Number of samples retained for further analysis | Number of total samples before discarding | Number of discarded (Percent) |
|---|---|---|---|
| Virginia | | | |
| Aug-2012 | 40 | 47 | 7 (15%) |
| Mar-2013 | 28 | 30 | 2 (7%) |
| Aug-2013 | 31 | 32 | 1 (3%) |
| Sep-2013 | 57 | 58 | 1 (2%) |
| Oct-2013 | 36 | 47 | 11 (23%) |
| New Jersey | | | |
| Apr-2013 | 47 | 47 | 0 (0) |
| Nov-2013 | 48 | 48 | 0 (0) |

**Determination of Pre- and Post-selection Time Points for the Association Study**

For this association study, the control population will be oysters before selection by

disease and the case (selected) population will be oysters after selection by disease. At

the Cape Shore, NJ site, August 2012 and March 2013 sample collections were both

considered as possible candidates for the control, unselected population given that

very little mortality was observed during that time period (Supplementary Figure 2).

In contrast, significant overwinter mortality (~50%) was observed at York River,

Virginia between August 2012 and March 2013, making the August 2012 sampling a less viable candidate for the control population at that site, since the use of this sample as a control would not allow to differentiate between disease-related mortality and over-winter mortality. However, the March 2013 collection from York River, VA contained only 30 individuals (Table 3), which is a relatively small sample size for detecting significant allele frequency shifts. Population pairwise multi-locus $F_{ST}$ between samples collected at different time points after deployment at the Virginia site were calculated using the set of 20 markers to determine the effect of mortality on population structure in samples from selected time points (Table 4). The $F_{ST}$ value between the Aug-12 and Mar-13 samples (spanning over-winter mortality; $F_{ST}$ = 0.01511, $p$= 0.05025 was almost the same as the $F_{ST}$ value between Mar-13 and Oct-13 (spanning disease-related mortality; $F_{ST}$ =0.0148, $p$= 0.06413), suggesting that the over-wintering mortality posed a selection on the oysters that is comparable in strength to the selection from disease-related mortality. Thus, Mar-13 was chosen as the sole pre-selection time point (control group), despite of the low sample size (n=30), while Oct-13 was chosen as the post-selection time point (case group). The equivalent time points (Apr-13 and Nov-13) were chosen as control and case groups for oysters deployed in New Jersey (pairwise multi-locus $F_{ST}$ = 0.0244, $p$ = 0.0003).

**Table 4**. Population multi-locus pairwise $F_{ST}$ for oyster samples collected at different time points after deployment in the Virginia site. Numbers in bold are statistically significant ($p$-value<0.05).

|        | Aug-12      | Mar-13   | Aug-13  | Sep-13 | Oct-13 |
|--------|-------------|----------|---------|--------|--------|
| Aug-12 | 0           |          |         |        |        |
| Mar-13 | **0.01511** | 0        |         |        |        |
| Aug-13 | 0.00505     | 0.00972  | 0       |        |        |
| Sep-13 | 0.01012     | 0.00858  | 0.00013 | 0      |        |
| Oct-13 | **0.01887** | 0.0148   | 0.00693 | 0.0072 | 0      |

**Identifying Markers Showing Allele Frequency Shifts after a Disease-related Mortality Event**

Fisher's exact test was conducted comparing the allelic distributions between pre- and post-selection time points. In the samples collected in Virginia, 3 out of 20 markers had significantly different allelic distributions between the pre and post-disease-related mortality event, while 9 out of 20 were significantly different in oysters collected in New Jersey (Table 5).  Two markers (RUCV 68 and RUCV 270) had significant shifts in allele distributions in both sites, suggesting similar selection forces (probably mortality due to Dermo and MSX diseases, based on disease prevalence and intensity, supplementary Table 1) at the 2 sites. Two other markers (RUCV 97 and Cv02i23) with previous evidence of association had significant different allele distribution between pre- and post-disease-related mortality event in one of the two sites only. Interestingly, several markers without previous evidence association also detected a significant shift in allelic distributions (Table 5). Locus-by-locus AMOVA results agreed closely with the Fisher's exact test results in the New

Jersey site. Both tests detected statistically significant differences in genotypes for the same loci in pre- and post-mortality populations, with the exception of RUCV1 and Cvi02i23 (which were significant by the Fisher's exact test, but showed no significant $F_{ST}$ ) and Cvi2j24 (which was significant by $F_{ST}$ but not the Fisher's test) (Supplementary Table 2). The locus-by-locus AMOVA did not yield the same markers that had significantly different allele distribution as the Fisher's exact test for the Virginia samples, with only two markers showing significant $F_{ST}$ values (RUCV 46 and Cvi2m10). However, looking at $F_{ST}$ values alone, markers that were detected as significant in the Fisher's exact tests in Virginia samples (RUCV 68 and RUCV 270) showed $F_{ST}$ values on the higher part of the range (between -0.010 and 0.33) for samples in this site.

