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Population Structure of *Zostera marina* (Eelgrass) on the Western Atlantic Coast Is Characterized by Poor Connectivity and Inbreeding

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Abstract

Zostera marina (eelgrass) can be found in the North Atlantic on the coast of Europe and on the east and west coasts of North America. Over the last 30 years, this once robust species has been reduced to sparse patchy populations due to disease and anthropogenic effects. In order to better understand the consequences of this devastation on the population genetics of the species, we have analyzed the population structure of western Atlantic *Z. marina*, employing microsatellite DNA polymorphisms. Although high fixation index values suggest moderate genetic differentiation among most of the *Z. marina* sites, population diversity was low. This lack of diversity was supported by a general dearth of observable heterozygotes in these sites; mean observed heterozygosity values (0.14–0.46) were lower than the mean expected heterozygosity values (0.57–0.81). Additionally, the mean F_{IS} (coefficient of local inbreeding) values in these sites were positive, again indicating a surfeit of homozygotes. Allelic richness suggests that Chesapeake Bay has the greatest internal genetic diversity of the sites studied. Inbreeding seems prevalent in these American populations, suggesting possible reproductive fitness problems in the future. There is evidence of demographic bottlenecking and particularly low genetic diversity in Long Island. Northern Maine had the highest effective population size, suggesting a possible use in future restoration projects.

Key words: eelgrass, genetic diversity, microsatellites, population genetics, *Zostera marina*

Introduction

Zostera marina is the most common eelgrass species in the temperate coastal areas (Homziak et al. 1982) of Western Europe, eastern North America ranging from Arctic Canada to North Carolina (Setchell 1935), and as far south as the Baja Peninsula on the North American West Coast. The eelgrass beds serve as wildlife habitats and provide stability for coastal systems through reduction in water velocity, increased wave attenuation, and stabilization of sediments (Fonseca and Fisher 1986; Almasi et al. 1987). *Zostera marina* is also highly sensitive to environmental alterations, and negative changes in the vitality and distribution of these vascular plant beds generally signal a decline in water quality.

Many *Z. marina* populations have experienced diebacks due to a wide variety of environmental and genetic stresses. Populations of *Z. marina* around the world experienced catastrophic losses of beds in the 1930s due to eelgrass wasting disease (Rasmussen 1977). *Zostera marina* meadows along the Atlantic coasts of North America and Europe were decimated during this period with more than a 90% loss of area (Muehlstein 1989).

In North America, *Z. marina* in Barnegat Bay, NJ, over the last 25 years has experienced a 62% loss in bed coverage (Bologna et al. 2000). In the 1980s, *Z. marina* in the tributaries of Chesapeake Bay fell victim to a combination of wasting disease (Orth and Moore 1983) and nutrient loading (Short et al. 1986). In North Carolina, the *Z. marina* has been steadily declining due to nutrient loading from brown tides that increases the turbidity of the water and starves the plants of light. Long Island *Z. marina* beds have also been affected by brown tides and the shellfish industry (Dzurica et al. 1989; Vaudrey and Getchis 2006). In Waquoit Bay, MA, nitrogen loading, due to microalgal canopies, has inhibited the growth

of eelgrass (Hauxwell et al. 2001). Finally, throughout the southern Maine coastline, wasting disease has continued to cause multiple diebacks over the last century, the most recent in the mid-1980s (Short et al. 1986).

Localized west Atlantic *Z. marina* genetics have been examined in the past (Williams and Orth 1998; Reusch et al. 1999; Reusch et al. 2000; Olsen et al. 2004; Rhode and Duffy 2004; Campanella et al. 2009) to determine gene diversity, but no comprehensive studies have been performed specifically probing sites from the southernmost habitat of *Z. marina* in North Carolina northward. The primary goal of this present study was to survey the genetic patterns of the species along that coast.

Additionally, we have long-term interest in improving restoration practices for *Z. marina* along the western Atlantic coast. It has been proposed in other restoration efforts that differences in survival rates may be linked to the genetics and stock origin of the plants. Williams and Orth (1998) presented evidence that transplants retain donor stock genetic identity, which may have limited the success of prior efforts through reduced genetic diversity. Williams (2001) demonstrated that elevated genetic diversity increased transplant survival, and Hughes and Stachowicz (2004) showed that genetic diversity can increase resistance of *Z. marina* populations to physical disturbance. As such, our secondary research objectives for this project were to assess the population genetic structure of *Z. marina* along the western Atlantic coast and determine how genetic diversity and effective population size may impact future restoration activities.

