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An Analysis of the Population Genetics of Restored Zostera Marina Plantings in Barnegat Bay, New Jersey

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An analysis of the population genetics of restored *Zostera marina* plantings in Barnegat Bay, New Jersey

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Abstract Within Barnegat Bay, New Jersey, eelgrass (*Zostera marina*) populations have declined by 62 % over the last 20 years. To better understand the consequences of this devastation, we have previously employed microsatellite DNA polymorphisms to analyze the population structure of *Z. marina* within Barnegat Bay, as well as along the eastern United States seaboard. We have restored populations of *Z. marina* in Barnegat Bay over the last 10 years to help assess the best planting conditions and ecotypes that might be used in long-term restoration strategies. In this study, we examined the genetic health of the restored populations compared to that of the donor eelgrass populations within the bay. Using microsatellites, we can identify which parental founding ecotypes survived the restoration process over multiple generations. The frequency of observed heterozygotes, although higher than in the natural populations, still indicates reduced levels of diversity and connectivity. The inbreeding frequency is high in the restored populations, but lower than what is seen in the native populations. All restored populations have effective population values >50, suggesting a high probability of survival in the short term.

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Introduction

Restoration ecology is employed to return ecological balance to disturbed ecosystems by re-introduction of local species that may have been lost over time. It is important that the replacement populations are genetically diverse (McKay et al. 2005; Falk et al. 2006). Genetic diversity must be present in these populations primarily because this variation helps organisms manage environmental unpredictability. Without a minimum level of genetic diversity, restored populations may eventually suffer declines, or even extirpation, due to the changing environmental parameters and ever-present anthropogenic stresses that often cause declines in the first place. Sufficient diversity must be present for the population to have a "genetic buffer" from which it may draw as needed to survive as a group. Loss of diversity also increases the probability of consanguineous breeding, leading to reduced heterozygosity, inbreeding depression, and diminished overall survival.

Eelgrass, *Zostera marina*, is one of the most widely distributed seagrass species in the world. This species serves as an essential habitat and provides important ecosystem functions ranging from primary production to reduction of physical forces such as wave action, water flow, particle deposition, and sediment stabilization (Fonseca and Fisher 1986; Almasi et al. 1987). Eelgrass is a sensitive indicator of long-term water quality due to its high light requirements. Coastal eutrophication often leads to elevated harmful algal blooms that can smother and kill grass beds (Bologna et al. 2007), while coastal development

often leads to turbidity increases and loss of light. As such, alterations in the health and distribution of these vascular plants generally signal a decline in water quality (Dennison et al. 1993; Kennish et al. 2007; Kennish 2011). Seagrass decline has occurred in many shallow, temperate and tropical regions of the world (Short et al. 1988; Walker and McComb 1992; Short and Burdick 1996; Valiela et al. 2000; Hauxwell et al. 2003; Orth et al. 2006). The wasting disease epidemic of the 1930s caused a massive world-wide collapse in *Z. marina* coverage (den Hartog 1987). Consequently, it is highly probable that genetic diversity of this plant species underwent a concurrent decline along with population size, resulting in bottlenecks, founder effects and complete loss of small populations within its global range. Populations that *have* recovered subsequently have limited regenerative genetic stock with which to repopulate coastal regions (Campanella et al. 2010a, b). In some cases, local *Z. marina* die-offs have occurred in a matter of months (Bologna et al. 2001) with subsequent recovery taking years (Bologna et al. 2007).

Zostera marina along the Atlantic coast of the United States has suffered significant declines (Orth and Moore 1983; Short and Burdick 1996; Lathrop et al. 2001; Hauxwell et al. 2003; Keser et al. 2003; Campanella et al. 2010b). The complete destruction of these populations would have significant ecological impacts. Some efforts to restore *Z. marina* in coastal estuaries have been quite successful (Leschen et al. 2010; Orth et al. 2010), but often restoration efforts have been limited in long-term survival due to the intrinsic environmental changes that have occurred in many of these systems (Treat and Lewis 2006).

Within New Jersey, restoration efforts have had varying levels of success (Reid et al. 1993; Bologna and Sinnema 2006, 2012). Over the last 10 years, we have established live transplants at a number of sites in Barnegat Bay with varying degrees of initial planting unit survival (6–100 % survival) (Bologna and Sinnema 2012). However, this restoration work was completed prior to any genetic stock analysis of the wild *Z. marina* populations in New Jersey (Campanella et al. 2010a). Plantings of multiple ecotype donors from the region around Barnegat Bay were done in a "blind" fashion with no information on the genetic state of any of the donor populations involved.