**Table 5.** Markers showing a significant shift in allele distribution between samples collected pre- and post-disease-related mortality event as detected using the Fisher's exact test (*Pc*<0.01, *Pc* is the *P* value after correction by multiplying it by the number of alleles for each marker). Markers with a dash showed no significant shifts. Markers **in bold** or marked with a (*) mapped to disease-resistant QTLs or were near a marker showing a significant shift in allele frequency post mortality in previous studies. LG: Linkage group.

| PLEX | Marker (Linkage Group according to Guo et al. 2008) | York River, Virginia | Cape Shore, New Jersey |
|------|-----------------------------------------------------|----------------------|------------------------|
| A | RUCV 1 | - | 0.0000642 |
| | RUCV 3 | - | 0.000422 |
| | RUCV 46 | - | - |
| | RUCY 28 | - | 0.0000696 |
| D | RUCV 11 | - | 0.00144 |
| | RUCV 27 | - | 0.000138 |
| | **Cv02i23 (LG2)** | - | 0.000081 |
| | **RUCV 66** | - | - |
| E | RUCV 24 | - | - |
| | **RUCV 97 (LG2)** | - | 0.0000341 |
| | RUCV 68 | 0.00169 | 0.0000376 |
| | **Cvi2j24 (LG2)** | - | - |

| | | | |
|---|---|---|---|
| F | RUCV 23 | - | - |
| | **Cvi2i4 (LG2)** | - | - |
| | RUCV 18 | - | - |
| | RUCV 270* | 0.0038 | 0.0000944 |
| G | **RUCV58 (LG2)** | - | - |
| | **Cvi1g4 (LG9)** | - | - |
| | Cvi1g3 | 0.00411 | - |
| | **Cvi2m10 (LG6)** | - | - |

In order to minimize the detection of false-positives and to provide additional support for detected associations, a closer inspection of the allele frequency shifts was conducted for those markers that showed significantly different allelic distribution in either one or both sites. Markers that shifted in frequency in the same direction in both sites, suggesting a response to a similar selection force (the disease-related mortality event), were of particular interest. For each marker, the allele showing the highest shift in the same direction in allele frequency in both sites was tested using the Fisher's exact test to determine the significance of the shift. Alleles showing significant shifts in allele frequency pre and post-disease-related mortality in the same direction in both sites included RUCV 270 (allele 545) and RUCV 27 (203), while the allele frequency shift in RUCV 28 (228), RUCV 97 (271) and RUCV 3 (292) was only significant in the New Jersey site. Cvi1g3 (240) was only significant in the Virginia site.

**Table 6**. Allele frequency for markers that had significantly different allelic distribution between pre- and post-disease-related mortality. Shading indicates the

allele with the highest frequency shift in the same direction in samples from both sites. * indicates statistical significance (Fisher's exact test, *p*-value<0.05).

