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Polymorphism in a serine protease inhibitor gene and its association with disease resistance in the eastern oyster (*Crassostrea virginica* Gmelin)

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ABSTRACT

Serine protease inhibitors (SPIs) are a superfamily of structurally related but functionally diverse proteins found in almost all organisms ranging from viruses to humans. Some of them play important roles in host defense. A recently identified SPI from the eastern oyster (*Crassostrea virginica*), cvSI-1, has been shown to inhibit the proliferation of the Dermo pathogen *Perkinsus marinus* in vitro, although direct evidence linking it to disease resistance is lacking. In this study, we identified polymorphism in the *cvSI-1* gene and studied its association with improved survival after disease-caused mortalities and in disease-resistant eastern oyster strains. Full-cDNA sequence of *cvSI-1* was sequenced in a diverse panel of oysters, revealing 12 single-nucleotide polymorphisms (SNPs) in the 273 bp coding region: five were synonymous and seven non-synonymous. The Dn/Ds ratio, 1.4, suggests that *cvSI-1* is under positive selection. Selected SNPs were genotyped in families before and after disease-caused mortalities as well as in disease-resistant and susceptible strains. At SNP198, the C allele consistently increased in frequency after mortalities that are caused primarily by Dermo and possibly also by MSX. Its frequency in the disease-resistant strain is significantly higher than that in the susceptible strains and the base population from which the selected strains were derived. These results indicate that polymorphism at *cvSI-1* is associated with Dermo (possibly also MSX) resistance in the eastern oyster. SNP198 is a synonymous mutation, and its association with disease resistance may be due to its close linkage to a functional polymorphism nearby.

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1. Background

The eastern oyster (*Crassostrea virginica* Gmelin), which supports important fishery and aquaculture industries in the United States, is seriously affected by two major diseases: MSX (caused by the protozoan *Haplosporidium nelsoni*) and Dermo (caused by the protozoan *Perkinsus marinus*) [1]. Each of the two diseases may kill up to 90% of the naive oysters. These two diseases are considered to be one of the leading causes for the collapse of the oyster populations and fisheries in the mid-Atlantic region [2]. The identification of oyster genes that are involved in disease resistance should improve our understanding of host defense in oysters and contribute to our efforts to develop disease-resistant stocks.

Serine protease inhibitors (SPIs) are a superfamily of structurally related proteins that are found in almost all organisms ranging from viruses to humans [3,4]. They have diverse functions and are involved in many biological pathways and processes. As suggested by their name, most SPIs inhibit the proteolytic activity of serine proteases. Any process that involves serine proteases, including digestion, blood clotting, inflammation and immune responses, may be regulated by SPIs [5]. Some SPIs such as HSP47 may not have inhibitory effects on proteases but function as storage and chaperone proteins [6]. In immune response, SPIs may regulate the Toll pathway [5] or directly inhibit serine proteases from invading pathogens that are often key virulence or pathogenic factors [7,8]. Thus, some SPIs may be important for disease resistance in oysters.

A serine protease inhibitor, cvSI-1, has been identified and characterized in the eastern oyster [9,10]. It has strong inhibitory effects on serine proteases of the oyster pathogen *P. marinus* and the proliferation of the pathogen itself in vitro. *CvSI-1* is expressed at higher levels in oysters selected for Dermo resistance than in unselected controls, although the selected oysters were from Louisiana and unselected oysters were from Maine, and there was no change in *cvSI-1* expression level in either population upon *P. marinus* challenge [10]. Thus, while it is possible or even likely that cvSI-1 is involved in host defense against Dermo, direct evidence

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linking it to improved survival or Dermo resistance is lacking. To further understand if cvSI-1 is indeed involved in disease resistance at organism level, we studied polymorphism in the *cvSI-1* gene and analyzed its possible association with improved survival or disease resistance in the eastern oyster. We sequenced the full coding sequence of *cvSI-1* in a diverse panel of oysters and identified 12 single-nucleotide polymorphisms (SNPs). Selected SNPs were genotyped in families before and after disease-caused mortality, as well as in disease-resistant and susceptible strains derived from the same base population. Here we present evidence that variation in *cvSI-1* is associated with disease resistance in the eastern oyster.

2. Materials and methods

2.1. Oysters, families and populations

Thirty oysters were used for resequencing *cvSI-1* and SNP discovery. The oysters were taken at random from three different geographic populations: Mobile Bay (Alabama), Delaware Bay (New Jersey) and Martha's Vineyard (Massachusetts), ten from each population.

To determine if variation at *cvSI-1* is associated with improved survival under diseases, selected SNPs were genotyped in three families before and after disease-caused mortalities. The three families were F2 (HB2 and XG3) or backcross (BCMF) families created with selected disease-resistant and susceptible strains (Guo et al., unpublished), so that disease-resistance genes might be segregating. Oysters from the three families were deployed at Cape Shore, NJ, where Dermo is enzootic. Mortalities were monitored and samples were regularly collected. Before and after mortality samples were identified on mortality curves and used for this study. They bracketed 50–80% mortalities that were mainly caused by Dermo.