Differences in survival rates may be linked to the genetics and ecotypic origins of donor plants (Williams and Orth 1998). It is thought that transplants retain donor stock genetic identity. If part of that identity was an initial lack of genetic diversity, then long-term survival of transplants would concomitantly be uncertain. Conversely, genetically diverse donors would hypothetically lead to diverse restorees. This hypothesis is supported in the literature (Williams 2001; Hughes and Stachowicz 2004; Reusch et al. 2005), but has not been experimentally tested.

In the study presented here, we are examining the genetic health of five restored *Z. marina* populations that we have generated over the last 10 years (Sinnema and Bologna 2009; Bologna and Sinnema 2012). We compare the genetic diversity of these restored populations to the natural populations that are presently in Barnegat Bay. In a previous study, Campanella et al. (2010a) assessed the natural population genetic structure of *Z. marina* in New Jersey and determined how the genetic diversity and effective population size affected the genetic quality of these sites. We found that the Barnegat Bay ecotypes were highly inbred and not genetically diverse. Additionally, five of the eight Barnegat populations studied (Ham Island, Manahawkin Bay, Shelter Island, Marsh Elder, Harvey Cedar Sedge) showed evidence of historical bottlenecks.

The present study investigates three major questions. First, is it possible to identify, using genetic methods, which parental ecotypes survived the restoration process over multiple generations? Second, what is the genetic diversity of the restored populations relative to the parent populations that gave rise to them? Finally, what evidence is there to support the hypothesis that the most successful restorations arise from multiple ecotype donors that are highly diverse?

Methods

Plants and collection

Individual *Z. marina* plants were collected at five successfully restored sites within Barnegat Bay, New Jersey (Fig. 1). These sites received *Z. marina* transplants using either a peat-pot technique or the bundled-stapled planting unit technique (see Bologna and Sinnema 2012 for details). The five sites include Cedar Creek South planted 2001 (39°51′46.68″N, 74°7′42.06″W), Cedar Creek South planted 2002 (39°51′44.88″N, 74°7′44.22″W), Mordecai Island planted 2001 (39°33′38.34″N, 74°15′03.90″W), Sedge Island planted 2001 (39°33′46.83″N, 74°17′32.83″W), and Sedge Island planted 2008 (39°33′52.26″N, 74°17′30.00″W). Original planting occurred using planting units spaced at 1 or 0.75 m (Mordecai Island only) intervals in a gridded design. For Cedar Creek 2001 and 2002 the original planting area was 196 m^2, Sedge Island 2001 transplant area was 441 m^2, while Mordecai Island was 182.5 m^2. For the Sedge Island 2008 site, 1,764 m^2 of *Z. marina* were planted at 1 m spacings among 36 7 m × 7 m grids within a 4,788 m^2 area (133 m × 36 m). The interspersed gaps were then seeded with seed stock collected earlier in the year. While all sites relied upon live donor transplants for restoration activities (Table 1), the Sedge Island sites also received seeds within the restored area that

