A Pilot Study of Genetic Resilience and Diversity Among Eelgrass (*Zostera marina*) Populations in the New England Coastal Region

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Introduction

Eelgrass: A Foundation Species

There has been and continues to be considerable interest in the global population status of the marine angiosperm *Zostera marina*. Since the 1930s, there has been a substantial decline in the overall abundance of this aquatic plant, which may have significant ecological consequences on estuarine environments (Muehlstein et al.1991; Short 2009).

Z. marina is the most prevalent seagrass found in shallow coastal waters across the entire Northern Hemisphere (Olsen et al. 2004). It serves a multitude of important ecological functions such as primary production in coastal environments, nutrient uptake and storage, oxygen production, water filtration, stabilization of coastal erosion, and it serves as a nursery habitat for a variety of coastal marine fauna. (Olsen et al. 2004; Short 2009). Decline in the *Z. marina* populations affects not only the species itself, but also all other organisms that are dependent upon its presence (Short 2009).

In the early twentieth century, *Z. marina* populations in Atlantic North America and Europe experienced a sharp and drastic decline in population quantities, the result of a wasting disease caused by the pathogenic protist *Labyrynthula zosterae* (Muehlstein et al. 1991). Since that time, the species has experienced numerous other stressors, mostly anthropogenic in nature, leading to instability in genetic structure of many populations (Olsen et al. 2004). Nutrient loading from human development has led to eutrophication in some areas, causing macroalgal blooms that block sunlight access to eelgrass meadows. Since eelgrass depends upon clear water

for sunlight, eutrophication has greatly influenced the survival of eelgrass meadows (Grilo et al. 2012; Short 2009). A loss of 15% of the world's seagrasses was reported between 1994 and 2004 alone (Olsen et al. 2004). Aside from sunlight quality, eelgrass meadows respond to a variety of other environmental factors. Water qualities such as nutrient availability, siltation, temperature and salinity all influence metabolic properties of the plant, and drastic changes in any of these properties can result in metabolic stress and possible death. Other physical factors also present great risks to eelgrass bed survival, such as water turbulence, boating or fishing activities and increased wave exposure, all of which leave the plant vulnerable to uprooting or burial (Short 2009).

Because eelgrass is such a vulnerable species, methods for restoration management must be implemented to arrest the species' decline and to repopulate afflicted meadows. Restoration management of the species requires a multi-step approach from a variety of disciplines (Short et al. 2010). Genetic testing must first be completed on a wide range of populations geographically to determine genetic diversity within and among populations. Field studies and mesocosm experimental testing must also be performed on a variety of populations to test for stressor tolerance. Collectively, these data will provide a better understanding of genetic and environmental interactions on the health of eelgrass that will aid in site selection for restoration donors (Short et al. 2010).

A Genetic Approach to Population Analysis

This project focused on a subset of the genetic component to a larger Nature Conservancy Study of eelgrass populations in Southern New England and Long Island Sound, with a focus on

genetic structure of eelgrass populations in the New England coastal region. The purpose of genetic analysis for this project is to offer an evidence-based approach to determining population structure from a molecular perspective. Molecular ecology techniques are widely employed in conservation biology to assess genetic variability within and among populations (Beebee and Rowe 2008). Populations with high genetic diversity are thought to have higher potential resilience to cope with environmental stressors. A population that is low in genetic diversity can often be explained as the result of a population bottleneck in the past (Beebee and Rowe 2008). A population bottleneck occurs when a population size is drastically reduced, as occurred with *Z. marina* populations in the past (Beebee and Rowe 2008; Muehlstein et al. 1991).

Since the late twentieth century, genetic methods of comparison have been used in systematics and population ecology. Mendel postulated that unit factors, now called genes, are passed from parent to offspring. Alternative forms of a single gene are alleles. Many genes are distributed across multiple chromosomes. Diploid species have pairs of chromosomes in sexual reproduction. With sexual reproduction, every individual inherits one allele from each parent; over multiple genes, the combination of alleles represents a distinct genetic composition, or genotype (Beebee and Rowe 2008). The Hardy-Weinberg equilibrium predicts that for a given gene in a large population of randomly mating individuals, both allele frequencies and genotypic frequencies remain constant, That is, relative proportions of homozygotes (two copies of the same allele) and heterozygotes (contrasting alleles) should remain unchanged. (Beebee and Rowe 2008). Population bottlenecks, non-random mating, and/or adaptive selection change the frequencies of alleles and the ratios of homozygotes. Mendelian genetics and The Hardy Weinberg Principle became the basis for evolutionary and population genetic studies. Modern

methods of genetic analysis still apply these theories, which are complemented by molecular marking techniques for population structure analysis (Beebee and Rowe 2008).

Markers for Genetic Diversity

A common origin for genetic variation between individuals in a population is mutations in part(s) of the DNA. Genetic mutations arise from a variety of sources; they may occur as a result of radiation or chemical damage, or be caused by errors in DNA replication mechanisms. Genetic variation is measured by determining genotypic frequencies among individuals at specific genomic regions known as molecular markers. Many different types of molecular markers exist, and each type has advantages for different types of analysis. Codominant molecular markers, those from which alleles are inherited from both parents in sexual reproduction, are frequently used because they allow for distinction between heterozygous and homozygous individuals (Beebee and Rowe 2008). Modern codominant molecular markers used extensively in genetic analysis today include microsatellites. Microsatellites, or Simple Sequence Repeats (SSRs), are typically neutral genetic markers (e.g. genes are not under adaptive selection pressure). SSRs tend to be hypervariable, with high mutation rates resulting from 'slippage' of DNA replication enzymes. They acquire mutations by a Stepwise Mutation Model, where a mutation typically changes the number of repeats by +/- one repeat units at a time. Because of the frequency of mutation, microsatellites offer a precise indication for genetic variability between individuals. A critical assumption for molecular markers used in population genetic analysis is that the markers are neutral with respect to natural selection, unbiased by the presence

of adaptive alleles. Microsatellites, or Simple Sequence Repeats (SSRs) offer that quality (Beebee and Rowe 2008)).

http://faculty.vetmed.ucdavis.edu/faculty/gclanzaro/lab/images/microsatellite_DNA.png

Figure 1: Microsatellite with tandem repeats units of $[GT]_n$. Stepwise mutation of 2[GT] units from A → B and B→ C

Reusch et al. (1999) isolated seven microsatellite-containing marker regions (loci) to be used for genetic analysis of *Zostera marina* (Reusch et al. 1999). Physical proximity between these markers on the chromosome was an important factor in determining the most favorable loci; those dispersed between different chromosomes will not suffer from linkage disequilibrium. Primer pairs designed for these loci allowed for multiplexing polymerase chain reactions. Each of the seven microsatellite loci identified contained ≥10 repeat units in length. These SSR provided statistically significant measures of the allele frequencies and genetic diversity for eelgrass populations, something that previously used analytical methods did not adequately provide (Reusch et al. 1999).

Genetic Diversity Within Populations

Polymerase chain reaction (PCR) amplification of the seven microsatellite markers allows for chromatogram analysis of size profiles for each locus, indicating allele types. Since the markers are co-dominant, each locus can be identified as either heterozygous or homozygous based on the allele sizes detected (Reusch et al. 2000) With the genotype data for each locus, a multi-locus genotype (MLG) can be determined. Characterization of multi-locus genotypes for the seven loci allow for determination of whether individual samples represent genetic individuals (genets) or morphological individuals of a particular genet (ramet) (Short et al. 2010.). Figure 2 shows two genets of eelgrass. "A" is a distinct genetic individual, or genet, from "B" and "C". Plants labeled "B" and "C" represent ramets of a single genet, because they are individual shoots from the same plant, a result of vegetative reproduction.