Materials and Methods

Plant Collection

Twenty individual *Z. marina* plants were collected at 9 sites along the western Atlantic coast. These 9 sites included Barnegat Bay Inlet (39°46′N, 74°08′W); Peconic Bay of Long Island (40°56′N, 72°26′W; Brad Peterson, State University of New York, Southampton, New York); Chesapeake Bay (37°13′N, 76°23′W; Ken Moore, Virginia Institute of Marine Science, Virginia); Northern Maine and Southern Maine (44°38′N, 67°20′W, and 43°4′N, 70°33′W, respectively; Bob Steneck and Chris Rigard, University of Maine); Wells, Maine (43°19′N, 70°33′W; James Dochtermann, Wells National Estuarine Research Reserve, Massachusetts); Waquoit, MA (41°34′N, 70°31′W; Chris Weidmann, Waquoit National Estuarine Research Reserve, Massachusetts); Bogue Sound, North Carolina (35°54′N, 75°48′W; Mark Fonseca, National Oceanographic and Atmospheric Administration, North Carolina); Egg Harbor, Alaska (59°38′N, 151°32′W; Rick Foster, Kachemak Bay Research Reserve, Alaska). To ensure that we were not gathering clonal samples, all plants were collected 5 m apart within eelgrass beds. Samples were collected at equal intervals across each of the locations. The 5-m collection separation was assumed to be acceptable because it exceeded the intervals used in other studies. Reusch et al. (1999) collected samples at approximately 3-m intervals, Olsen et al. (2004) samples were collected 1–1.5 m apart, and Coyer et al. (2008) collected at 1- to 2-m separations. Tissue samples were shipped or transported on ice to Montclair State University from all locations. Samples were then separated, numerically labeled, and stored at −80 °C until DNA extraction.

DNA Extraction and Microsatellite Amplification

Total DNA was extracted from 0.3–0.5 g of *Z. marina* leaf tissue, using the DNeasy DNA extraction kit according to the manufacturer's directions (Qiagen Corporation, Valencia, CA). DNA was extracted from 20 individuals within each site. DNA concentration was determined by UV absorbance on a Nanodrop ND-1000 UV spectrophotometer (Nanodrop Technologies, Wilmington, Delaware), and samples were stored at −80 °C until polymerase chain reaction (PCR) amplification was performed.

PCR was used to amplify 7 microsatellite loci from the extracted *Z. marina* DNA. Primers for these 7 amplified loci were developed by Reusch et al. (1999): ZosmarGA2 (AJ009900), ZosmarGA3 (AJ009901), ZosmarCT3 (AJ009898), ZosmarCT12 (AJ249303), ZosmarCT17 (AJ249307), ZosmarCT19 (AJ249304), and ZosmarCT20 (AJ249306). Primers were fluorescently labeled with either Carboxyfluorescein (FAM) or 6 - carboxy - 2′,4, 4′,5′,7, 7′ -Hexachlorofluorescein (HEX) dyes (Invitrogen Corp, Carlsbad, CA).

Reactions were carried out using 10 ng DNA in RNase-free/DNase-free 0.2-μl tubes with 15–30 nmoles labeled primers. Reaction mixes were all kept at 4 °C until 10 μl of Choice Taq Mastermix DNA Polymerase (Denville Scientific, Inc, Denville, NJ) was added. Amplification was performed in a Mastercycler gradient thermocycler (Eppendorf, Inc, Hamburg, Germany). The PCR program employed consisted of a 1-min denaturing step at 95 °C, followed by 30 cycles of the following times and temperatures: 15 s at 95 °C, 15 s at 55 °C, and 30 s at 72 °C. Amplified PCR products were then stored at −20 °C until later analysis.

Microsatellite Allele Size Analysis

Allele sizes of microsatellite PCR products were determined using an ABI Prism 310 DNA Sequencer (Applied Biosystems Corp, Foster City, CA). The PCR products were diluted 1:10 with sterile water. Diluted product (0.5 μl) was added to an aliquot of 30 μl of formamide and 0.5 μl of the molecular weight standard ROX 500 (Applied Biosystems Corp). Samples were analyzed for allele sizes on the sequencer for 30 min using POP4 polymer (Applied Biosystems Corp) and the D Filter setting. GeneMarker v1.51 software (SoftGenetics Corp, State College, PA) was used to evaluate the microsatellite allele sizes from raw data and score alleles for homo/heterozygosity.

Statistical Analysis of Data

Clonal diversity (*C*) was calculated by the method of Olsen et al. (2004) and expressed as a function of the number of ramets collected and the number of genets detected based

on all 7 loci employed. The spatial scale of the collection was always 5 m or greater between samples. Mean allelic richness (AR) was estimated using Rarefac (Petit et al. 1998). The sizes of the adjusted populations ranged from 11 to 20 genets with an average adjustment to 15.1 genets.

Mean estimated gene flow values (N_m), F statistics, as well as observed heterozygosity (H_{obs}) and expected heterozygosity (H_{exp}) were calculated with POPGENE32 under the codominant marker settings (Yeh and Boyle 1997). The isolation by distance (IBD) analysis was performed using the isolation by distance online web analysis program V3.15 (Jensen et al. 2005). The IBD program performs a Mantel test to evaluate the relationships between the various analyzed sites and provides a reduced major axis regression analysis on the graphic output. Effective population sizes (N_e) were calculated based on linkage disequilibrium by NeEstimator (Peel et al. 2004). Hill (1981) demonstrated that for neutral loci unlinked with selected loci in a randomly mating isolated population, linkage disequilibrium would come exclusively from genetic drift and could be used to estimate N_e. The program BOTTLENECK was used to estimate likelihood of population bottlenecks (Piry et al. 1999). Finally, effective population sizes (N_e) were calculated based on linkage disequilibrium by NeEstimator. Principal coordinate analyses (PCoAs) were performed using Microsat genetic distance data in GENALEX6 (Peakall and Smouse 2006). The matrix containing the allele sizes was calculated using chord distances employed for the neighbor-joining tree. Program parameters were set to employ a triangular distance matrix, covariance standard, and included data labels. GENALEX6 was also employed to generate the F_{ST} (Fixation Index) pairwise comparison for each population. FreeNA (Chapuis and Estoup 2007) was used to analyze and correct the data set for putative null alleles.