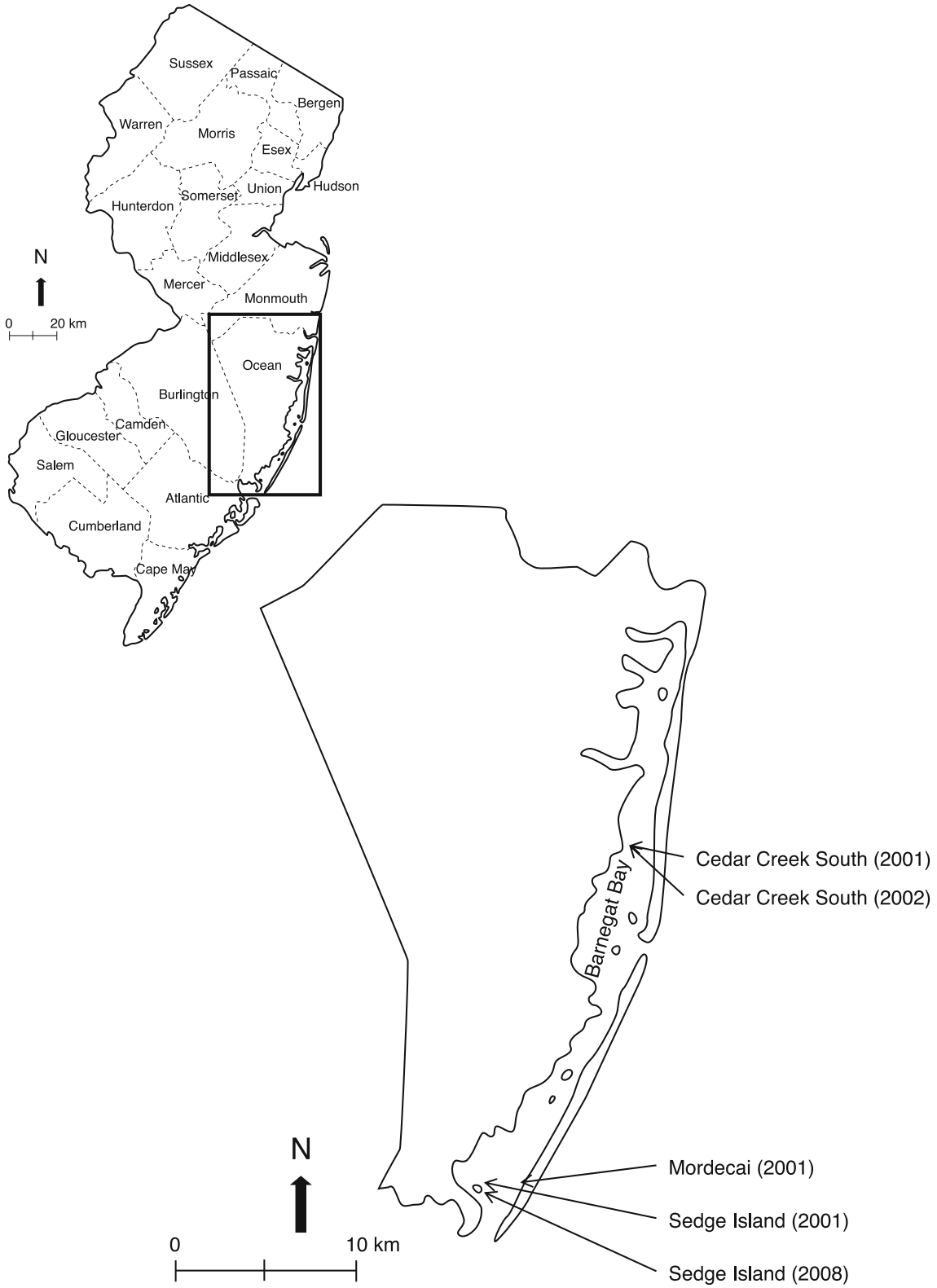

Fig. 1 The geographic collection sites for the restored populations studied in New Jersey

had been collected and coalesced from numerous regions within Barnegat Bay. The 2001 Sedge Island planting had seeds generally collected in the Marsh Elder, Shelter Island, and Ham Island regions of the bay, while the Sedge Island planting in 2008 had a broader collection of seeds from throughout the bay. During 2008 when the initial Sedge

Table 1 Clonal diversity (*C*) in the *Z. marina* populations studied

Population	Number of ramets	Number of genets	Clonal diversity	Live plant founder(s)
Cedar Creek South 2001	32	31	0.96	Barnegat Bay Inlet
Cedar Creek South 2002	37	37	1.00	Barnegat Bay Inlet
Sedge Island 2001	32	30	0.93	Ham, Marsh Elder, Shelter Island
Mordecai Island 2001	34	33	0.97	Ham, Marsh Elder, Shelter Island
Sedge Island 2008[a]	38	38	1.00	Ham, Marsh Elder, Shelter Island

Clonal diversity (*C*) was determined from the number of ramets sampled and the number of genets detected based on all the loci employed

[a] Sedge Island 2008 was additionally sown with an uncharacterized assortment of seed from across the Barnegat Bay

planting occurred, approximately 250,000 seeds were dispersed onto the site from a large, bay-wide coalescence of seeds. This site was additionally seeded in 2009 with approximately 100,000 seeds during a yearly monitoring event to increase spatial coverage and potential genetic diversity on the site (Sinnema and Bologna 2010).