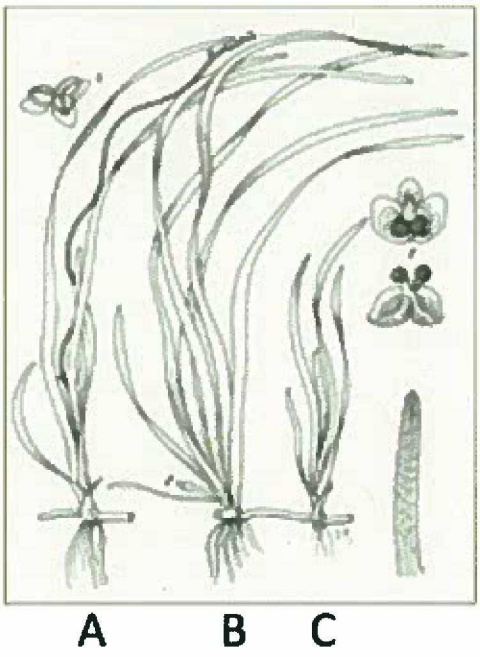

Figure 2: Genets and Ramets of Eelgrass

Allelic richness and heterozygosity are measures of genetic diversity that can be determined for a locus across a set of populations, or for a population across a set of loci. Allelic richness, calculated by the rarefaction method as described by Petit, et al. (1998) indicates the mean number of alleles found per locus for populations normalized for number of individuals. Heterozygosity is given two values: H_e, or expected heterozygosity, and H_o, observed heterozygosity. Expected heterozygosity is the proportion of heterozygous genotypes expected based on measured allele frequencies, assuming Hardy-Weinberg equilibrium. Observed heterozygosity is simply the proportion of genes found to be heterozygous, as determined by genotyping by sizing alleles by capillary electrophoresis. (Beebee and Rowe 2008). H_O and H_e allow for calculation of F-statistics to determine degree of inbreeding within and between populations, where F_{IS} is a measure of inbreeding within a population, and F_{ST} is a measure of genotypic differentiation between subpopulations relative to the total population (Beebee and Rowe 2008).

Materials and Methods

Field Collection of Shoot Tissue:

Zostera marina samples were collected from Great Bay, Nanny Island and Little Harbor in New Hampshire, and from Duck Harbor, Falmouth, and Nantucket in Massachusetts. Eelgrass samples were collected and provided by Fred Short and Holly Bayley from the Jackson Estuarine Laboratory and by Seagrass Net.

The collection method used was a "random walk" sampling design. Individual plant shoots were collected randomly at 2 meters apart in a zigzag pattern within the meadow. The individuals were taken to an off-location site for separation. Samples were stored at 4˚C for a minimal amount of time prior to processing. For each sample, the innermost piece of vegetative tissue, ~3cm in length, was removed to avoid possible contamination by epiphytes. This tissue was blotted dry and stored in a 1.7mL tube on silica drying crystals. Samples were stored at room temperature until DNA extraction (Olsen 2004).

Genomic DNA Extraction from *Z. marina* shoot tissue:

The DNA extraction procedure used was an adaptation by Coyer, et. al. from a high-throughput genomic DNA extraction and purification procedure described by Elphinstone (Elphinstone et al 2003).

Cluster racks of 96 1.2mL tubes (USA Scientific™) were assembled. A single ball bearing (3/16 inch diameter, GoldSpec™) was placed into each tube. *Z. marina* leaf samples were removed

from silica gel and cut into ~5mm pieces with ethanol-sterilized scissors. Approximately six cut tissue samples from each individual were placed in cluster tubes containing ball bearings (BBs). Cluster racks containing samples were frozen at -80°C for approximately two days. The frozen samples were removed from freezer and immediately placed in Retsch MM 400 mixer mill without delay to avoid thawing of tissue. The samples were pulverized in the mixer mill at a frequency of 25 sec^{-1} for 10-second intervals until a fine green powder was obtained. The ball bearings were removed from each tube with a magnet.

Cetyl Trimethyl Ammonium Chloride (CTAB) solution was prepared by mixing 42.3mL of 2% CTAB and 106µL β mercaptoethanol. 400µL of the CTAB solution was added to the freshly ground tissue samples. The tubes were incubated for one hour at 55°C with mixing. To this solution, 400µL Chloroform: Isoamyl Alcohol (24:1) was added to each tube. The resulting solution was mixed by inversion carefully, and then centrifuged at 3000rpm for 20 minutes. After mixing, organic and aqueous layers were formed. The aqueous layer was on top and contained the DNA and water-soluble cell material. 150µL of the aqueous solution and 150µL of saturated Sodium Iodide were added to a silica filter plate (Millipore™, cat. #MSFBN6B10). The filter plate was centrifuged with a waste plate at 1000rpm for 15 minutes and at 2000rpm for 10 minutes. The filter plate was washed once with 150µL wash buffer (see below) and centrifuged at 3000 rpm for 10 minutes with a waste plate. The plate was set to dry for 30 minutes to evaporate all ethanol. Elution buffer (see below) was heated in a water bath at 55°C. 100µL of elution buffer was added to the filter plate and allowed to incubate for five minutes. The plate was centrifuged at 1000rpm for 5 minutes and at 2000rpm for 5 minutes, with a collection plate to collect the eluted DNA. The eluted DNA collected was stored at 4°C indefinitely with each sample well capped.

NanoDrop Spectrophotometric Analysis of Genomic DNA:

A ThermoScientific NanoDrop 2000c™ was connected by USB cable to a PC computer. Product software was used for all sample analyses.

The lid to the NanoDrop instrument was flipped open to reveal a slot designated for a cuvette for large volume measurements and a small hub designated for micro volume measurements. The small hub was wiped with a dry Kimwipe to remove unwanted foreign matter. 1.0µL of genomic DNA sample was removed from storage at 4°C and pipetted onto the top of the measurement hub. The lid was closed slowly. Upon closing the lid, data collection was selected on the Nanodrop software. Within seconds, the program displayed values for OD 260, OD 280, OD 260/280 and OD 260/230. The data was saved as a file within the program.

Polymerase Chain Reaction (PCR) Amplification of Genomic DNA:

Dilution of stock DNA Primers:

Forward and reverse DNA primers were designed for the seven microsatellite loci and purchased from various suppliers (Reusch et al. 1999). The primers were each diluted to 100µM, then aliquoted into small tubes and diluted to 5.0µM using the volumes listed in Table 1.. Note: only 8/16 of the primers listed in Table 1 were diluted. Other primers listed were diluted by Sarah Weigel in the Klein lab and did not require dilution from stock sample.

Primer	Initial Amount	Volume Stock	Volume H20	Final Concentration	Fluorescent Label
19F	26.9 nmoles	18.6 uL	81.41 uL	5 uM	Green
19R	42.44 nmoles	11.78 uL	88.22 uL	5 uM	
23F	32 nmoles	15.63 uL	84.38 uL	5 uM	Green
23R	39.39 nmoles	12.69 uL	87.31 uL	5 uM	
35F*	29.1 nmoles	17.2 uL	82.82 uL	5 uM	Green
35R*	37.24 nmoles	13.43 uL	86.57 uL	5 uM	
17DF	36.98 nmoles	13.52 uL	86.48 uL	5 uM	Blue
16R	43.5 nmoles	11.49 uL	88.51 uL	5 uM	
GA16F					Yellow
17DR					
GA2F					Blue
GA2R					
GA12F					Blue
GA12R					
GA20F					Blue
GA20R					

Table 1: Primer Dilution Volumes (*GA35F and GA35R primers were used for PCR but the GA35 locus was not used for subsequent analysis)

PCR Preparation (for 20μL PCR reactions):

A Bench hoods designed for cell culture, equipped with UV light germicidal and HPA-filtered airflow was used in the processing of all PCR reagents. The hoods were cleaned and all plastic tips and tubes were exposed to Ultra Violet (UV) light germicidal for approximately 15 minutes prior to use.