Results

Allele Frequency Data and Diversity

We sampled 180 ramets from the western Atlantic coast along with the geographic out-group from Alaska and performed analyses with 7 microsatellite loci revealing a total of 136 genets collected (Table 1). Of the sites studied, North Carolina appeared to have the highest clonal diversity (C = 1.00) and Long Island the lowest (C = 0.55) (Table 1).

Chesapeake Bay had the highest AR of the other sites with a value of 9.5 (Table 1). The Chesapeake Bay value probably reflects long residence as well as pronounced outcrossing with other western Atlantic sites. Alaska also has a high AR (7.6). The lowest mean was found in our northernmost sample Northern Maine (4.50). The mean AR of all the sites analyzed was 6.3 that is a bit higher than Olsen et al (2004) found in Canadian sites (ca. 4) along the Atlantic coast. The total number of alleles per locus ranged from 2 to 17 (Table 2). Across all sites, the *GA2* allele had the largest mean number of alleles (8.2), whereas the *CT19* allele had the fewest (4.4). Across all loci, the Chesapeake Bay site had the largest mean number of alleles (10.8) and Northern Maine the lowest (4.5). We can conclude that all the populations are genetically diverse at the allelic level based on the AR found there.

On average, the expected number of heterozygotes (H_{exp}) was consistently higher for each allele than the observed number of heterozygotes (H_{obs}) (Table 2). This result suggests that in general the sites are outcrossing very little. There were few exceptions to this observation. The Alaskan out-group demonstrated higher values of H_{obs} for *GA3* (0.95) and *CT19* (0.90) than H_{exp}. The Waquoit site showed a similar trend with *CT19* (H_{obs} = 0.25). The mean H_{obs} (0.46) for Alaska was the highest for any of the sites. Southern Maine had the lowest average H_{obs} values at 0.14. Although all the western Atlantic sites demonstrated loci that were completely homozygous with H_{obs} values of 0, the only site to manifest loci that were monomorphic was Southern Maine. Because the *CT12* and *CT19* loci in Southern Maine were monomorphically homozygous, no H_{exp} or F_{IS} (coefficient of local inbreeding) values could be calculated.

We calculated the coefficient of local inbreeding (F_{IS}) (Weir and Cockerham 1984) in order to further examine the hypothesis that there is little outcrossing among these sites (Table 2). At all loci and in all sites (except for *GA3* and *CT19* in Alaska, Waquoit, and Northern Maine), it was found that the calculated F_{IS}' was positive, indicating an excess of homozygotes at the sites. This result supports low levels of diversity in the sites studied. The Long Island site seems to be particularly affected with a lack

Table 1. Clonal diversity and AR in the *Zostera marina* populations studied

Population	No. of ramets	No. of genets	C	Mean AR	Location
Alaska	20	19	0.95	7.63	59°38′N, 151°32′W
Northern Maine	20	14	0.70	4.50	44°38′N, 67°20′W
Southern Maine	20	12	0.60	5.42	43°4′N, 70°33′W
Wells, Maine	20	17	0.85	6.35	43°19′N, 70°33′W
Waquoit, MA	20	12	0.60	5.00	41°34′N, 70°31′W
Long Island	20	11	0.55	6.70	40°56′N, 72°26′W
Barnegat Bay Inlet, NJ	20	14	0.70	6.59	39°46′N, 74°08′W
Chesapeake Bay	20	17	0.85	9.57	37°13′N, 76°23′W
North Carolina	20	20	1.00	5.18	35°54′N, 75°48′W

Clonal diversity (C) was determined as the ratio of the number of genets detected to the number of ramets sampled, based on all of the loci employed.

Table 2. Within-population genetic diversity in all the population of eelgrass examined in this study