To further demonstrate that polymorphism at *cvSI-1* is associated with disease resistance, SNPs were genotyped in disease-resistant (NEH) and susceptible (FMF and UMFS) strains, as well as the wild base population from which these strains were derived. NEH was a disease-resistant strain selected at Rutgers University since 1960 that had shown strong resistance to MSX [11] and moderate resistance to Dermo [12]. FMF and UMFS were selected strains from Frank M. Flower Oyster Company (NY) and University of Maine, respectively. They had been selected for superior growth and resistance to JOD (juvenile oyster disease, caused by *Roseovarius crassostreae*), but both were susceptible to Dermo and MSX.

2.2. Resequencing and SNP discovery

For resequencing, total RNA was extracted from gills or adductor muscles of 30 oysters from the panel using the Promega SV Total RNA Isolation Kit. cDNA was synthesized with the Qiagen QuantiTect Reverse Transcription Kit. Resequencing primers were designed using Primer Premier 5.0 based on *cvSI-1* sequence (gi|71796854|gb|DQ092546.1|) in GenBank covering the entire coding sequence (Table 1). PCR was performed in a 20 μL reaction mixture consisted of 100 ng cDNA, 2 μL 10 × PCR Buffer (20 mM Tris–HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 0.4 μL dNTP (2.5 mmol/L), 0.8 μL of each primer (5 μmol/L), 1 μL bovine serum albumin (0.5 g/L) and 1 U Go*Taq* DNA polymerase (all from Promega). Reaction conditions for PCR were as follows: 95 °C for 5 min; 35 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. PCR products were purified and directly sequenced with ABI 3730 sequencers at the High-Throughput Genomics Unit (HTGU), University of Washington. Known positive and negative controls were included for sequencing, and all samples were sequenced in both directions.

Table 1
Primers used to amplify *cvSI-1* coding region and for HRM genotyping in the eastern oyster.

Name	Sequence (5′→3′)	Product size (bp)	Tm (°C)
SPI-2	F:TATTAAGAAAACAATGGACGTAG R:GATTTATTTCAAAACAAGAAAACC	354	60
SNP94HRM	F:GGTTTGCACCAGTAATGA R:CCCTTTTACATAGCCACTT	72	55
SNP198HRM	F:TTGTCAACAACTGCTTACACCT R:CCGTGCAGACTGCATGTTC	63	55

Sequence alignment, annotation and SNP discovery were conducted using VECTOR NTI Advance 10.1.1 (Invitrogen).

2.3. SNP assay design, genotyping and analysis

For genotyping, genomic DNA was extracted from adductor muscles of each oyster with the Omega Biotek D3373-02 E.Z.N.A. Mollusc DNA Kit, quantified on a nanodrop spectrophotometer and verified for product integrity on agarose gels. Two SNPs (Table 1) were selected for genotyping and characterization with the high resolution melting (HRM) assay using Syto® 9 green fluorescent stain. The HRM assay is based on the amplification of a short DNA fragment where different alleles of an SNP give different melting profiles detectable on a real-time PCR system. HRM primers were designed using Primer Premier 5.0 following the CorProtocol for HRM analysis [13]. Product size was set between 60 and 100 bases. Primer size is set to be between 18 and 27 bases long, with melting temperatures between 57 and 63 °C, and GC content between 20% and 80%. Default values were used for other parameters such as max 3′ stability and max mis-priming. Secondary structure of the amplicon was analyzed using the DINAMelt Server (http://www.bioinfo.rpi.edu/applications/hybrid/twostate-fold.php) [14].

PCR mixture consisted of 10 ng of genomic DNA, 200 nM of each primer, AmpliTaq Gold® 360 Master Mix (containing 0.5 μM Syto® 9, Applied Biosystems Inc.) and PCR grade water in a volume of 20 μL. PCR cycling and HRM analysis were performed on an Applied Biosystems® 7500 Fast real-time PCR system (Applied Biosystems Inc.). PCR amplification was conducted with the following protocol: 95 °C for 5 min; 45 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and final incubation at 72 °C for 10 min. For melting curve analysis, PCR products were heated from 65 °C to 95 °C, at the rate of 1 °C per second. Normalized melting curve, temperature-shift curve and difference plots were generated with the High Resolution Melting Analysis 2.0 software provided with the ABI 7500 Fast real-time PCR system. Genotypes were automatically called or manually assigned when necessary. Known genotypes identified by sequencing were included as positive controls. The software GENEPOP on the web (http://genepop.curtin.edu.au/) was used to identify deviations from Hardy–Weinberg equilibrium. Frequency difference between populations was examined by chi-square test.

3. Results

3.1. Sequences and SNPs

Of the 30 oysters used for resequencing, 12 oysters failed in PCR amplification or sequencing reaction, and 18 oysters produced high quality sequences covering all three geographic populations. Analysis of the 18 sequences revealed 12 SNPs in the 273 bp coding region, corresponding to about one SNP per 23 bp (Fig. 1). Minor allele frequency ranged from 0.13 to 0.42 (Table 2). Of the 12 SNPs, five were synonymous and seven were non-synonymous (Table 2).

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