A small plastic tube (1.7mL or 2.0mL) was labeled as 'Master Mix'. HPLC-grade water was placed in a separate small plastic tube to be used in the master mix. 10x KCl reaction buffer, 10x dNTP mix, 5mM $MgCl_2$, all relevant primer pairs (5uM each) were removed from -20˚C freezer and placed in a cold block in ambient air to thaw passively. When all reagents were thawed, each was vortexed briefly to create homogeneous solutions. The solutions were pipetted into the master mix tube in volumes outlined in Table 2 and Table 3 for 5-plex and 4-plex reactions. Note

that the total volume is dependent upon number of reactions. The number of reactions used to calculate total volume was always approximately ten percent higher than the actual number of reactions. For standard 96-well plates, a total of 104 reactions were used to calculate the total volume.

Fluorescent Label Color	Reagent Name	Volume/Reaction (uL)	# Reactions	Total Volume (uL)
	10x KCl Reaction buffer	2	104	208
	10x dNTP mix	2	104	208
Blue	GA12-F	0.3	104	31.2
N/A	GA12-R	0.3	104	31.2
Green	GA19-F	1.1	104	114.4
N/A	GA19-R	1.1	104	114.4
Blue	GA20-F	0.6	104	62.4
N/A	GA20-R	0.6	104	62.4
Blue	GA17D-F	0.8	104	83.2
N/A	GA17D-R	0.8	104	83.2
Yellow	GA16-F	0.8	104	83.2
N/A	GA16-R	0.8	104	83.2
	50 uM $MgCl_2$	1.2	104	124.8
	Taq DNA Polymerase	0.15	104	15.6
	HPLC-H_2O	5.45	104	566.8
	Total Volume	**18**		**1872**

Table 2: PCR master mix solution for 5-plex PCR reactions

Label Color	Reagent Name	Volume/Reaction (uL)	# Reactions	Volume in MM (uL)
	10x KCl Reaction buffer	2	104	208
	10x dNTP mix	2	104	208
Blue	GA2-F	0.7	104	72.8
	GA2-R	0.7	104	72.8
Green	GA23-F	0.9	104	93.6
	GA23-R	0.9	104	93.6
Green	GA35-F	1.1	104	114.4
	GA35-R	1.1	104	114.4
	$MgCl_2$	1.2	104	124.8
	Taq DNA Polymerase	0.15	104	15.6
	HPLC-H_2O	7.25	104	754
	Total Volume	**18**		**1872**

Table 3: PCR master mix solution for 3-plex PCR reactions

The Bioline™ Taq DNA polymerase was the last reagent added to the master mix. It was kept at -20°C until needed. Taq polymerase was not vortexed prior to addition to the master mix. Once completed, the master mix was mixed gently to allow the polymerase to mix well into solution. The master mix was kept on ice until used. A bacteriological hood equipped with UV light as a germicidal was exposed to UV light for approximately 15 minutes. Inside the bacteriological hood, 18.0 µL of the master mix solution was transferred into 0.2mL PCR tubes, which were placed in a frozen PCR cold block.

Genomic DNA samples stored at 4°C were removed from refrigeration and mixed on a gyrating platform for 20 minutes prior to use. 2.0µL of each DNA sample used was added individually to each tube containing 18.0µL PCR master mix. When all DNA samples were added, the PCR tubes were capped, labeled with sample number, and centrifuged at 350rpm for five seconds to ensure all droplets containing DNA were into solution.

Thermal Cycling:

After centrifugation, the PCR tubes were placed in the BioRad C1000™ thermal cycler. The lid on the thermal cycler was allowed to preheat to 105°C prior to closing the lid on top of the tubes. The protocol titled "ZM56REG" was used for all samples. The thermal cycling conditions and timing are outlined in Table 4. When the thermal cycling reactions ended, the samples were kept at 4°C indefinitely in the thermal cycler.

Step #	Process	Temperature (°C)	Time (s)
1	Initial Heat	94°C	180
2	Denaturation	94°C	30
3	Annealing	56°C	20
4	Extension	72°C	60
5	Repeat steps (2,3,4) x35		
6	Final Extension	72°C	300
7	Hold	4°C	Forever

Table 4: Thermal Cycler Protocol for PCR Amplification

When the samples were removed from the thermal cycler, the tubes were placed in a rack, wrapped in aluminum foil to avoid light exposure to fluorescent tags, and stored at 4°C.

2% Agarose Gel Electrophoresis of PCR-Amplified DNA:

An Erlenmeyer flask was prepared with 1.0g Invitrogen UltraPure™ agarose and 50mL 0.5x TE running buffer. 6.0μL Sybr Safe DNA gel stain was added and the mixture was heated by microwave in 5-second increments for a total of approximately 45 seconds until all solid was completely dissolved into solution. The solution was allowed to cool for two minutes. Agarose gel was poured into casting tray, well comb was placed into position and gel was allowed to

polymerize for 30 minutes. Well comb and casting tray were removed and the gel was turned 90° for electrophoresis with the wells situated at the cathode end of the electrophoresis chamber. 0.5x Tris-EDTA (TE) running buffer was added to the chamber to "fill line".

DNA from PCR products was selected for electrophoresis. 15µL of PCR-amplified DNA was mixed with ~3µL Bromophenol Blue gel loading dye. The DNA-dye solution was mixed in a pipette repeatedly until a homogeneous solution was made. 15mL of the DNA solution was pipetted into the wells of the agarose gel. 6µL of Bioline™HyperLadder II size marker standard was pipetted into the first well for size comparison. The lid for the electrophoresis chamber was applied and the power supply was adjusted to 70mV. DNA was allowed to migrate from the wells toward the anode end of the gel for approximately one hour, or until the Bromophenol Blue dye migrated 2/3 the length of the gel.

The power supply was stopped and the gel was removed from the chamber. It was transferred to a UV light box for imaging. A digital image of the gel was taken under UV light exposure. The "no flash" and "macro" settings were applied. The images were saved and the gel was disposed of.

Capillary Electrophoresis of PCR Amplicons:

The PCR products obtained were stored at 4°C until ready for fragment analysis. 6µL of each sample was transferred into 0.2mL PCR tubes. The tubes were labeled and an excel spreadsheet template found at http://dnacore.unh.edu/ was created to outline the sample name, DNA type, volume submitted, etc. The template was submitted to the Hubbard Genome Center (HGC) on the DNAcore website for the University of New Hampshire. Upon online submission, a

submission number was received. The PCR products were wrapped in foil, labeled with the submission number, and placed in a submission refrigerator at 4˚C for the HGC staff.

The sample solutions (see Table 5) were loaded onto a 384-well plate and placed into the ABI 3130 Capillary Genetic Analyzer. The capillaries were filled with POP-4™ polymer (Performance Optimized Polymer) by Applied Biosystems. The size standard used was GeneScan™ 500 Rox™.

Contents	Volume (uL)
Amplified sample	1.00
Size standard	0.5
Hi-Di™ Formamide	8.5

Table 5: Contents used for Capillary Electrophoresis

Analysis of Fragment-Analyzed Chromatograms:

The Hubbard Genome Center uploaded an output file containing the ABI3130 Automated Sequencer results. The output file was opened with GeneMapper™ software for chromatogram analysis. Chromatograms were obtained by following GeneMapper™ protocol. When obtained, the chromatograms were separated by locus. Samples were scored by visual assessment of the quality of peaks obtained. Only tall, rounded peaks were chosen as valid scores. The peaks scored were given numerical value for fragment size. A chromatogram with one peak indicated homozygous alleles. Chromatograms with two distinct peaks and two fragment size values were heterozygous. The fragment size values were saved for all valid scores, and exported to an excel spreadsheet indicating fragment size values for all individuals and for all loci.

Genetic Analysis:

Statistical analyses were performed using the fragment size values as the first input file. The following software programs were used according to software-specific instructions:

-CONVERT™
-FSTAT 2.9.3.2™
-GDA Genetic Data Analysis by Paul Lewis™
-GENALEX 6 ™

Results

The results presented include *Z. marina* samples from populations in Duck Harbor, MA and Great Bay, NH. Due to difficulties encountered with data analysis for those populations, data analysis will be presented for five previously surveyed populations. Tissue samples from Great Bay and Little Harbor in New Hampshire were analyzed for allele sizes by Sarah Weigel in the Klein Lab at the University of New Hampshire. Allele sizes for samples from Nanny Island, NH, Falmouth, MA and Nantucket, MA were analyzed by Alyssa Mixon in the Olsen and Coyer Lab at the University of Groningen in The Netherlands.