Populations	*GA2*	*GA3*	*CT3*	*CT12*	*CT17*	*CT19*	*CT20*	Mean
Alaska								
a	13	17	8	7	3	12	4	9.1
H_{obs}	0.50	0.95	0.15	0.05	0.40	0.90	0.30	0.46
H_{exp}	0.92	0.92	0.71	0.14	0.47	0.89	0.63	0.67
F_{IS}	0.49	**−0.02**	0.78	0.66	**−0.01**	**0.003**	0.47	0.30
F_{ST}	0.04	0.02	0.19	0.57	0.81	−0.06	0.26	0.26
Northern Maine								
a	3	2	6	5	9	2	5	4.57
H_{obs}	0.00	0.28	0.57	0.00	0.42	0.42	0.21	0.27
H_{exp}	0.45	0.25	0.80	0.69	0.86	0.50	0.48	0.57
F_{IS}	1.00	**−0.13**	0.29	1.00	0.51	0.16	0.56	0.48
F_{ST}	0.52	0.72	0.08	0.22	0.11	0.44	0.49	0.36
Southern Maine								
a	6	5	7	1	13	1	5	5.42
H_{obs}	0.25	0.00	0.25	0.00	0.50	0.00	0.00	0.14
H_{exp}	0.69	0.72	0.85	**NT**	0.95	**NT**	0.75	0.79
F_{IS}	0.65	1.00	0.71	**NT**	0.48	**NT**	1.00	0.77
F_{ST}	0.27	0.22	0.02	1.00	0.03	1.00	0.22	0.39
Wells, Maine								
a	7	9	5	10	5	5	7	6.85
H_{obs}	0.00	0.29	0.23	0.52	0.23	0.05	0.52	0.26
H_{exp}	0.77	0.81	0.62	0.87	0.72	0.66	0.83	0.75
F_{IS}	1.00	0.64	0.63	0.40	0.68	0.91	0.37	0.66
F_{ST}	0.19	0.11	0.27	0.02	0.25	0.25	0.13	0.17
Waquoit, MA								
a	6	3	6	4	7	3	6	5.00
H_{obs}	0.16	0.00	0.33	0.00	0.50	0.25	0.00	0.17
H_{exp}	0.78	0.63	0.68	0.43	0.83	0.23	0.78	0.62
F_{IS}	0.79	1.00	0.52	1.00	0.41	**−0.06**	1.00	0.66
F_{ST}	0.18	0.31	0.21	0.52	0.15	0.74	0.19	0.32
Long Island								
a	8	7	8	10	3	4	8	6.80
H_{obs}	0.25	0.00	0.00	0.00	0.40	0.65	0.35	0.23
H_{exp}	0.75	0.79	0.88	0.60	0.90	0.73	0.82	0.78
F_{IS}	0.69	1.00	1.00	1.00	0.48	0.06	0.46	0.67
F_{ST}	0.23	0.12	0.05	0.09	0.30	0.14	−0.02	0.13
Barnegat Bay Inlet								
a	11	8	8	5	2	5	9	6.80
H_{obs}	0.40	0.25	0.10	0.00	0.20	0.40	0.55	0.27
H_{exp}	0.89	0.80	0.81	0.18	0.61	0.76	0.79	0.69
F_{IS}	0.62	0.83	0.91	1.00	0.75	0.44	0.55	0.72
F_{ST}	0.04	0.13	0.15	0.42	0.83	0.10	0.03	0.24
Chesapeake Bay								
a	15	13	14	17	3	3	11	10.8
H_{obs}	0.50	0.25	0.40	0.00	0.35	0.15	0.15	0.26
H_{exp}	0.94	0.88	0.92	0.62	0.95	0.44	0.89	0.81
F_{IS}	0.50	0.67	0.55	1.00	0.63	0.65	0.81	0.67
F_{ST}	0.03	0.06	0.005	0.01	0.28	0.40	−0.11	0.09
North Carolina								
a	5	5	4	9	4	5	7	5.57
H_{obs}	0.30	0.25	0.30	0.00	0.30	0.05	0.55	0.25
H_{exp}	0.52	0.72	0.58	0.87	0.72	0.74	0.77	0.70
F_{IS}	0.43	0.66	0.49	1.00	0.59	0.93	0.29	0.62
F_{ST}	0.44	0.20	0.32	0.02	0.25	0.16	0.19	0.22

Multiply sampled ramets have been excluded from these calculations. Bold values indicate statistically significant loci with a putative heterozygous excess. A Bonferroni correction was applied to determine F_{IS} significance. a = allele number; NT = no test done because locus was entirely monomorphic.

of heterozygotes because it had high F_{IS} values of 1.00 at the *GA3, CT3,* and *CT12* loci. The Alaska site indicated larger numbers of heterozygotes with 2 alleles *GA3* (−0.02) and *CT17* (−0.01) having negative values for F_{IS} (Table 2). Among the Western Atlantic sites, Northern Maine had the lowest mean F_{IS} value at 0.48.

The positive F_{IS} values identified suggested a movement away from Hardy–Weinberg Equilibrium for the populations

under study. If we assume that the positive F_{IS} values are indeed significant, then this is evidence that inbreeding is occurring among these isolated populations. This conclusion is further supported by the observation that at least one fixed genetic locus (Table 2) can be observed for almost every population. Additionally, the low effective population sizes observed (Table 4) correlate with this conclusion.

The fixation index (F_{ST}) was also calculated to examine overall genetic differentiation and the heterogeneity of gene frequencies in the sites studied (Nei 1977). Chesapeake Bay has the lowest mean F_{ST} value at 0.09 with 4 loci out of 7 below an F_{ST} of 0.05 (*GA2, CT3, CT12,* and *CT20*) (Table 2). These low F_{ST} values would suggest little differentiation in the Chesapeake Bay plants, based on Wright's (1978) qualitative guidelines. However, the Southern and Northern Maine sites with mean F_{ST}s of 0.39 and 0.36, respectively, have "very great" genetic differentiation. Most of the sites have mean F_{ST} values that fall into the "moderate" range of genetic differentiation (0.09–0.15). The negative F_{ST} values found for 2 loci (*CT19* and *CT20*) suggest that the true F_{ST} measures for those loci are probably not significantly different from 0 and indicate a limited role for those loci in the genetic differentiation of the sites involved (Table 2).

Pairwise F_{ST} were also calculated between each group (Table 3). These values indicate genetic differentiation between each examined site and further support the relationships suggested by the F_{ST} values in Table 2. Northern and Southern Maine appear to be "very greatly" differentiated (0.338) from each other, despite their relative geographic proximity, whereas Chesapeake Bay and Waquoit reveal themselves in the "moderate" range (0.093) (Table 3).