To ensure that we were not gathering clonal samples, individuals were collected approximately 5 m apart within the restored beds. *Zostera marina* from the restored sites was collected using the same technique as natural populations in Campanella et al. (2010a, b). While clonal collection was possible using this technique, clonality was assessed in the statistical analyses of all populations. Tissue samples were 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 to 0.5 g of *Z. marina* leaf tissue, using the DNeasy DNA extraction kit according to the manufacturer's directions (Qiagen Corporation, Valencia, CA, USA). DNA was extracted from 30 to 40 individuals within each population. DNA concentration was determined by UV absorbance on a Nanodrop ND-1000 UV Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and samples were stored at −80 °C until polymerase chain reaction (PCR) amplification was performed.

The PCR was used to amplify seven microsatellite loci from the extracted *Z. marina* DNA. Primers for these seven 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 FAM or HEX dyes (Invitrogen Corp., Carlsbad, CA, USA).

Reactions were carried out using 10 ng DNA in RNase/DNase-free 0.2 μL tubes with 15–30 nmol labeled primers. Reaction mixes were all kept at 4 °C until 10 μL of Choice Taq Mastermix DNA Polymerase (Denville Scientific, Inc., Denville, NJ, USA) 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 3130 Genetic Analyzer (Applied Biosystems Corp., Foster City, CA, USA). The PCR products were diluted 1:10 with sterile water. 0.5 μL of the diluted product 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, USA) was used to evaluate the microsatellite allele sizes from raw data and score loci for homo/heterozygosity.

Statistical analysis of data

Clonal diversity (*C*) was determined employing the method of Olsen et al. (2004) and calculated by dividing the number of genets detected by the number of ramets sampled, based on all seven loci with the spatial scale between each ramet sampled being approximately 5 m. Redundant multilocus genotypes were removed from all further data analyses.

Observed (H_O) and expected heterozygosities (H_E) were calculated with GENALEX 6 under the codominant marker settings (Peakall and Smouse 2006). The probability (*P*) of significant deviation from Hardy–Weinberg equilibrium and F_{IS} were also calculated employing GENALEX 6.

STRUCTURE 2.3.3 (Pritchard et al. 2000) was employed to infer population structure through clustering of similar genotypes. STRUCTURE was run using default settings

with a "burnin" period of 10,000 and 10,000 Markov chain Monte Carlo (MCMC) repetitions after burnin.

The correct number of clusters (K) was obtained by testing K values 3 through 14 and performing ten repeats for every K value. Estimated log probabilities of data for each value of K were evaluated using ΔK, which is the rate of change in log probability between each K value (Evanno et al. 2005).

Microsat 2.0 (Minch, E., 1995, Stanford University) was utilized to calculate allelic heterogeneity and generate genetic distance matrices, based on the allele size data. Principal coordinate analyses (PCoAs) were performed using the Microsat genetic distance data in GENALEX6. Program parameters were set to employ a triangular distance matrix, and included data labels.

The program BOTTLENECK was used to estimate likelihood of population bottlenecks (Piry, S., 1999, French Institut National de la Recherche Agronomique). In the bottleneck analysis, the two phase mutation model (TPM) (DiRienzo et al. 1994) or the infinite allele model (IAM) (Kimura and Crow 1964) of microsatellite changes was employed. The TPM model is generally preferred over the IAM, because it assumes that microsatellites mutate at a constant rate, without respect to their repeat lengths. Moreover, there is no bias in TPM toward expansion or contraction, so microsatellites grow or contract unconstrained over time (DiRienzo et al. 1994; Luikart and Cornuet 1998).

The two-tailed Wilcoxon test (Cornuet and Luikart 1996) was employed because it assumes a "two-tailed" distribution over a population, is relatively powerful, and can be used with as few as four polymorphic loci and any number of individuals. It also provides the more conservative alpha of 0.025 as opposed to the one-tailed test with an alpha of 0.05. Since it was unclear when and if bottlenecks had taken place in our populations, it seemed wiser to use the more conservative alpha of the two-tailed test and an initial null hypothesis of H_E equal to H_O.

Effective population sizes (N_e) were calculated based on linkage disequilibrium by NeEstimator (Peel, D., 2004, Queensland Government, Department of Primary Industries and Fisheries, Brisbane). 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.