NanoDrop2000™Spectrophotometry of Genomic DNA Extract

DNA concentrations were measured to determine the efficacy of DNA extraction for a set of samples. It was presumed that the concentration of DNA in a sample was directly proportional to the amount of DNA obtained from an extraction procedure.

	Sample ID	Nucleic Acid Conc.	Unit	A260	A280
1	451	69.5	ng/µl	1.39	0.693
2	452	83.7	ng/µl	1.674	-0.003
3	453	79.3	ng/µl	1.586	0.144
4	454	61.8	ng/µl	1.236	0.085
5	4457	34.9	ng/µl	0.697	-0.062
6	4458	59.5	ng/µl	1.189	-0.045
7	4466	48.5	ng/µl	0.971	0.021
	Mean	*62.46*			
8	2062	149.3	ng/µl	2.986	0.08
9	3232	103.5	ng/µl	2.069	0.029
10	3234	80.4	ng/µl	1.608	0.108
11	3236	57.2	ng/µl	1.143	0.066
12	2022	28.9	ng/µl	0.579	-0.029
13	2034	28.6	ng/µl	0.572	-0.027
14	2042	60.7	ng/µl	1.213	-0.038
15	0.1x TE	-1.8	ng/µl	-0.035	-0.06
16	0.1x TE	1	ng/µl	0.02	-0.032
17	3100	72.3	ng/µl	1.446	0.074
18	3099	34.3	ng/µl	0.686	-0.028
19	3098	75.5	ng/µl	1.509	0.372
20	2017	29.6	ng/µl	0.593	-0.048
21	2026	66.7	ng/µl	1.334	-0.047
22	2027	67.2	ng/µl	1.343	-0.029
23	2031	37.9	ng/µl	0.757	-0.063
	Mean	*63.72*			

Table 6: Concentration of Nucleic Acid in eelgrass DNA Extracts

Table 6 contains data from the NanoDrop2000 spectrophotometer for DNA extract samples. Rows labeled 1-7 contain nucleic acid concentrations for Great Bay (GB) and Duck Harbor (DH) samples. Rows 1-8 contain nucleic acid concentrations for positive control samples (samples that amplified by PCR). The mean nucleic acid concentration values for each group are reported. A260 values are absorbance values at a wavelength of 260nm, the wavelength at which DNA absorbs light. A280 values represent absorbance values at wavelength of 280nm, or light absorbance by proteins in solution.

Agarose Gel Electrophoresis of PCR-Amplified DNA:

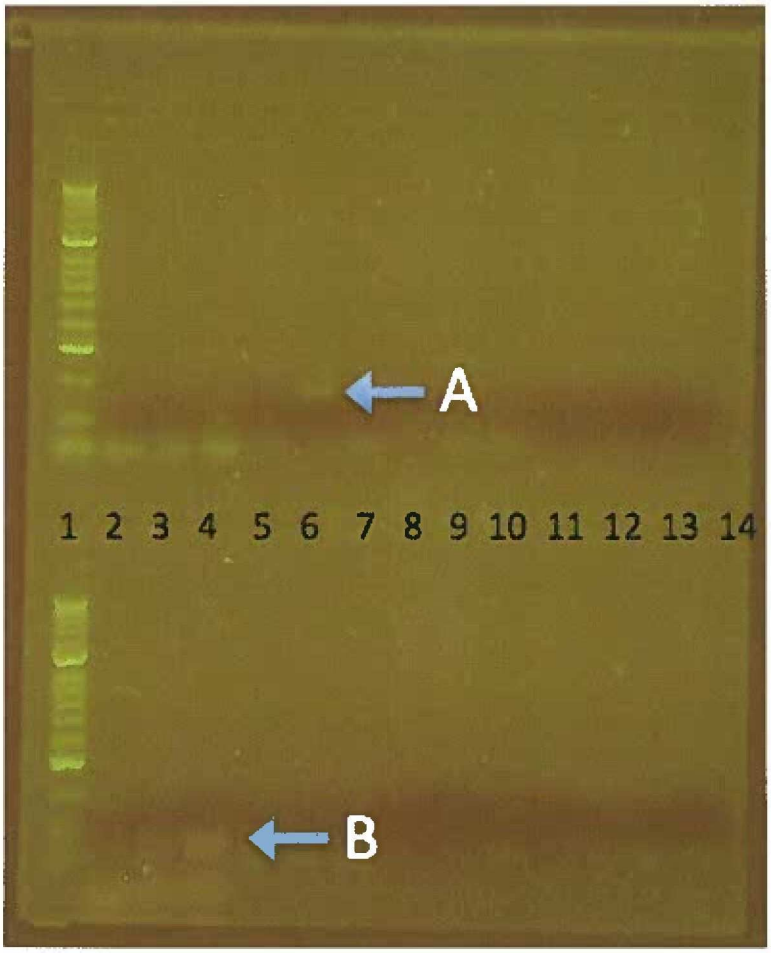

Figure 3: DNA Migration on 2% Agarose Gel

Figure 3 shows DNA migration on a 2% Agarose gel by electrophoresis for PCR-amplified DNA. Note: Amplification was erratic with relatively few lanes exhibiting bands of higher molecular size than the primer/dimers. "A" shows positive amplification (~200base bairs in size) for sample #2022 at locus GA16. "B" shows sample #2020 for loci GA2, GA12, and GA19 in well 2, 3, and 4, respectively. These bands may represent positive amplification (~90 base pairs in size). Further analysis is needed to confirm amplification. Tther wells show no evidence of amplification.

Chromatogram Fragment Analysis of PCR-Amplified DNA:

Fragment analysis for all samples PCR amplicons was performed on an ABI 3130 Automated Sequencer. This method separates amplified microsatellite fragments by size and uses a laser to detect fluorescent tags at each locus. The resulting chromatogram shows peaks that correspond to allele sizes at each locus measured. One peak represents a homozygous individual, whereas two peaks represent a heterozygote.

Chromatogram without fragment traces:

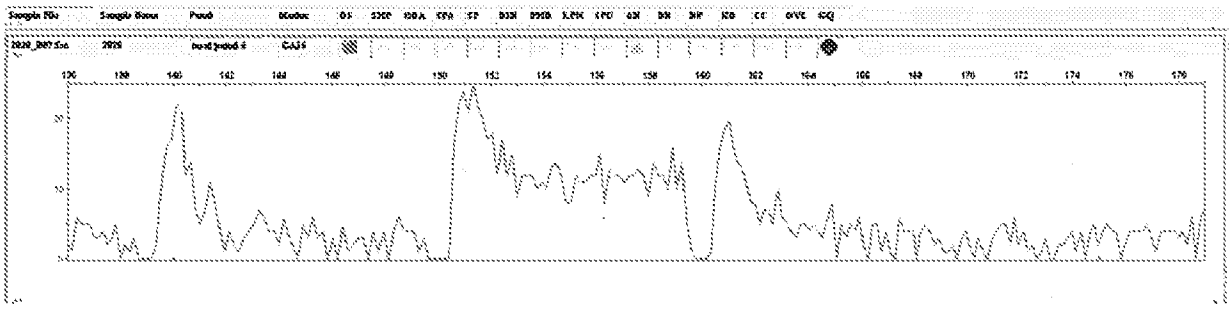

Figure 4: Fragment Size Chromatogram with no Peak Values

Figure 4 is a chromatogram created using GeneMapper™ from fragment size data obtained from the ABI 3130 Automated Sequencer at the Hubbard Genome Center for sample #2020 at locus GA16. The chromatogram shows no distinct peak values for fragment size, indicating improper amplification by PCR or faulty detection of fluorescent tags by the sequencer.