Population Bottlenecks and Effective Population Size (N_e)

The presence of historical demographic population bottlenecks was calculated employing the 2-tailed Wilcoxon test with the two-phase mutation model (DiRienzo et al. 1994) (Table 4). Because a 2-tail test was applied, a stringent α value of 0.025 was used to designate a cutoff value for the likelihood of bottlenecks. Little evidence of bottlenecks was found in the sites examined, except for the Long Island group (Table 4). Brown tides have historically distressed Long Island *Z. marina* beds, lending support to bottlenecking in the region (Dzurica et al. 1989).

The effective population sizes (N_e) with 95% confidence intervals were estimated using linkage disequilibrium for all sites to better characterize their genetic diversity (Table 4). The Long Island site (N_e = 32.3) had the lowest value observed with a 95% confidence interval of 18.2–100.3 individuals. North Carolina had the highest calculable effective population size (N_e = 210.2). Northern Maine demonstrated the highest N_e value of infinity, indicating that the linkage disequilibrium method could not distinguish the very large N_e from infinity (Peel et al. 2004).

Effective population size is an indicator of clonal richness and plant "bed longevity." There seemed to be no geographic correlation of clonal richness from north to south, and the N_e values vary quite a bit among the sites examined. Chesapeake Bay, North Carolina, and Wells seem to possess high levels of clonal diversity based on their N_e values, whereas Long Island appears to have low clonal diversity (Table 4) with dominance of a few larger clones. All the other sites seem to have inconsistent levels of clonal diversity. The mixture of larger and smaller clones provides evidence that some outside recruitment is occurring.

Isolation by Distance and PCoA

PCoA (Figure 1), which allows a multicoordinate comparison of all individuals in all sites, suggests that the Maine sites (Northern, Southern, and Wells) have differentiated themselves somewhat from the other west Atlantic sites. Northern and Southern Maine are found in the left-hand quadrants with no individuals drifting to the right-hand sectors. The waters of Northern Maine are significantly colder and rougher than Southern Maine, giving the northern plants a more taxing environment in which to reproduce. The genetic differences between the plants at the 2 sites are not unexpected (Figure 1). This observation is further supported by the results in Table 3 that suggest that Northern and Southern Maine are genetically differentiated by a great degree.

Wells, Maine, is found in the upper quadrants only and primarily in the upper left quadrant with Southern Maine. The rest of the sites primarily maintain themselves in the right-hand quadrants. These results suggest a general lack of

Table 3. Pairwise population F_{ST} values

	Barnegat Bay Inlet	Long Island	Chesapeake Bay	Alaska	Waquoit	Wells	South Maine	North Maine	North Carolina
Barnegat Bay Inlet	**0.000**	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Long Island	0.134	**0.000**	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Chesapeake Bay	0.109	0.093	**0.000**	0.001	0.001	0.001	0.001	0.001	0.001
Alaska	0.297	0.254	0.245	**0.000**	0.001	0.001	0.001	0.001	0.001
Waquoit, Mass.	0.152	0.147	0.093	0.316	**0.000**	0.001	0.001	0.001	0.001
Wells, Maine	0.218	0.168	0.176	0.251	0.225	**0.000**	0.001	0.001	0.001
South Maine	0.332	0.279	0.253	0.354	0.357	0.250	**0.000**	0.001	0.001
North Maine	0.342	0.273	0.248	0.363	0.349	0.267	0.338	**0.000**	0.001
North Carolina	0.268	0.207	0.215	0.275	0.264	0.188	0.316	0.333	**0.000**

F_{ST} values are below the diagonal. Probability values based on 999 permutations are shown above the diagonal. Pairwise calculations were generated with the computer program GENALEX6.

Table 4. Bottlenecks and effective population size (N_e)

Populations	Bottleneck significance index	Effective population size (N_e)	Confidence interval
Alaska	0.054	78.6	40.2–572.0
Northern Maine	0.587	∞	53.1–∞
Southern Maine	0.156	33.0	14.5–∞
Wells, Maine	0.296	33.2	20.1–77.0
Waquoit, MA	0.687	33.5	14.8–∞
Long Island	**0.007**	32.3*	18.2–100.3
Barnegat Bay Inlet	0.070	56.9	25–∞
Chesapeake Bay	0.078	63.7	36.9–191.3
North Carolina	0.296	210.2	43.1–∞

An estimate of population bottlenecks was determined employing the two-phase mutation model (values below the α value of 0.025 (bold) support the occurrence of bottlenecks). Values of "∞" indicate values too large to calculate. Multiply sampled ramets have been excluded from these calculations. Asterisk indicates the lowest effective population size.

structure among these sites, except with the Maine *Z. marina*. This lack of structure supports outcrossing in the more southern sites. This is especially prevalent in Chesapeake Bay that can be seen in 3 different quadrants of the graph, supporting the hypothesis that this site is a nexus at which high gene flow occurs.

The IBD test (Figure 2) examined the association between the genetic and geographic distances for all the Atlantic sites, without Alaska included. No significant relationship was found to be present between genetic and physical distance ($r = 0.33$, $P = 0.070$). The lack of positive correlation indicates that samples are not spatially genetically structured and that isolation by distance does not play a role. Note that we did find a clear isolation by distance relationship between the east and west coast sites, when we included Alaska in the IBD analysis (data not shown).