Results

Allelic frequency data and diversity

One hundred and seventy-three ramets were sampled from the restored Barnegat Bay populations and analyzed with seven microsatellite loci revealing a total of 169 genets (Table 1). Of the populations studied, Cedar Creek South 2002 and Sedge Island 2008 had the highest clonal diversity ($C = 1.00$), while Sedge Island 2001 had the lowest ($C = 0.93$) (Table 1).

The total number of alleles per locus ranged from 3 to 15 (Table 2). Across all populations, the GA2 locus had the largest mean number of alleles (10.4, calculated from data in table), while the CT17 locus had the smallest (5.6). The CT17 locus also had the lowest mean number of alleles in the natural populations studied in Campanella et al. (2010a). This result suggests that at least some alleles have been carried across generations even in the "hybridized", restored populations. Across all loci, the Sedge Island 2008 population had the largest mean number of alleles (10.7).

In addition to allele number, we examined the frequency of rare alleles that have been carried over from the natural populations (Table 3). The natural population allelic frequencies employed in the analysis came from Campanella et al. (2010a). We found that rare alleles *were* passed along from the natural populations to the restored populations. We defined rare alleles as any allele observed in the natural populations at a frequency of 0.05 or lower. The natural populations Ham Island and Harvey Cedar Sedge were the donors for the largest number of rare alleles found in the restored populations. The Sedge Island 2008 restored population was the largest recipient of rare alleles with ten (Table 3), and the Cedar Creek South 2002 population the smallest recipient with three alleles. Although it is unclear if we are observing the results of outcrossing to transfer these alleles, in the case of at least one rare allele of CT17, we are probably seeing genetic fixation and drift from the natural populations. The CT17 allele (158) has apparently drifted from the natural populations because it is no longer rare in the restored Cedar Creek South 2001 (allele frequency 0.709) and Sedge Island 2001 (allele frequency 0.216) populations. The only locus to show no evidence of any rare alleles being transmitted was CT19 (Table 3).

Without exception, the expected number of heterozygotes (H_E) was consistently higher for each locus than the observed number of heterozygotes (H_O) for all restored populations (Table 2). The mean H_O was highest for Sedge Island 2008 (0.50) and lowest for Mordecai (0.26). Note that Sedge Island 2008 received substantial seed supplements beyond the live transplants. The difference between the expected and observed heterozygotes frequencies was lowest in the Sedge Island 2001 population ($\Delta = 0.18$). The reduced H_O frequencies suggest inbreeding and a lack of genetic diversity. However, these values are higher than in the natural populations studied (Campanella et al. 2010a). The overall mean H_O for the natural Barnegat Bay populations was 0.28 (Campanella et al. 2010a), and the overall mean H_O for the restored populations is 0.36. CT17

Table 2 Within-population genetic diversity in all of the populations of restored eelgrass studied

Populations	GA2	GA3	CT3	CT17	CT12	CT19	CT20	Mean
Cedar Creek South 2001								
a	10	5	5	3	4	6	6	5.5
H_O	0.38	0.06	0.20	0.00	0.48	0.58	0.51	0.31
H_E	0.85	0.60	0.41	0.45	0.53	0.49	0.43	0.53
F_{IS}	0.54	0.89	0.52	1.00	0.093	−0.16	−0.20	0.38
P	0.000	0.000	0.000	0.000	0.000	0.000	0.998	0.142
Cedar Creek South 2002								
a	9	7	8	8	5	4	6	6.7
H_O	0.56	0.29	0.48	0.00	0.054	0.56	0.37	0.33
H_E	0.74	0.46	0.67	0.83	0.66	0.55	0.42	0.61
F_{IS}	0.23	0.36	0.27	1.00	0.91	−0.03	0.10	0.40
P	0.000	0.000	0.000	0.00	0.000	0.140	0.312	0.064
Sedge Island 2001								
a	9	8	6	3	5	7	8	6.5
H_O	0.63	0.33	0.23	0.00	0.16	0.44	0.62	0.34
H_E	0.85	0.65	0.65	0.12	0.34	0.46	0.60	0.52
F_{IS}	0.25	0.49	0.64	1.00	0.51	0.02	−0.03	0.41
P	0.00	0.00	0.00	0.00	0.00	0.437	0.998	0.205
Sedge Island 2008								
a	12	12	10	10	15	7	9	10.7
H_O	0.86	0.33	0.44	0.00	0.83	0.54	0.55	0.50
H_E	0.80	0.75	0.54	0.84	0.83	0.57	0.62	0.70
F_{IS}	−0.07	0.56	0.18	1.00	−0.007	0.05	0.11	0.26
P	0.997	0.000	0.827	0.000	0.624	0.817	0.352	0.398
Mordecai Island 2001								
a	12	10	10	4	6	6	7	7.8
H_O	0.54	0.45	0.03	0.00	0.06	0.61	0.18	0.26
H_E	0.86	0.71	0.85	0.50	0.33	0.61	0.54	0.62
F_{IS}	0.36	0.36	0.96	1.00	0.82	0.01	0.65	0.59
P	0.000	0.000	0.000	0.000	0.000	0.005	0.000	0.000