Chromatogram with fragment traces:

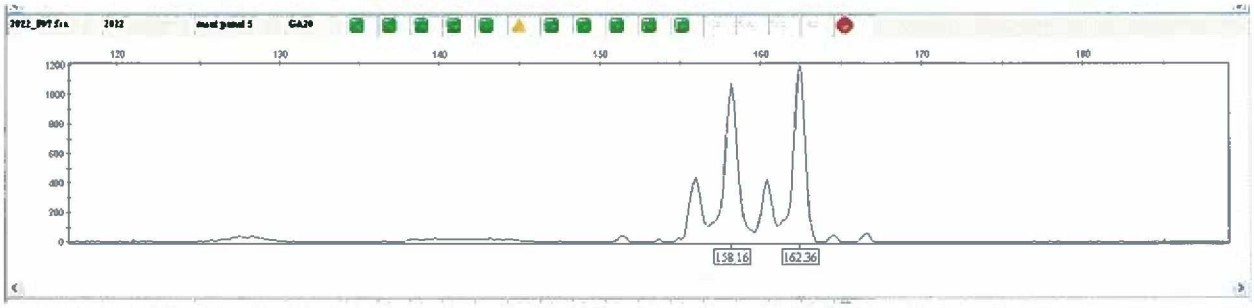

Figure 5: Fragment Size Chromatogram with Peak Values:

Sample #2022, locus GA20

Figure 5 is a chromatogram with two distinct peak values at 158 base pairs and 162 base pairs. The peaks correlate to fragment size of each allele at the locus measured. The locus in Figure 5 contains two peaks, indicating that the individual is heterozygous at the particular locus.

Master List of Scored Allele Sizes by Locus (separated by population):

POP	INDV	GA2	GA2	GA23	GA23	GA12	GA12	GA16	GA16	GA17D	GA17D	GA19	GA19	GA28	GA28
MA1	104	110	110	161	169	128	130	166	168	194	196	138	138	162	166
MA1	107	110	110	161	161	130	130	166	168	194	196	136	138	160	162
MA1	109	110	110	161	161	128	130	168	168	194	192	136	138	162	162
MA1	112	110	110	161	169	130	130	164	166	194	194	136	136	162	162
MA1	116	110	116	161	163	130	130	166	166	194	194	138	146	162	162
MA1	117	110	110	161	161	130	130	166	168	194	194	138	138	168	168
MA1	119	110	110	161	163	128	128	172	172	196	198	138	138	164	170
MA1	120	110	110	161	161	128	128	172	172	196	196	138	138	164	170
MA1	124	110	116	161	161	128	128	166	174	192	198	138	138	164	164
MA1	125	110	110	161	167	130	130	168	168	194	194	138	146	162	162
MA1	127	106	110	161	161	128	130	166	168	194	194	138	138	162	162
MA1	129	106	110	161	161	126	128	172	174	194	198	136	138	164	170
MA1	130	110	110	161	161	128	128	166	168	188	194	136	146	168	164
MA1	131	110	110	161	161	126	128	172	172	194	194	136	146	164	164
MA1	132	102	110	163	163	130	130	166	168	194	194	138	138	162	168
MA1	134	106	106	161	161	130	130	166	168	190	194	138	138	168	168
MA1	136	102	110	161	161	128	128	170	172	194	196	138	146	160	164
MA1	141	110	110	167	167	128	128	170	172	194	194	138	146	164	164
MA1	142	106	110	161	167	128	128	168	172	188	196	138	146	162	164
MA1	144	110	110	163	163	128	128	168	168	194	194	138	138	162	166
MA1	147	110	110	161	163	130	130	166	168	194	194	138	138	162	166
MA1	149	106	106	161	161	128	128	168	168	188	200	138	146	164	164
MA1	150	110	114	163	167	128	128	168	172	188	196	138	138	164	164

Table 7: Allele Sizes by locus for Population MA1 (Nantucket, MA). Values obtained by Alyssa Mixon from the Olsen and Coyer Lab.

POP	INDV	GA2	GA2	GA23	GA23	GA12	GA12	GA16	GA16	GA17D	GA17D	GA19	GA19	GA20	GA20
MA3	4054	112	112	161	169	130	130	168	168	196	196	138	138	162	162
MA3	4055	108	112	161	161	130	130	166	168	194	200	138	138	162	162
MA3	4057	110	112	159	169	130	130	168	168	190	198	138	146	162	168
MA3	4058	108	112	161	161	130	130	168	168	194	194	138	138	162	162
MA3	4059	108	112	161	161	130	130	168	168	194	204	138	138	162	162
MA3	4060	108	108	165	165	130	130	166	168	194	196	138	138	160	162
MA3	4061	112	112	163	163	130	130	166	168	194	194	138	138	160	162
MA3	4062	108	112	163	163	130	130	168	168	194	194	138	138	140	162
MA3	4063	108	112	161	161	130	130	168	168	196	196	138	138	160	162
MA3	4064	108	112	165	165	138	138	166	168	194	194	138	138	162	162
MA3	4065	108	112	163	163	130	130	166	168	196	196	138	138	162	164
MA3	4069	112	112	163	163	130	130	166	168	196	196	138	138	162	168
MA3	4071	108	108	165	165	130	130	166	168	196	200	138	138	162	162
MA3	4072	108	108	161	161	130	130	166	168	196	196	138	138	160	162
MA3	4073	112	112	161	161	130	130	166	166	194	194	138	138	162	162
MA3	4074	110	110	163	163	130	130	166	168	194	196	138	138	162	162
MA3	4076	112	112	159	163	130	130	168	168	192	194	138	138	160	162
MA3	4079	108	110	165	165	130	130	168	168	194	196	138	138	162	162
MA3	4080	110	110	171	171	130	130	166	166	194	194	138	138	162	162
MA3	4084	108	110	161	161	130	130	168	168	192	194	138	138	160	162
MA3	4085	108	108	159	163	130	130	168	168	194	196	138	138	160	162
MA3	4086	108	108	161	161	130	130	166	168	194	198	138	138	160	162
MA3	4087	108	108	163	163	130	130	166	168	194	194	138	138	162	162
MA3	4090	110	110	165	165	130	130	166	168	196	196	138	138	160	162
MA3	4091	108	114	161	161	130	130	166	168	194	194	138	138	162	162
MA3	4092	108	114	161	161	130	130	166	168	194	194	138	138	162	162
MA3	4094	108	114	165	165	130	130	164	166	194	198	138	138	162	164
MA3	4098	108	110	165	169	128	128	166	168	194	194	138	138	158	162
MA3	4097	110	110	159	169	128	128	166	168	194	198	138	138	160	162
MA3	4099	110	110	159	169	128	128	166	168	194	196	138	138	160	162
MA3	4100	108	108	161	161	130	130	168	168	194	194	138	138	162	162

Table 8: Allele Sizes by locus for Population MA3 (Falmouth, MA). Values obtained by Alyssa Mixon from the Olsen and Coyer Lab.

POP	INDV	GA2	GA2	GA23	GA23	GA12	GA12	GA16	GA16	GA17D	GA17D	GA19	GA19	GA20	GA20
NI	1	108	108	163	171	130	130	166	168	194	194	135	138	162	162
NI	5	108	114	167	169	128	130	166	168	194	196	138	146	158	162
NI	8	110	110	169	171	130	130	168	168	194	194	138	138	160	162
NI	14	110	112	167	169	130	134	168	170	192	194	146	146	160	162
NI	15	110	112	171	171	130	130	168	168	192	194	146	146	162	162
NI	17	110	112	169	171	128	130	168	172	192	192	146	146	162	162
NI	19	112	112	169	169	128	130	168	168	194	194	138	146	162	162
NI	21	110	112	167	171	130	130	168	168	192	198	146	146	160	162
NI	22	112	116	171	171	130	130	166	168	194	196	138	146	160	164
NI	23	110	112	169	170	130	130	168	168	194	196	146	146	162	168
NI	24	110	112	167	173	120	120	166	168	196	196	138	146	158	162
NI	27	110	112	167	169	130	130	156	168	194	194	146	146	160	160
NI	31	104	110	165	169	130	130	158	170	194	194	146	146	168	170
NI	33	108	112	167	171	128	130	168	166	196	196	138	146	162	164
NI	35	108	112	171	171	130	130	168	170	196	198	146	146	164	164
NI	36	110	118	173	175	130	130	164	168	196	196	138	148	162	164
NI	37	110	112	163	167	130	130	168	168	192	194	138	146	162	162
NI	38	110	110	169	175	130	130	168	168	194	194	138	138	158	162
NI	42	108	110	171	171	130	130	166	168	194	194	138	146	160	162
NI	43	120	124	171	171	130	130	152	168	196	196	138	146	160	162
NI	47	110	110	112	114	130	130	164	168	196	196	146	146	162	162
NI	49	110	112	169	169	130	130	168	168	194	196	146	146	162	168
NI	50a	110	110	167	171	130	130	166	172	196	196	138	146	162	164

Table 9: Allele Sizes by locus for Population NI (Nanny Island, NH). Values obtained by Alyssa Mixon from the Olsen and Coyer Lab.