Null Allele Analysis

Null alleles are a serious issue in all microsatellite studies. These "invisible" alleles can lead to a skewing or a misinterpretation of the data. We performed a statistical analysis using the FreeNA program of Chapuis and Estoup (2007) to determine what this impact might be on our data. We directed FreeNA to employ 10 000 bootstrap iterations to perform its calculations. The average null allele frequency for all loci and populations was estimated to be 0.2431 with values ranging between 0.0000 and 0.4567. Additionally, an estimate was calculated of Weir's (1996) global F_{ST} as well as an F_{ST} value using the excluding null alleles (ENAs) correction described by Chapuis and Estoup (2007) (Table 5). The result suggests that null alleles are having little effect on the F_{ST} values that we have estimated. The ENA-corrected values still indicate the same trend of moderate (0.14909) to great (0.32859) genetic differentiation. This result supports our conclusion of genetic isolation and that we are actually distinguishing a limited number of observed heterozygotes in these *Z. marina* populations. It also suggests that null alleles, although potentially present, have little impact on our results.

Discussion

Zostera marina Population Genetics in the Western Atlantic

Our research assessed for the first time the microsatellite-based genetic differences of *Z. marina* sites along the eastern

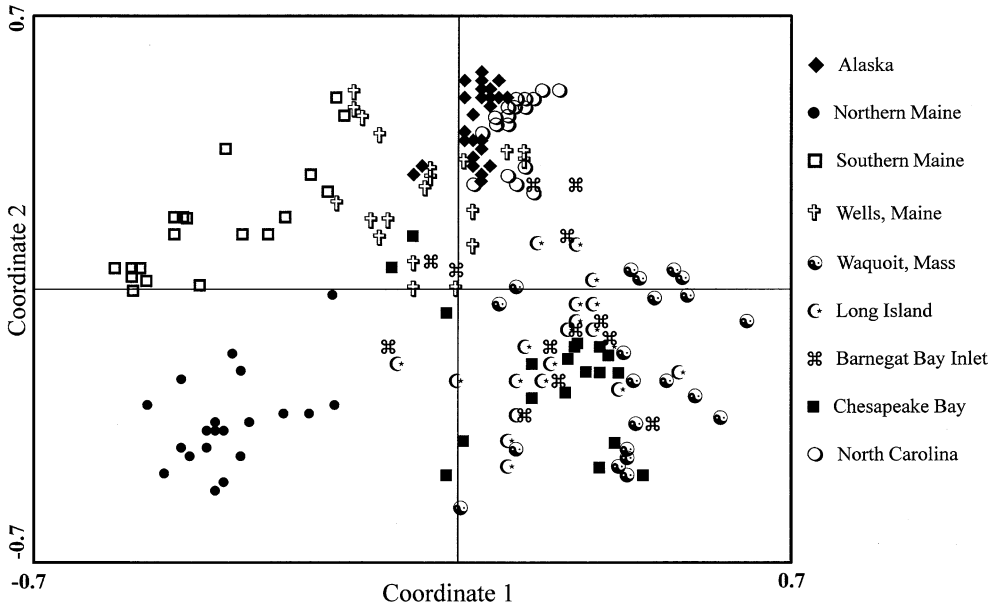

Figure 1. Associations among *Zostera marina* individuals in all the populations revealed by PCoA performed on genetic distance estimates calculated from microsatellite data of 7 loci.

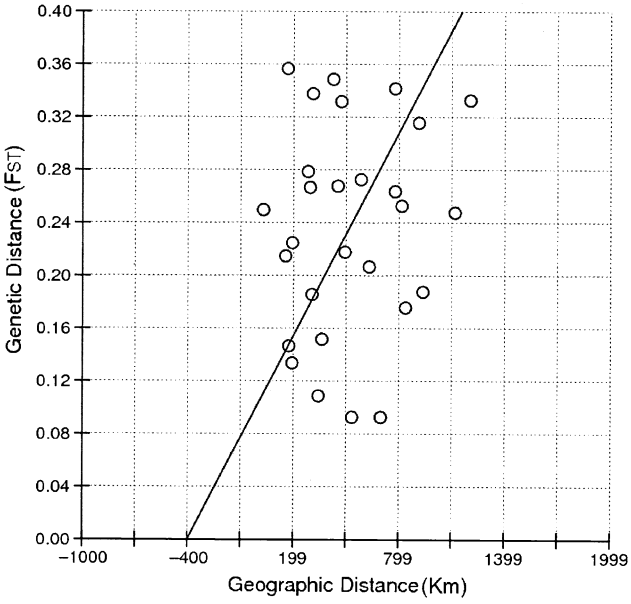

Figure 2. Isolation by distance analysis. The graph indicates genetic distance (F_{ST}) based on seven microsatellite loci vs. linear geographic distance (kilometers) for all possible pairwise combinations of *Zostera marina* within each eelgrass population studied on the west Atlantic coast. Alaska was not included in this analysis.

seaboard of the United States. These sites vary with respect to environmental stressors such as temperature elevations, macroalgal blooms, and recurrent brown tides (Orth and Moore 1983; Short et al. 1986; Dzurica et al. 1989; Hauxwell et al. 2001; Bologna et al. 2001; Gastrich et al. 2004).