| Marker | Allele | Virginia | | | New Jersey | | |
|---|---|---|---|---|---|---|---|
| | | Pre-selection frequency | Post-selection frequency | Change | Pre-selection frequency | Post-selection frequency | Change |
| RUCV 28 | 222 | 0% | 0% | 0% | 2% | 2% | 0% |
| | 224 | 2% | 13% | 11% | 3% | 0% | -3% |
| | 226 | 2% | 5% | 3% | 7% | 15% | 8% |
| | 228 | 21% | 10% | -11% | 16% | 0% | -16%* |
| | 232 | 13% | 19% | 7% | 25% | 19% | -6% |
| | 234 | 8% | 13% | 5% | 0% | 4% | 4% |
| | 236 | 0% | 0% | 0% | 4% | 6% | 2% |
| | 238 | 19% | 11% | -7% | 14% | 34% | 20% |
| | 240 | 13% | 15% | 2% | 2% | 4% | 2% |
| | 242 | 4% | 10% | 6% | 9% | 5% | -4% |
| | 244 | 17% | 5% | -12% | 15% | 8% | -7% |
| | 248 | 0% | 0% | 0% | 2% | 2% | 0% |
| | 250 | 2% | 0% | -2% | 0% | 0% | 0% |
| RUCV 3 | 284 | 35% | 35% | 0% | 43% | 26% | -18% |
| | 288 | 9% | 9% | 0% | 9% | 2% | -7% |
| | 292 | 31% | 46% | 14% | 28% | 60% | 31%* |
| | 296 | 2% | 0% | -2% | 7% | 6% | 0% |
| | 300 | 20% | 10% | -10% | 11% | 4% | -7% |
| | 308 | 2% | 0% | -2% | 2% | 2% | 0% |
| RUCV 27 | 197 | 4% | 12% | 8% | 2% | 2% | 0% |
| | 199 | 2% | 6% | 4% | 2% | 1% | -1% |
| | 201 | 0% | 3% | 3% | 9% | 15% | 6% |
| | 203 | 29% | 10% | -18%* | 16% | 0% | -16%* |
| | 205 | 0% | 0% | 0% | 0% | 2% | 2% |
| | 207 | 21% | 38% | 17% | 26% | 21% | -5% |
| | 209 | 0% | 0% | 0% | 2% | 4% | 2% |
| | 211 | 7% | 0% | -7% | 2% | 6% | 4% |
| | 213 | 23% | 26% | 3% | 17% | 38% | 20% |
| | 215 | 0% | 3% | 3% | 5% | 2% | -3% |
| | 217 | 7% | 1% | -6% | 5% | 5% | 0% |
| | 219 | 4% | 0% | -4% | 12% | 2% | -10% |
| | 223 | 4% | 0% | -4% | 2% | 2% | 0% |
| RUCV 68 | 321 | 0% | 0% | 0% | 0% | 1% | 1% |
| | 327 | 56% | 54% | -1% | 20% | 38% | 18% |
| | 330 | 17% | 1% | -15% | 17% | 24% | 8% |
| | 333 | 0% | 7% | 7% | 0% | 8% | 8% |
| | 336 | 0% | 1% | 1% | 0% | 0% | 0% |
| | 339 | 28% | 36% | 8% | 63% | 29% | -34% |
| RUCV 18 | 135 | 6% | 4% | -1% | 4% | 6% | 2% |
| | 137 | 37% | 26% | -11% | 29% | 49% | 20% |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 139 | 56% | 65% | 10% | 64% | 37% | -27% |
| | 141 | 2% | 4% | 2% | 3% | 7% | 4% |
| **RUCV 270** | 529 | 4% | 3% | -1% | 0% | 11% | 11% |
| | 545 | 0% | 13% | 13%* | 5% | 21% | 17%* |
| | 549 | 18% | 8% | -10% | 2% | 2% | 0% |
| | 553 | 6% | 11% | 5% | 5% | 0% | -5% |
| | 561 | 0% | 3% | 3% | 1% | 4% | 3% |
| | 565 | 4% | 0% | -4% | 0% | 0% | 0% |
| | 569 | 30% | 40% | 10% | 60% | 41% | -19% |
| | 577 | 14% | 2% | -12% | 15% | 7% | -7% |
| | 581 | 24% | 19% | -5% | 13% | 13% | 0% |
| **Cv02i23** | 359 | 2% | 0% | -2% | 4% | 1% | -3% |
| | 363 | 25% | 36% | 11% | 28% | 19% | -9% |
| | 371 | 14% | 17% | 2% | 15% | 5% | -10% |
| | 375 | 45% | 40% | -4% | 46% | 47% | 1% |
| | 379 | 4% | 3% | -1% | 0% | 17% | 17% |
| | 387 | 0% | 1% | 1% | 0% | 0% | 0% |
| | 403 | 2% | 0% | -2% | 2% | 4% | 2% |
| | 451 | 0% | 0% | 0% | 1% | 0% | -1% |
| | 459 | 0% | 1% | 1% | 0% | 0% | 0% |
| | 463 | 0% | 0% | 0% | 1% | 5% | 4% |
| | 475 | 4% | 1% | -2% | 3% | 0% | -3% |
| | 479 | 5% | 0% | -5% | 0% | 1% | 1% |
| | 483 | 0% | 0% | 0% | 0% | 1% | 1% |
| **RUCV97** | 245 | 13% | 9% | -4% | 6% | 12% | 6% |
| | 247 | 6% | 1% | -4% | 5% | 2% | -3% |
| | 249 | 0% | 0% | 0% | 0% | 1% | 1% |
| | 251 | 0% | 1% | 1% | 5% | 2% | -3% |
| | 253 | 4% | 1% | -2% | 4% | 1% | -3% |
| | 255 | 9% | 12% | 3% | 14% | 13% | -1% |
| | 257 | 17% | 3% | -14% | 9% | 8% | -1% |
| | 259 | 28% | 32% | 5% | 24% | 24% | 0% |
| | 261 | 9% | 7% | -2% | 7% | 0% | -7% |
| | 263 | 2% | 6% | 4% | 11% | 5% | -6% |
| | 267 | 4% | 3% | -1% | 3% | 10% | 7% |
| | 269 | 2% | 0% | -2% | 0% | 1% | 1% |
| | 271 | 0% | 3% | 3% | 0% | 13% | 13%* |
| | 275 | 4% | 3% | -1% | 1% | 0% | -1% |
| | 277 | 4% | 12% | 8% | 7% | 0% | -7% |
| | 287 | 0% | 3% | 3% | 2% | 8% | 6% |
| | 319 | 0% | 3% | 3% | 0% | 0% | 0% |
| **Cvi1g3** | 240 | 17% | 0% | -17%* | 2% | 0% | -2% |
| | 246 | 0% | 1% | 1% | 6% | 0% | -6% |
| | 255 | 4% | 3% | -1% | 5% | 10% | 5% |
| | 258 | 50% | 63% | 13% | 59% | 55% | -4% |
| | 261 | 2% | 0% | -2% | 0% | 4% | 4% |
| | 264 | 27% | 33% | 6% | 28% | 31% | 3% |