POP	INDV	GA2	GA2	GA23	GA23	GA12	GA12	GA16	GA16	GA17D	GA17D	GA19	GA19	GA20	GA20
GB	5550	112	112	161	167	130	132	167	167	195	195	137	145	162	162
GB	5551	112	112	165	171	130	130	161	165	195	195	145	145	160	164
GB	5552	112	114	169	173	130	130	167	167	193	195	145	145	162	164
GB	5553	112	114	169	173	130	130	167	167	193	195	145	145	162	164
GB	5554	112	114	165	169	130	130	167	167	193	195	145	145	162	162
GB	5555	112	114	169	173	130	130	167	167	193	195	145	145	162	164
GB	5556	108	112	161	165	130	130	167	167	195	195	145	145	162	162
GB	5557	112	112	163	165	130	130	167	169	195	195	137	137	160	162
GB	5558	108	112	169	169	130	130	165	169	191	195	137	145	162	162
GB	5559	108	112	165	169	130	132	-9	-9	195	195	145	145	160	162
GB	5560	112	114	169	173	130	130	151	169	193	195	145	145	162	164
GB	5561	112	114	169	171	130	132	165	167	195	195	137	137	146	162
GB	5562	112	114	163	169	130	130	-9	-9	195	195	137	137	160	162
GB	5563	112	114	167	169	128	130	167	167	195	197	145	145	164	164
GB	5564	112	116	167	169	130	130	-9	-9	195	195	137	145	162	162
GB	5565	112	112	165	171	130	130	165	167	195	195	145	145	160	164
GB	5566	112	116	165	169	128	130	167	169	195	195	145	145	162	162
GB	5567	108	112	165	167	130	130	167	169	195	195	145	145	162	162
GB	5568	110	114	167	169	130	130	167	167	195	195	137	145	160	162
GB	5569	112	112	165	173	130	130	167	167	195	197	137	145	162	166
GB	5570	108	114	165	171	130	130	-9	-9	195	195	137	145	162	166
GB	5571	112	112	165	167	130	130	165	169	195	195	137	145	162	162
GB	5572	112	112	169	171	128	130	167	167	195	195	145	145	160	162
GB	5573	112	112	165	169	130	130	167	169	195	195	145	145	160	164
GB	5574	112	112	169	171	128	130	167	167	195	195	145	145	160	162
GB	5575	112	114	167	171	128	130	163	167	193	195	137	145	158	162
GB	5576	112	112	161	173	130	130	167	169	195	195	137	145	162	166
GB	5577	112	112	161	173	130	130	167	169	195	195	137	145	162	166
GB	5578	112	114	169	171	128	130	167	167	195	195	145	145	164	166
GB	5579	112	116	163	169	128	130	167	169	195	195	137	145	162	162
GB	5580	112	116	163	173	130	130	167	167	195	195	137	137	162	162
GB	5581	112	110	163	165	130	130	167	167	195	199	137	145	162	162
GB	5582	112	116	163	173	130	130	167	167	195	195	137	137	162	162
GB	5583	108	116	167	169	128	130	167	167	197	199	145	145	162	162
GB	5584	112	114	169	169	130	130	167	169	195	195	137	145	144	162
GB	5585	110	114	165	167	130	130	165	167	195	195	145	145	158	162
GB	5586	110	114	165	167	128	130	165	167	193	197	137	149	162	164
GB	5587	110	112	165	167	130	130	167	167	193	195	145	149	162	162
GB	5588	110	114	165	171	130	130	165	169	195	195	137	145	160	160
GB	5589	112	114	165	169	130	130	165	167	195	195	137	145	160	164
GB	5590	110	114	169	171	130	130	165	167	195	195	137	145	160	162
GB	5591	112	116	163	173	130	130	167	167	195	195	137	137	162	162
GB	5592	112	116	163	173	130	130	167	167	195	195	137	137	162	162
GB	5593	112	116	167	169	130	130	167	167	195	195	137	145	162	162
GB	5594	108	114	169	171	128	130	167	169	195	195	137	145	160	166
GB	5595	104	112	163	167	130	130	167	167	195	199	137	145	162	162
GB	5596	112	114	165	167	128	130	167	169	193	196	137	145	162	164
GB	5597	112	112	163	171	130	130	167	171	195	195	145	145	162	162
GB	5598	104	112	163	167	130	130	167	167	195	199	137	145	162	162
GB	5599	110	114	169	171	130	130	165	167	195	195	137	145	160	162

Table 10: Allele Sizes by locus for Population GB (Great Bay). Values obtained by Sarah Weigel from the Klein Lab.

POP	INDV	GA2	GA2	GA23	GA23	GA12	GA12	GA16	GA16	GA17D	GA17D	GA19	GA19	GA20	GA20
LH	5600	112	114	161	165	130	130	167	167	195	197	137	137	152	162
LH	5601	108	118	159	165	130	130	165	167	195	195	137	145	162	162
LH	5602	110	118	163	169	130	130	167	167	195	197	137	145	154	162
LH	5603	108	112	163	165	130	130	167	167	195	195	137	145	162	162
LH	5604	108	118	159	165	130	130	165	167	195	195	137	148	162	162
LH	5605	108	118	159	165	130	130	165	167	195	195	137	145	162	162
LH	5606	112	118	165	165	130	130	167	167	195	197	145	145	162	162
LH	5607	110	112	171	175	130	132	167	167	195	197	145	145	162	164
LH	5608	112	114	161	165	130	130	167	167	195	197	137	137	152	162
LH	5609	112	114	161	165	130	130	167	167	195	197	137	137	152	162
LH	5610	108	118	159	165	130	130	167	167	195	195	137	145	162	162
LH	5611	112	114	161	165	130	130	167	167	195	197	137	137	152	162
LH	5612	112	114	161	165	130	130	167	167	195	197	137	137	152	162
LH	5613	110	118	163	165	130	130	167	167	195	195	137	137	164	166
LH	5614	112	118	163	165	130	130	167	167	195	195	137	145	164	164
LH	5615	112	118	163	163	130	130	163	167	195	195	137	145	164	164
LH	5616	108	118	159	165	130	130	165	167	195	195	137	145	162	162
LH	5617	112	114	161	165	130	130	167	167	195	197	137	137	152	162
LH	5618	112	118	163	165	130	130	167	167	195	195	137	145	164	164
LH	5619	112	118	163	165	130	130	167	167	195	195	137	145	164	164
LH	5620	112	114	161	165	130	130	167	167	195	197	137	137	152	162
LH	5621	108	112	165	165	130	130	167	171	195	195	137	145	168	164
LH	5622	112	118	163	165	130	130	167	167	195	195	137	145	164	164
LH	5623	112	114	161	165	130	130	167	167	195	197	137	137	152	162
LH	5624	110	118	163	165	130	130	167	167	195	195	137	137	164	166
LH	5625	112	114	161	165	130	130	167	167	195	197	137	137	152	162
LH	5626	112	114	161	165	130	130	167	167	195	197	137	137	152	162
LH	5627	112	114	161	165	130	130	167	167	195	197	137	137	152	162
LH	5628	112	112	163	171	130	130	167	167	195	195	145	145	162	162
LH	5629	112	112	163	171	130	130	167	167	195	195	145	145	162	162
LH	5630	110	118	163	165	130	130	167	167	195	195	137	137	164	166
LH	5631	112	112	161	165	130	130	167	167	195	197	137	137	152	162
LH	5632	112	114	161	165	130	130	167	167	195	197	137	137	152	162
LH	5633	112	118	163	165	130	130	163	167	195	195	137	145	164	164
LH	5634	108	118	159	165	130	130	165	167	195	195	137	145	162	162
LH	5635	112	114	161	165	130	130	167	167	195	197	137	137	152	162
LH	5636	112	112	163	171	130	130	167	167	195	195	145	145	162	162
LH	5637	112	118	163	165	130	130	167	167	195	195	137	145	164	164
LH	5638	112	118	165	165	130	130	167	167	195	195	137	137	164	164
LH	5639	112	118	165	165	130	130	167	167	195	195	137	137	164	164
LH	5640	112	118	165	165	130	130	167	167	195	195	137	137	164	164
LH	5641	108	112	168	175	130	132	165	167	195	197	137	145	162	166
LH	5642	108	112	165	168	130	130	167	171	195	195	137	145	168	164
LH	5643	108	118	160	165	130	130	167	167	195	195	137	145	162	162
LH	5644	108	118	159	165	130	130	165	167	195	195	137	145	163	163
LH	5645	110	112	165	169	130	130	165	167	195	195	137	145	161	163
LH	5646	108	118	159	165	130	130	165	167	195	195	137	145	163	163
LH	5647	108	112	165	165	130	130	167	171	195	195	137	145	168	165
LH	5648	112	114	161	165	130	130	167	167	195	197	137	137	162	163