The fixation index (F_{ST}) was calculated to examine overall genetic differentiation and the heterogeneity of gene frequencies in the sites studied (Nei 1977). Although our F_{ST} values suggest moderate genetic differentiation within most of the *Z. marina* sites (Table 2), at the same time the sites studied demonstrated that they are not internally genetically diverse. Increased genetic differentiation translates as reduced gene flow. The F_{ST} is the proportion of the total genetic variance contained in a subpopulation relative to the total genetic variance (Wright 1978). Values of F_{ST} can range from 0 to 1. The higher the F_{ST}, the greater the implied differentiation of a population from other populations. Wright (1978) defined "moderate differentiation" as being F_{ST} values between 0.05 and 0.15; values <0.05 were defined as evincing low levels of genetic differentiation and "higher" levels of gene flow.

Table 5. Global F_{ST} values calculated using FreeNA and employing 10 000 bootstrap iterations.

Local	Global F_{ST} without ENA	Global F_{ST} with ENA
GA2	0.19180	0.14909
GA3	0.16118	0.15739
CT3	0.10222	0.08555
CT12	0.43319	0.41275
CT17	0.21304	0.19906
CT19	0.33859	0.32859
CT20	0.20073	0.17590

F_{ST} determination was performed with and without a null allele correction using the method of Weir (1996).

The generally "high" F_{ST} values found in this study support the paucity of observed versus expected heterozygotes. The Atlantic sites had mean H_{obs} values (0.14–0.46) that were far lower than the H_{exp} values (0.57–0.81) (Table 2). Additionally, the mean F_{IS} values in all sites are positive, again indicating a surfeit of homozygotes over heterozygotes (Table 2). Our own results agree with Rhode and Duffy (2004), who found Chesapeake Bay populations to have high positive F_{IS} values and few heterozygotes. The *CT12* allele shows complete homozygosity in several sites with F_{IS} values of 1.00 (Northern Maine, Waquoit, Long Island, Barnegat Bay Inlet, Chesapeake Bay, and North Carolina) (Table 2).

Inbreeding coefficients were found to be highly positive and significant. Inbreeding appears evident in every population examined with at least one locus fixed to homozygosity in each group of plants examined. Although there is evidence of some outcrossing (Figure 1, Table 4), the local meadows appear in general to be relatively isolated from one another. The low heterozygosity genotypes appear to be well adapted to their environments. Though we have no experimental evidence to support this hypothetical adaptation, we did observe population sizes (N) in the field greater than the minimal theoretical effective population size of 260 (Palstra and Ruzzante 2008). If effective population sizes are low, then inbreeding will continue to be an issue and increase, whether the meadows are large or small in size.

The site that seems most severely affected with inbreeding and isolation is off Long Island. Long Island is the only site to clearly show evidence of bottlenecking (Table 4); eelgrass population declines have been observed off Long Island for decades (Dennison et al. 1989; Keser et al. 2003). It is unclear whether the bottlenecks indicated in Long Island are demographic or genetic in nature. Although the major die-offs for *Z. marina* occurred in the 1930s, it is possible that not enough time has passed yet to actually observe the effects of a genetic bottleneck (Johnson and York 1915; Dennison et al. 1989; Koch and Beer 1996; Keser et al. 2003). Because *Z. marina* is potentially a long-lived plant, unless there has been major population turnover, it is possible that the bottleneck we are observing is demographic. If Long Island indeed demonstrates a genetic bottleneck, then it is rare because European populations, like the majority of our own populations, show little or no evidence of bottlenecks (Olsen et al. 2004).

The Long Island site appears to have undergone substantial population declines, inducing a population bottleneck and showing low genetic diversity based on a mean F_{IS} (0.67), low H_{obs} (0.23), and the lowest N_e (32.3) and clonal diversity values ($C = 0.55$) of any site studied (Tables 2, 3). Keser et al. (2003) examined *Z. marina* sites at 3

locations in Long Island Sound from 1985 to 2000. Statistically significant declines in eelgrass abundance, which most likely resulted in genetic bottlenecks, were observed at all 3 locations.

The North Carolina *Z. marina* demonstrate the low clonal diversity that is to be expected at this location. Previous studies (Thayer et al. 1984; Fonseca and Bell 1998) support this observation. Because of the recurring warm water environment found at the southernmost *Z. marina* habitat, it appears that the North Carolina group are not perennial plants but annuals that die out from season to season as the water warms. This life cycle contributes to the site's low clonal diversity.

We are aware that our results do not necessarily agree with those of other Atlantic populations of *Z. marina* that have been studied. We observed few heterozygotes from our Atlantic sites. We have suggested that this observation is primarily due to inbreeding and genetic isolation of the various sites. An alternate hypothesis might be that we have a great number of null alleles that we are simply unable to observe. We employed both FreeNA (Chapuis and Estoup 2007) and Microchecker (Van Oosterhout et al. 2004) to determine the probability of null alleles in our loci. Both programs found that most of our homozygous marker sites have potential null alleles present. Microchecker predicted that Alaska has the fewest potential loci with null alleles present (3/7). North Carolina, Wells, and Chesapeake Bay are the worst with all loci (7/7) potentially having nulls. FreeNA indicated that even though these null alleles may be present, they should have little impact on our results or conclusions (Table 5). Because the distribution of F_{ST} values was many times skewed and not normal, we performed a nonparametric analysis using the Kruskal–Wallis test (Sokal and Rohlf 1969) on the F_{ST} values generated by FreeNA for each locus with and without ENA. At an α of 0.05, none of the deviations were statistically significant.