**Homology Search**

As all RUCV markers were developed from coding sequences (ESTs), there is a chance that they are part of the functional genes. A homology search with tblastx in the non-redundant database (Stephen et al. 1997) was conducted for markers that showed significant shift in allelic distribution in either one site or two sites. Nucleotide blast was also conducted for markers that did not detect a hit initially with tblastx. RUCV 68 was found to be homologous to the 40S ribosomal protein S28 (Table 7). RUCV 27 and RUCV 28 showed identity with a predicted cell-number regulator 3-like protein that contained a PLAC8 (Placenta-specific 8) domain in the closely related species *C. gigas*. Cv02i23 found was found to be homologous to another hypothetical protein in *C. gigas,* but tblastx did not detect any putative conserved domains (Table 7).

**Table 7**. Results of homology search for markers that showed significant shift in allelic distributions in at least one site. In **bold** or marked with a (*) mapped to disease-resistant QTLs or were near a marker showing a significant shift in allele frequency post mortality (*) in previous studies.

| Marker | Accession number | Query length | EST homology | Domain | E-value | Query cover | Identity |
|---|---|---|---|---|---|---|---|
| RUCV 68 | gi|31908118 | 720 | 40S ribosomal protein S28 [Crassostrea gigas] | S1_S28E | 3e-23 | 20% | 100% |
| RUCV 270* | gi|31902968 | 841 | No hit | - | - | - | - |
| **RUCV 97** | gi|14581013 | 687 | No hit | - | - | - | - |
| **Cvi1g3** | gi|49618833 | 218 | No hit | - | - | - | - |
| **Cv02i23** | gi|49618841 | 331 | Hypothetical protein CGI10008026 [Crassostrea gigas] | - | 8e-16 | 70% | 69% |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| RUCV 3 | gi\|31908953 | 903 | No hit | - | - | - | - |
| RUCV 28 | gi\|14581194 | 622 | Predicted: cell number regulator 3-like [Crassostrea gigas] | PLAC8 | 1e-54 | 52% | 94% |
| RUCV 27 | gi\|14581203 | 607 | Predicted: cell number regulator 3-like [Crassostrea gigas]] | PLAC8 | 1e-64 | 53% | 92% |
| RUCV 1 | gi\|39726421 | 305 | No hit | - | - | - | - |
| RUCV 11 | gi\|31906314 | 791 | No hit | - | - | - | - |