Table 11: Allele Sizes by locus for Population LH (Little Harbor, NH). Values obtained by Sarah Weigel from the Klein Lab.

GenClone™ Analysis of Genetic Diversity by Population:

Population	n (number of individuals)	g (genets)	R (genotypic richness)
MA1	23	23	1.00
MA3	31	29	0.93
NI	23	23	1.00
LH	49	21	0.42
GB	50	41	0.82

R = (g-1)/(n-1), where g = # genets and n = # individuals

Table 12: Genotypic and Allelic Richness of Populations

Table 12 shows values of genotypic and allelic richness for the five populations analyzed. Number of genets refers to the number of genetic individuals in a population. Genotypic richness is a function of the number of genets per population size. Allelic richness indicates the mean number of alleles per locus for each population.

GDA™ Genetic Data Analysis:

GENETIC DIVERSITY BY LOCUS				
LOCUS	A	He	Ho	f
GA2	11	0.78	0.64	0.18
GA23	12	0.85	0.61	0.28
GA12	7	0.34	0.18	0.49
GA16	15	0.78	0.56	0.28
GA17D	15	0.75	0.39	0.48
GA19	7	0.73	0.4	0.45
GA20	17	0.67	0.58	0.13
MEAN	12	0.7	0.48	0.31

Table 13: Genetic Diversity at Loci Across all Five Populations

Table 13 lists values for genetic diversity at each locus, averaged over all five populations measured. Allelic Diversity (A) is the mean number of alleles per locus over seven loci. H_e is the expected heterozygosity at each locus; H_o is the observed heterozygosity for each locus.

GENETIC DIVERSITY BY POPULATION				
POPULATION	A	He	Ho	f
MA1	5.43	0.58	0.45	0.22
MA3	4.71	0.47	0.32	0.32
NI	6.14	0.59	0.53	0.09
GB	6.14	0.54	0.57	-0.07
LH	4.57	0.49	0.5	-0.01
MEAN	5.4	0.53	0.48	0.11

Table 14: Genetic Diversity Within Populations Across all Seven Loci

Table 14 lists values for allelic richness, expected heterozygosity (H_e), averaged over seven loci, and observed heterozygosity (H_o) averaged heterozygosisty each population, averaged over all seven loci Allelic Diversity (A) is the mean number of alleles per locus. H_e is the expected heterozygosity at each locus; H_o is the observed heterozygosity for each locus. (f) represents F_{IS} values for each population, a measure of inbreeding. The inbreeding coefficient is a function of the difference between H_e and H_o. . $F_{IS} = 0$ indicates no inbreeding within a population, whereas F_{IS}= 1 indicates full inbreeding.

PRIVATE ALLELES	
LOCUS	POPULATIONS WITH PRIVATE ALLELES
GA2	MA1, NI
GA23	NI
GA12	MA3, NI
GA16	GB, MA1, NI
GA17D	GB, MA1, MA3
GA19	GB, MA1, NI
GA20	GB, LH, MA3

Table 16: Private Alleles Among Seven Loci

Private alleles, as in Table 16, represent unique alleles found at each of the seven loci for certain populations. Every population had at least one private allele at one or more locus.

FSTAT™ Statistical Analysis of Population Relatedness:

FST VALUES=RELATEDNESS BETWEEN POPULATIONS										
	MA1		MA3		NI		GB		LH	
MA1	0.000		0.164	*	0.185	*	0.374	*	0.399	*
MA3	0.164	*	0.000		0.149	*	0.353	*	0.386	*
NI	0.185	*	0.149	*	0.000		0.294	*	0.338	*
GB	0.374	*	0.353	*	0.294	*	0.000		0.061	*
LH	0.399	*	0.386	*	0.338	*	0.061	*	0.000	

Table 15: F_{ST} Values Compared Between Five Populations

The F_{ST} statistic is a value given to estimate the genetic differentiation between two populations. F_{ST} values can be determined in Table 15 by comparing a population in the first column to another population in the first row. (Note: $F_{ST} = 0$ for comparison of the same population).

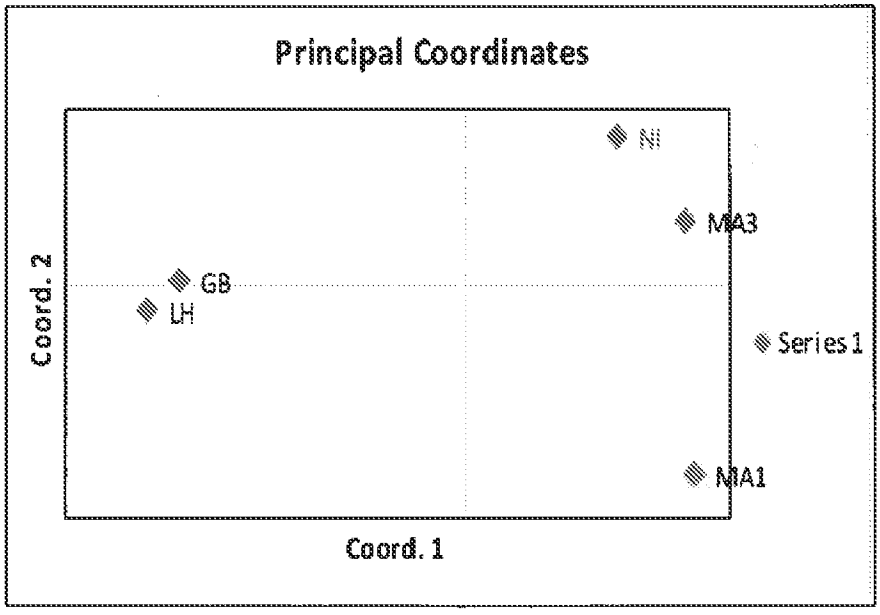

Figure 6: Principal Coordinates Graph for Five Populations

Figure 6 is a visual representation of relatedness between the five populations, as determined by principal coordinate analysis of all pairwise F_{ST} values (see Table 15).