Multiply sampled ramets have been excluded from these calculations

a, number of alleles, H_O, observed heterozygotes; H_E, expected heterozygotes; F_{IS}, coefficient of local inbreeding; *P*, probability (from Chi square test) of significant deviation from Hardy–Weinberg equilibrium

was the only monomorphic locus with no heterozygotes at all detected (Table 2).

Sedge Island 2008 was the only population close to Hardy–Weinberg equilibrium with five of the seven loci examined having non-significant ($P \gg 0.05$) deviations (Table 2). The population that seemed farthest out of HWE was Mordecai (Table 2), where every locus demonstrated a highly significant ($P \ll 0.01$) deviation from HWE. The other three restored populations generally deviated from HWE, with only one to two loci out of seven being in HWE. Among all the loci, CT19 and CT20 seem to be the most consistently in HW equilibrium.

We calculated the coefficient of local inbreeding (F_{IS}) (Nei 1977) to examine the level of inbreeding in the restored populations (Table 2). The mean F_{IS} frequencies for all the populations indicated inbreeding *is* taking place. The Mordecai population had the highest mean F_{IS} value (0.59) and presumably the greatest level of inbreeding. The Sedge 2008 population had the lowest mean F_{IS} (0.26) of any population in the study. Individual loci showed various levels of local inbreeding. CT19 had a negative F_{IS} for both Cedar Creek South 2001 (−0.16) and 2002 (−0.03) (Table 2). From previously published data (Campanella et al. 2010a), we found that the overall mean coefficient of local inbreeding for the natural Barnegat Bay populations was 0.64 and the overall mean F_{IS} for the restored populations in this study is 0.41. This result supports the conclusion that, although inbreeding is present, the restored

Table 3 Frequency of rare alleles among the natural and restored populations

	M/W	CCS01	CCS02	Sedge01	Sedge08	Mord	BBI	Ham Is	Sedg Is	Shelter	Oyster	Mana	Marsh	Harv Ced
GA2	220	–	0.013	–	0.013	–	–	0.025	–	–	–	–	–	–
	238	0.016	–	–	–	–	–	–	–	–	0.025	–	0.050	0.025
	240	0.048	–	–	–	0.015	–	–	–	0.025	–	–	–	–
	250	–	–	–	–	0.015	–	–	0.050	–	–	–	–	–
GA3	212	–	–	–	–	0.030	–	–	–	–	–	–	0.050	–
	228	–	–	0.033	–	–	–	0.025	–	–	–	–	–	–
	234	–	–	0.016	0.027	0.030	–	0.050	–	–	–	–	–	0.050
CT3	230	0.066	–	0.066	0.013	–	0.035	–	–	0.050	–	–	–	–
	234	–	–	–	–	0.030	0.035	–	–	–	–	–	–	0.050
	236	0.016	–	0.050	0.013	–	–	0.050	–	–	–	–	–	–
	248	0.013	–	–	0.013	–	–	–	–	–	0.025	–	–	0.050
	250	–	–	0.033	–	–	–	–	–	–	0.050	0.050	–	0.025
CT17	114	–	0.054	–	–	–	–	–	–	–	0.025	–	–	0.025
	156	–	–	–	0.027	0.030	–	–	0.025	0.025	–	–	–	–
	158	***0.709***	–	***0.216***	–	–	–	–	–	–	–	0.025	–	–
CT12	146	–	–	***0.100***	0.039	0.015	–	–	0.025	–	–	–	–	–
	158		–	–	0.039	–	–	–	0.025	–	–	–	–	–
CT19		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
CT20	152	0.016	–	–	0.041	0.031	–	0.025	–	–	–	–	–	–
	156	0.048	0.054	0.017	0.083	–	–	0.025	–	–	–	–	–	–