**DISCUSSION**

The aim of this study was to gather new or strengthen existing evidence supporting the association of previously published markers with disease-resistant phenotypes as performed in family-based studies by determining if these markers also experience a significant shift in allele frequency in an oyster stock experiencing Dermo and MSX-related mortality in 2 different sites. Among our panel of 20 microsatellites, it was expected that the 8 markers located in regions previously associated with disease resistance (Guo et al. 2008, Zhang, Guo and Gomez-Chiarri, unpublished) would have a higher probability of discriminating among populations pre- and post- a disease-related mortality event. However, none of these markers showed significant differentiation in allelic distribution before and after mortality at both the Virginia and New Jersey deployment sites. Of these 8 markers, only 2 could differentiate between populations pre- and post- the disease-related mortality event at one of the two sites (Cv02i23 and RUCV 97-New Jersey). Interestingly, a signal of potential selection was detected at two markers (RUCV 270 and RUCV 68) with no expectation of association with disease at both deployment sites and several markers (RUCV1, RUCV3, RUCV11, RUCV18, RUCV27, and RUCV28) at the New Jersey site.

In our study, RUCV 270 was the strongest candidate to have association with Dermo and MSX disease resistance in eastern oysters, since it showed a significant post-mortality shift in allelic distribution in the same direction in both sites. Allele 545 of RUCV 270 was particularly interesting, since it increased significantly in both populations after the mortality event, suggesting that it was under selection during this event. The EST sequence from which RUCV 270 is derived was not homologous to

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any sequences in the non-redundant nucleotide database based on tblastx search, suggesting that the EST sequence containing RUCV 270 does not code for known proteins. This is not unexpected since many microsatellite markers do not code for proteins, but they may experience shifts in allelic distribution in response to a disease-related mortality event because the functional genes under selection are nearby on the chromosome. RUCV 270 mapped to linkage group 10 (Guo et al. 2008) and there are 8 other genetic markers (2 MS and 7 AFLP markers) within 5cM of RUCV 270 (Zhang, Guo and Gomez-Chiarri, unpublished). However, none of these markers showed significant shifts in allele frequency after ROD mortality except Cvi12, the one closest to RUCV270 according to the genetic map (Figure 1). The next logical step is to fine-map this region and test more genetic markers in the close vicinity of RUCV 270 and Cvi12 for association with disease resistance, in order to determine if this is a disease-resistant QTL and ultimately identify the functional gene under selection.

Results of the Fisher's exact test suggested a high probability that allelic distribution at marker RUCV 68 was different before and after selection in both sites. However, closer examination of individual allele frequency shifts at this marker show no significant shifts in the same direction at the two deployment sites. The mostly differing response to selection of this alleles at the two deployment sites does not support an association of this marker with disease resistance.

Several of the markers used in this study were located in a region of linkage group 2 associated with disease-resistance that also contains the serine protease gene, a functional gene that has been associated with disease resistance (Guo et al. 2008).

Serine protease inhibitors have demonstrated ability to inhibit the proliferation of the Dermo pathogen, *Perkinsus marinus* (La Peyre et al. 2010). A polymorphism in the serine protease inhibitor promoter region has been shown to be associated with resistance to Dermo, and was verified comparing susceptible and resistant oyster lines (Yu et al. 2011, He et al. 2012). Of the 5 markers in linkage group 2 tested in this study, only Cv02i23 and RUCV 97, showed significant shifts in allele frequency after the mortality event in our study, and in only one of the sites (New Jersey). A lack of detection of a significant association with disease resistance may be due to the limitations of our study, including a small sample size, especially for the Virginia site (28 pre-selection samples and 36 post-selection samples), which would limit the power to detect noticeable post-mortality shifts. The Virginia site also experienced high over-wintering mortality (over 50%), and the fact that pre-winter samples have significantly differentiated genetic composition suggests that over-wintering already had a selection effect on the oysters. If the alleles associated with disease resistance were reduced to a low level by over-wintering selection, it would reduce our ability to detect them by analyzing post-mortality allele frequency shifts. More significant post-mortality shifts in oyster samples from New Jersey site were detected, which could be explained by the larger sample size and low over-wintering mortality. Moreover, using highly polymorphic microsatellite markers proved to be a bit problematic, as sample sizes allocated for each allele can be extremely small. Their favorably high variability needs to be backed up with a large sample size. In future studies, SNPs might be better candidates if a large sample size is not available. It would be interesting to genotype the polymorphism in the serine protease inhibitor gene of our samples to determine

whether it was associated with better survival and whether its change in allele frequency is in accordance with those detected for Cv02i23 or RUCV97.