Discussion/Conclusion

The research project proposed at the beginning of the 2011-2012 academic year was An Analysis of Genetic Resilience and Diversity Among Eelgrass (*Zostera marina*) Populations in the New England Coastal Region. This project was designed as an investigation to probe the genetic factors influencing genetic population structure for three populations: Two populations from Great Bay in New Hampshire and one population from Duck Harbor in Wellfleet, MA. The project was meant to serve as a compliment to a larger project sponsored by the The Nature Conservancy to study the genetic makeup of Eelgrass populations in Northeastern coastal and estuarine areas. The purpose of this study was to develop an understanding of the populations' genetic structures to aid in restoration management of the declining species.

Eelgrass samples from Great Bay and Duck Harbor were analyzed using the methods of DNA isolation, amplification and fragment analysis listed above, but very few results were produced for unknown reasons. A major portion of the project was devoted to investigations of various components of the DNA processing, including the DNA extraction to PCR amplification of the microsatellite loci.

The CTAB DNA extraction was the first method to undergo troubleshooting. Tissue samples were pulverized using a mixer-mill, and obtaining a powdery grind was essential to effective extraction of DNA from the samples. It was found that to obtain the optimal grind, 1.2mL microelution tubes by USA Scientific™ with $3/16^{th}$ inch ball bearings worked best. The tissue samples were also frozen at -80°C for two days, which caused the brittle samples to break

more easily. New solvents and solution used in the extraction were made to avoid use of contaminated materials. Different amounts of leaf tissue were used in an attempt to obtain a more concentrated DNA extraction. The NanoDrop2000 was used to evaluate the efficacy of the extractions (see Table 6). It was found that the average DNA concentration for Great Bay and Duck Harbor extracts was almost exactly equal to the DNA concentration for positive controls (samples that had positive results for fragment analysis). However, the NanoDrop spectrophotometer does not give information about the quality or type of DNA present in the solution.

Troubleshooting PCR amplification of extracted DNA was another labor and time-intensive portion of the project. Various attempts to troubleshoot the PCR reaction took place throughout the year. The Polymerase Chain Reaction is sensitive to many chemical and physical factors, and it was difficult to pinpoint problem areas. Different types of DNA samples were amplified, including positive and negative controls for comparison to Great Bay and Duck Harbor samples. The PCR reactions were repeated continuously, each time with a change in a parameter of the reaction. First, different concentrations of genomic DNA were used. Three different types of Taq polymerases were used (HotMaster, Qiagen HotStar™, Bioline). Higher fidelity was found with Bioline Taq. New forward and reverse DNA primers were used. To keep the PCR reagents cool, cold blocks were used instead of shaved ice during the PCR preparation. Prior to addition to the PCR master mix, genomic DNA samples were mixed on a gyrating platform for 20 minutes to ensure that all DNA was mixed into solution. When all DNA samples were added to PCR tubes, they were centrifuged at 350rpm for five seconds to ensure all droplets containing DNA were into master mix solution. The reactions were also carried out in different thermal cycler machines with various reaction conditions such as temperatures and cycle

numbers. Agarose gel electrophoresis of PCR-amplified samples was often used as a method to determine if the PCR reaction succeeded in proper amplification of samples. Strong bands for DNA amplicons were rarely found after electrophoresis, indicating in most cases that the amplification was unsuccessful or did not go to completion. Figure 3 shows a 2% agarose gel with bands, although it is difficult to determine if the bands shown are the desired PCR product or primer dimers.

To avoid false negative results from slab electrophoresis, PCR products were sent to the Hubbard Genome Center for fragment analysis. Results obtained for PCR amplicons were very sparse. Chromatograms rarely showed evidence of fragments. Fragment analysis of samples is imperative for genetic analysis. Without fragment analysis, inferences cannot be made about individual or population genetics. As a result of consistent negative fragment analysis results, the two Great Bay populations and the Duck Harbor population could not be used for genetic analysis, due to insufficient genetic data.

Although the fragment sizes could not be determined for Great Bay and Duck Harbor populations originally used for the project, fragment size data was available for other populations in the New England coastal region, including populations from Great Bay (GB), Little Harbor (LH) and Nanny Island (NI) in New Hampshire, and populations from Falmouth (MA1) and Nantucket (MA3) in Massachusetts. These populations serve well as replacement populations for genetic analysis, because they are similar geographically to the populations originally studied. Populations MA1, MA3, and NI were all analyzed for fragment sizes on an ABI Automated Sequencer at the University of New Hampshire by Sarah Weigel in the Klein lab, and GB and LH population samples were analyzed on an ABI Automated Sequencer in the Olsen and Coyer Lab at the University of Groningen in The Netherlands by Alyssa Mixon.

Genetic analysis was performed using various software programs previously described, which processed fragment size data (i.e., which samples were homozygous vs. heterozygous at particular loci) using statistical algorithms. The results obtained, found in Table 12 – Table 16 give information about the genetic diversity within and between populations for a set of loci. Table 12 shows the number of genets, or genetically unique individuals, within a population. When the number of genets is normalized for population size, genotypic richness can be determined for each population. GB and NI populations had a genotypic richness of 1, where all of the individuals sampled in these population are genetically unique. Little Harbor showed the lowest genotypic richness, with R = 0.42, indicating that more than half of the population is composed of multiple ramets, belonging to the same genets.

Frequency of inbreeding within populations was determined by comparing the Expected Heterozygosity (H_e) to the Observed Heterozygosity (H_O). An observed heterozygosity that is much smaller than expected heterozygosity can indicate a degree of inbreeding within a population, a value given by the statistic F_{IS} (labeled as 'f' in Table 14). A high F_{IS} value can indicate a high degree of inbreeding within a population. Populations MA1 and MA3 showed relatively high values for F_{IS} in comparison to other populations, indicating a moderate level of inbreeding within MA1 and MA3 populations. GB and LH showed very low values of F_{IS}, indicating very small amounts of inbreeding within these populations. Allelic diversity, indicating the mean number of alleles per locus, was also calculated for each population. The mean allelic richness for all populations was 5.4 alleles, and all populations had allelic richness values close to the mean. F_{IS} values were also determined by locus (Table 13). Because the populations were differentiated, the relatively large F_{IS} values seen per locus in Table 13 indicates subpopulation structuring.

Private alleles are those that are found to be unique to a certain population. As seen in Table 16, all five populations tested had unique alleles at one or more locus. Nanny Island had private alleles for five loci and Great Bay for four loci, whereas Little Harbor contained a private allele for only one locus. Private alleles indicate novel genetic diversity in population.

F_{IS}, as previously described is a measure of inbreeding within populations. Another F-statistic value used to measure genetic variation is F_{ST}. F_{ST} values indicate degree of genotypic differentiation between populations. F_{ST} values range from 0 to 1, where $F_{ST} = 1$ indicates that compared populations are fully separated and $F_{ST} = 0$ indicates that compared populations are identical. $F_{ST} > 0.2$ indicates strong separation between populations. Table 15 lists F_{ST} values for all of the five populations analyzed. The table shows that both MA1 and MA3 are strongly separated from GB and LH. This finding is expected because there is a large geographical gap between these New Hampshire and Massachusetts populations. NI however showed higher relatedness to both MA1 and MA3 than it did to GB or LH. This result was unexpected because of the geographical proximity of NI to both LH and GB populations. As previously mentioned, NI, MA1 and MA3 were all analyzed with an automated sequencer at the University of New Hampshire, whereas GB and LH were analyzed in The Netherlands. This unexpected result indicated a need for further testing to confirm that the degree of differentiation between NI and other New Hampshire populations was not a result of differences between the two sequencing machines used. The Principal Coordinates graph (Figure 6) shows F_{ST} relationships between populations spatially. As expected, GB and LH populations are very closely related to each other. MA1, MA3 and NI show somewhat less population structuring.

The five populations analyzed for genetic analysis all showed some degree of genetic and allelic diversity. Although MA1 and MA3 showed high levels of inbreeding as determined by

F_{IS}, it was determined that each of these populations harbor three private alleles, which offer novel genetic diversity to the populations. Little Harbor, Great Bay and Nanny Island all showed low degrees of inbreeding within the populations, which can also indicate genetic diversity. All populations besides LH showed high genotypic richness, indicating that the number of ramets within these populations is low, which keeps genetic variation high.

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