Rare alleles were defined as any alleles at a frequency of 0.05 or less in the natural *Z. marina* populations. Restored populations: CCS01, Cedar Creek South 2001; CCS02, Cedar Creek South 2002; Sedge01, Sedge Island 2001; Sedge08, Sedge Island 2008; Mord, Mordecai. Natural populations: BBI, Barnegat Bay Inlet; Ham Is, Ham Island; Sedg Is, Sedge Island; Shelter, Shelter Island; Oyster, Oyster Creek; Mana, Manahawkin Bay; Marsh, Marsh Elder; Harv Ced, Harvey Cedar Sedge. Populations in *bold* are the restored populations. Frequencies in *bold italics* are those in the restored populations that are no longer "rare"

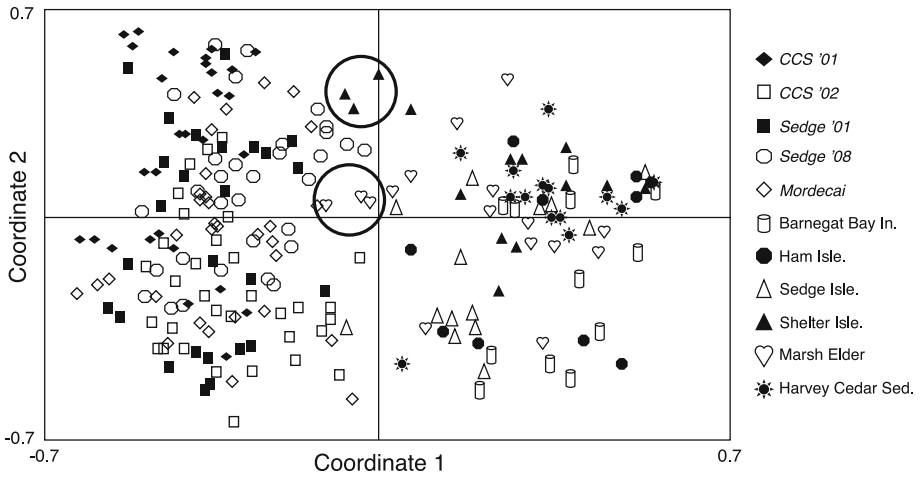

Fig. 2 Associations among *Zostera marina* individuals comparing the restored and wild populations are revealed by PCoA performed on genetic distance estimates calculated from microsatellite data of seven loci. *Circles* indicate congruence with likely parental ecotypes in the left quadrant. *Italics* in the legend indicates the restored populations. CCS, Cedar Creek South

populations are demonstrating *less* inbreeding relative to the natural populations.

Connectivity and founders

"Cluster" and "principle coordinate" (PCoA) analyses to examine connectivity were performed with the microsatellite genetic distance data, employing the restored and natural *Z. marina* populations (Campanella et al. 2010a) (Fig. 2). A PCoA is a statistical test that determines similarities and dissimilarities between sets of multivariate data and plots the concordance of those similarities on two axes. The analysis in this case reveals the connectivity of all the individuals in each population sampled against each other.

Based on the PCoA, the restored populations skew to the left quadrants of the coordinate map and appear more similar to each other than the donor and wild populations. However, indications of genetic origin are evident in the case of three of those restored populations. Mordecai and both of the Sedge Island restoration sites from 2001 and 2008 were transplanted with live individuals from Ham Island, Marsh Elder, and Shelter Island (Fig. 1). On the PCoA, these sites have Marsh Elder and Shelter Island donor individuals—indicated by circles—overlapping onto the left quadrants that essentially belong to the restored populations alone (Fig. 2). This result suggests that after multiple generations, the surviving offspring at these sites are most closely related to those particular ecotypes (Fig. 2).

The cluster analysis generated by STRUCTURE (Fig. 3a) examines each individual in a population and assigns portions of those populations to groupings based on genetic commonality. The optimal K value of the natural and restored *Z. marina* populations was estimated by employing the ΔK approach of Evanno et al. (2005) (Fig. 3b, c). We found the most likely number to be $K = 11$.