Five other markers without previous evidence of association showed significant allelic distribution shift in one site in our study. We cannot confidently say that these markers are associated with disease resistance, and more association study needs to be done for these markers. However, RUCV 3 in this group had the largest allele frequency shift in the same direction of all the affected markers, although no statistically significant allelic distribution shift was detected in the Virginia site. RUCV 27 and RUCV 28 matched to the same gene, and both showed allele frequency shift of more than 10% in the same direction in both sites, which indicated that they could be under selection. These 2 markers showed homology to a predicted cell-number-regulator-like protein in *C. gigas* that contains a PLAC8 domain, a cysteine-rich domain found in a variety of proteins in animals, plants, fungi and algae (Song et al. 2011). The study of PLAC8 containing proteins only started recently and variety of functions have been proposed or observed, including controlling fruit size, cell number, and heavy metal transport in plants (Song et al. 2011). Association studies with more populations need to be done to confirm whether RUCV 27 and RUCV 28 are associated with disease resistance, and investigate the potential functional role in disease resistance of PLAC8-containing proteins.

Marker-assisted selection in oysters is still in its very early stage. The main challenge is the lack of validated genetic markers associated with disease resistance. This study provides extra support for several candidate markers of disease-resistant markers identified by previous studies. The scientific community should prioritize

these markers in further investigations, with a particular focus on fine mapping the

QTL in linkage group 2 and determining if a disease resistant QTL could be present in

linkage group 10.

**APPENDIX**

**Field Evaluation of Disease Resistance in Oyster Lines.**

Our study took advantage of samples collected from a previous unpublished collaborative study funded by the Agricultural Research Service (ARS) was done to evaluate the performance of six lines (Table 1) of oysters deployed at five different locations along the east coast from September 2012 to November 2013 (Damariscotta River, Maine, Narragansett Bay and Ninigret Pond in Rhode Island, Cape Shore, New Jersey and York River, Virginia). Growth and survival were measured for 16 months and samples were collected in cases of high-mortality events for disease diagnosis and future genotyping. Strong genotype by environment interactions were observed: oysters from three of the northern lines (UMaine, NEH-RI, and Clinton) were significantly larger and had significantly higher survival rates than the southern lines (hANA and DEBY) in the Rhode Island sites, while the southern lines had a higher yield than the northern lines in the Delaware and Chesapeake Bay sites. The study found that line performance (yield) was mainly driven by survival. MSX and Dermo were proposed to be the main drivers behind the high mortality of northern lines in the Delaware and Chesapeake Bay sites. Supplementary figure 1 shows the cumulative mortality graph of the six oyster lines.

This study provides a good opportunity to conduct association studies for disease resistance since we have samples collected before and after mortality events to serve as control and case groups. Samples collected before mortality can be considered as the control population, which largely consists of susceptible oysters, with some resistant oysters in the mix. Samples collected after mortality can be considered as the

case population, as they had undergone selection from disease. When choosing which oyster line and site to use for this project, two criteria were used: 1) Oyster population with high mortality is preferred, since the selection on the genetic marker loci by disease would be easier to identify. However, populations with 100% mortality should be avoided as there would be no case populations. 2) High MSX and/or Dermo prevalence and intensity during mortality period is preferred, for disease resistance is the trait we are interested in. Oyster lines in Maine site suffered high mortality early in the study from a ROD outbreak and no oysters were left, so Maine site is excluded. NEH-RI line had high mortality in both Virginia and New Jersey site and still had survivors at the end of the study (Figure 2). qPCR (Quantitative Polymerase Chain Reaction) data confirmed high prevalence and intensity of Dermo and MSX in the summer mortality period (Supplementary Table 1). Other lines/sites did not have as high mortality rates, so NEH-RI line at Virginia and New Jersey were chosen for this study.

**Supplementary Figure 1**. Cumulative mortality data for six lines of oysters deployed

at five sites from Sept-2012 to Dec-2013.