The problem with null alleles is that because they cannot be directly detected, their presence can only be inferred, so it is unclear whether those null alleles are actually present in our data. Van Oosterhout et al. (2004) suggested that when all loci appear to have null alleles that indicates a population that is out of Hardy–Weinberg equilibrium due to the fact that it is not panmictic. This has nothing to due with null alleles per se. Lack of panmixia will distort the allelic frequencies in such a way as to mimic the widespread presence of null alleles.

From a practical point of view, this study presents important data that can be interpreted in several ways. First, it is possible that our studied sites are indeed genetically different with fewer heterozygotes, high inbreeding, and reduced levels of diversity than those in northeastern Canada and the North Atlantic. Alternatively, we may simply have a greater number of null alleles than those other populations have evinced. Finally, if our populations are not in Hardy–Weinberg equilibrium and are highly inbred, the lack of panmixia may be leading to the resultant false impression that we have a greater number of null alleles present. Given the positive F_{IS} values and the moderately high to very high F_{ST} values (Table 2), the third option is the most viable and our populations are likely to be inbred and isolated, with detectable, but little, outside recruitment (Table 4). Any of these situations represents an interesting case study in population genetics. Examination of larger and more numerous eastern seaboard *Z. marina* populations with greater numbers of loci may clarify what is actually occurring.

Management Implications

Because *Z. marina* restoration efforts have had limited success (Moore et al. 1996; Williams and Orth 1998; Bologna and Sinnema 2005; Orth et al. 2005; Bologna and Sinnema 2006), one purpose for our research was to suggest how genetic variation may help to advance restoration success. It is possible that "blindly and randomly" replacing lost populations with stock *Z. marina* populations whose genetic status is uncharacterized may have led to low success rates in the past. If populations such as the one from Long Island were used in restoration, its low genetic diversity could lead to its demise and the failure of the *Z. marina* reestablishment.

Hughes and Stachowicz (2004) previously demonstrated that genetic diversity can aid *Z. marina* populations in resisting various physical disturbances. They found that diverse eelgrass genotypes survived avian predation and utilized nitrogen resources better during recovery from grazing than the less genetically diverse experimental plantings. Reusch et al. (2005) combined six genotypes of *Z. marina* in the Baltic Sea under high heat conditions. Like Hughes and Stachowicz (2004), Reusch et al. (2005) found that the "more diverse assemblages" of eelgrass phenotypes survived the temperature stress and recovered much faster than monocultures did.

One corollary to these studies is that a lack of genetic diversity weakens a population's ability to overcome any physical disturbance, but Reusch and Hughes (2006) suggest that the phenomenon may not be so simple. They suspect that diverse genotypes only become advantageous to the populations when they are under stress or severe disturbance. It may be that genotype diversity has little effect except under stress, but one might argue that genetic diversity is important beyond potential "stress relief." Enough diversity must be present for the plants to have a "genetic buffer" from which they may draw as needed to subsist. Increased diversity reduces the chances of inbreeding among close relatives. Reproduction in inbred populations leads to further diminished heterozygosity, inbreeding depression, and reduced overall survival. Furthermore, genetic diversity is the basis for physical phenotypic diversity in form and function; without that source of variation physical form is limited and truncated.

An ideal population for use in restoration studies would have a high AR, H_{obs}, N_e, and F_{ST}, combined with no evidence of bottlenecks and a negative F_{IS}. There are no populations among those we studied that fit all these criteria. However, there are several possibilities to which we can turn. Northern Maine is one candidate to employ in

restoration studies. Despite its surfeit of homozygotes (mean F_{IS} =0.48) suggesting low AR (mean AR= 4.5), other data support a more diverse population than is initially apparent. Northern Maine had the largest (though immeasurable) effective population size, a mean F_{ST} of 0.36 indicating great genetic differentiation, and no evidence of bottlenecks (Tables 2 and 3). While Northern Maine seems to have little gene flow with the other sites due to its physical isolation, individuals still demonstrate some genetic commonality with those of Wells (Maine), Southern Maine, Long Island, Barnegat Bay, and Chesapeake Bay (Figure 1). The one caveat with employing Northern Maine is that it might not grow well in all the possible habitats and temperature environments where it might be replanted.

Another candidate for use in restoration is Chesapeake Bay. Chesapeake Bay had the largest mean AR of any of the sites examined (9.57) (Table 1). The Chesapeake Bay plants examined also demonstrated a great deal of gene flow with the other sites and were thus "highly connected." Plasticity for different temperature regimes could be tested with Chesapeake Bay plants to determine if they could grow effectively as far north as Maine.

We hypothesize that elevated genetic diversity from a single population may result in increased restoration success. As far as we know from the present literature, this hypothesis has never been tested. The inclusion of multiple donor populations does increase restoration success, but the specific genetic analyses of these survivors has not been performed adequately (Williams 2001; Hughes and Stachowicz 2004). If the elevated diversity is associated with the ability of transplants to endure under various conditions, then it is possible that few donor populations contribute to the survival of the transplants. This would lead to establishment of the bed but doom it in the long run if only a few genotypes survive. In the end, the employment of seed stock from multiple populations over several years may help alleviate the potential reduction of genetic survival. Our future restoration efforts will take into account this basic understanding. This research must be successful if we intend to make any advancement in our restoration efforts in coming years.

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