## Cumulative Percent Mortality
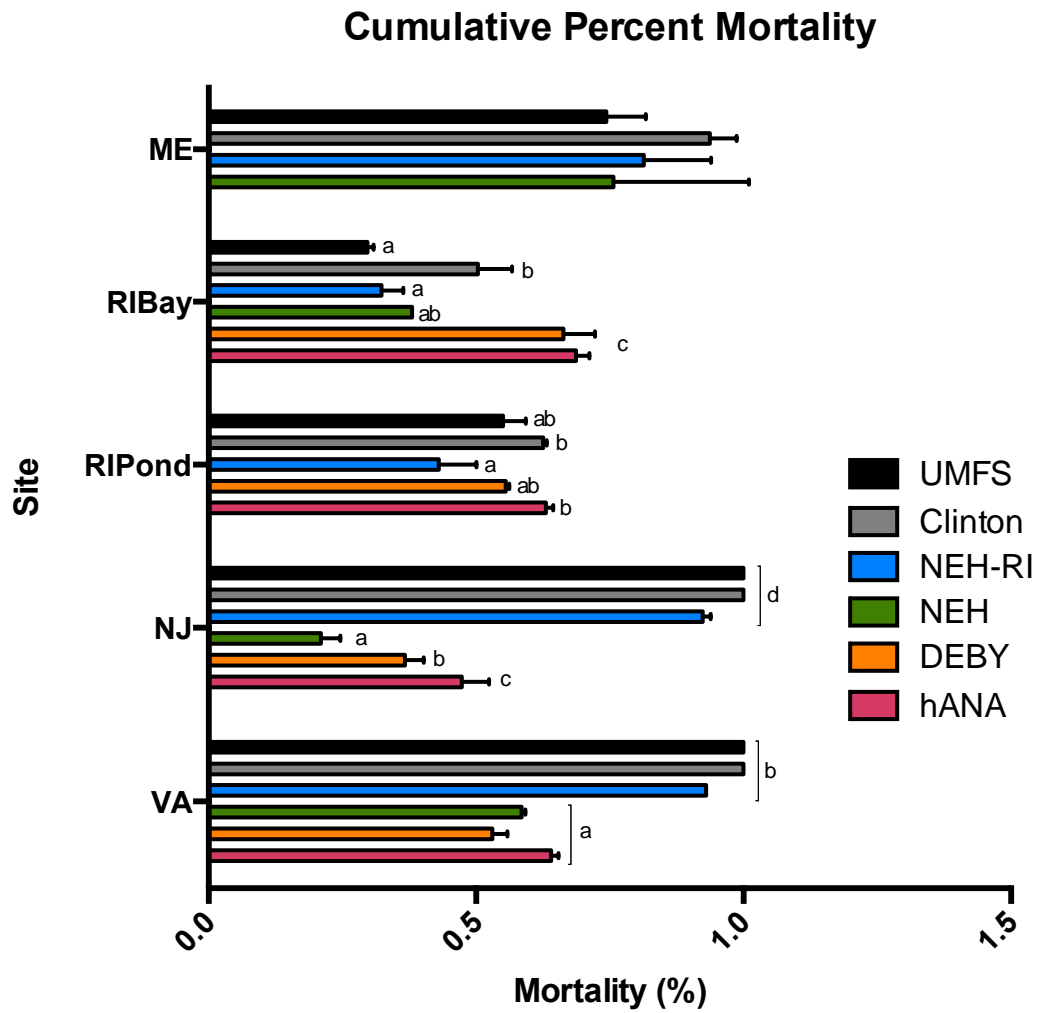
**Supplementary Figure 2.** Cumulative percent mortality experienced by oysters from the NEH-RI line deployed from August 2012 to November 2013 in sites in Virginia (York River) and New Jersey (Cape Shore).
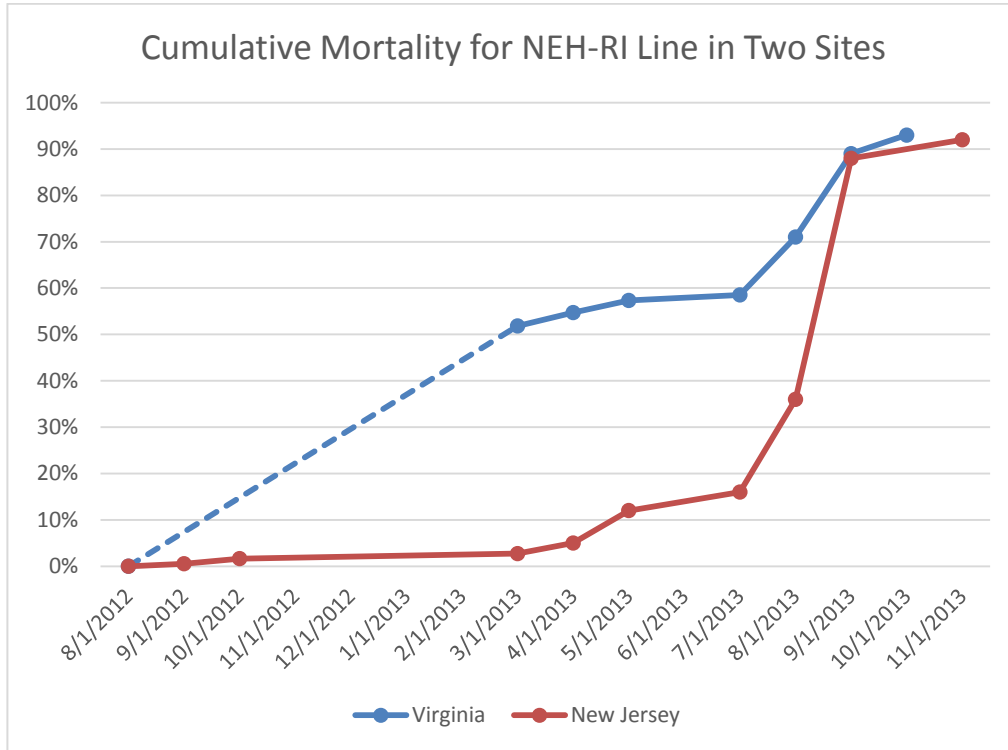
**Supplementary Table 1**. Dermo and MSX prevalence and intensity for NEH-RI line

at Virginia and New Jersey site during summer mortality period.

| Site | Time | Number of Samples Tested | Dermo Prevalence | Dermo Avg. Log Concentration | MSX Prevalence | MSX Avg. Log Concentration |
|------|------|------|------|------|------|------|
| Virginia | Sept-2013 | 60 | 100% | 5.06 | 58% | 4.04 |
| New Jersey | Sept-2013 | 9 | 100% | 3.22 | 100% | 3.1 |

**Supplementary Table 2.** Locus-by-locus pairwise difference for selected microsatellites between pre- and post- a disease related mortality event experienced by a population of NEH-RI oysters deployed in sites in Virginia (York River) and New Jersey (Cape May) (locus by locus AMOVA). Shaded results are statistically significant ($p < 0.05$)

| | Virginia | | New Jersey | |
|---|---|---|---|---|
| **Locus** | $F_{ST}$ | $p$ | $F_{ST}$ | $p$ |
| RUCV 3 | 0.011 | 0.4452 | 0.035 | 0.0306 |
| RUCV1 | -0.005 | 0.8208 | 0.029 | 0.0597 |
| RUCV 46 | 0.033 | 0.0497 | 0.020 | 0.1171 |
| RUCV 28 | 0.011 | 0.3457 | 0.040 | 0.0083 |
| RUCV11 | 0.023 | 0.0769 | 0.021 | 0.0238 |
| RUCV27 | 0.014 | 0.3708 | 0.040 | 0.0105 |
| Cv02i23 | -0.001 | 0.4979 | 0.024 | 0.0530 |
| RUCV66 | 0.031 | 0.0775 | 0.001 | 0.4450 |
| RUCV24 | 0.020 | 0.2117 | 0.017 | 0.1586 |
| Cvi2j24 | 0.006 | 0.2310 | 0.019 | 0.0235 |
| RUCV 68 | 0.024 | 0.2146 | 0.106 | 0.0004 |
| RUCV97 | 0.002 | 0.4394 | 0.016 | 0.0126 |
| Cvi2i4 | 0.022 | 0.0927 | 0.007 | 0.1385 |
| RUCV23 | 0.010 | 0.1481 | -0.001 | 0.5089 |
| RUCV18 | 0.002 | 0.3838 | 0.083 | 0.0023 |
| RUCV 270 | 0.024 | 0.2021 | 0.047 | 0.0230 |
| Cvi1g3 | 0.023 | 0.1022 | -0.002 | 0.5114 |
| Cvi1g4 | 0.012 | 0.3648 | 0.003 | 0.4672 |
| RUCV58 | -0.010 | 0.8974 | -0.001 | 0.5335 |
| Cvi2m10 | 0.031 | 0.0282 | 0.009 | 0.1669 |

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