In agreement with the PCoA analysis, restored populations Cedar Creek 2002, Sedge Island 2001 and 2008, and Mordecai all cluster together in group 11 (Fig. 3a), suggesting that they are more similar to each other than natural populations in clusters 1–8. Cedar Creek 2001 segregates into cluster 9, though Mordecai still clusters at an $\sim$3 % level with this population.

Also in agreement with PCoA results, both Sedge Island plantings ('01 and '08) and Mordecai cluster with the natural founder population of Shelter Island (Fig. 3) at a $>$1 % level. We see some additional clustering of these restorees with the Manahawkin Bay population, which supports our hypothesis that there is some outcrossing occurring. Note that our 1 % clustering cut-off appears justified, since our *Z. marina* outgroup Alaska showed no clustering above 1 % with any of the populations included in the study.

Population bottlenecks and effective population size (N_e)

The presence of population bottlenecks over the last decade was calculated employing the two-tailed Wilcoxon test with the TPM or IAM Models (Table 4). An α value of 0.025 was used to designate a cut-off value for the significance of bottlenecks. Both the Sedge Island 2001 and 2008 restored populations showed evidence of bottlenecks under the TPM model, while no bottlenecks could be detected under the IAM (Table 4).

The effective population sizes (N_e) with 95 % confidence intervals were estimated using linkage disequilibrium for all populations to better characterize their genetic diversity (Table 4). The Cedar Creek South 2001 population ($N_e = 52.9$) had the lowest value observed. Sedge

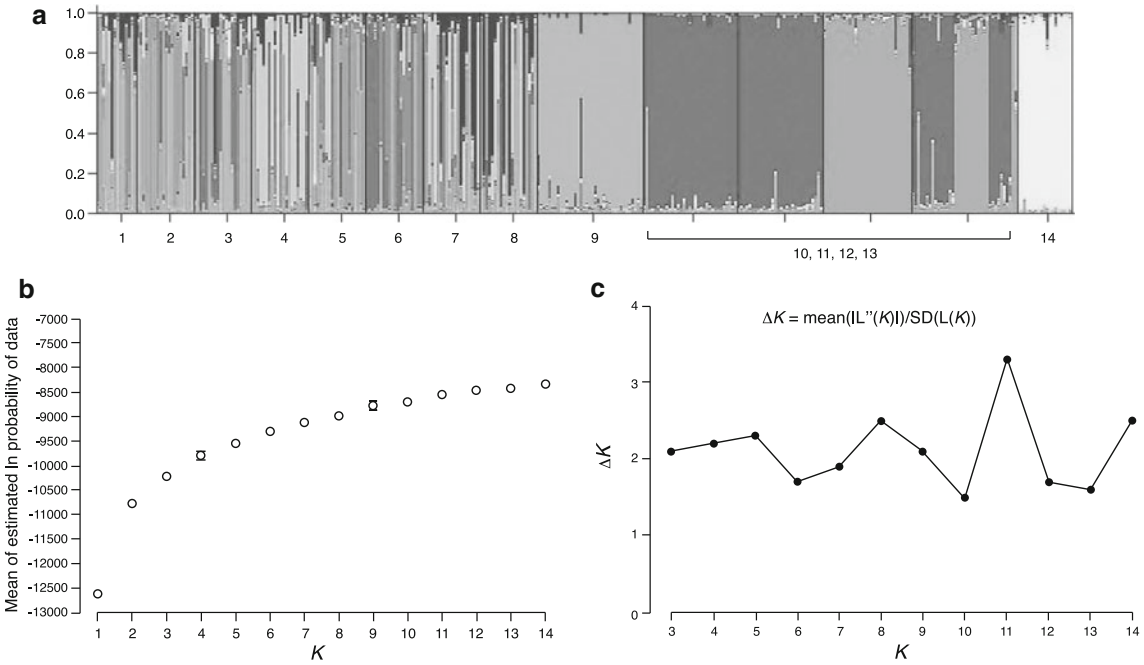

Fig. 3 **a** Estimated population structure of 14 natural and restored *Z. marina* populations using STRUCTURE for the $K = 11$ population. Each individual is characterized by a *thin vertical line* which is separated into K segments that represent the population group memberships. *Numbers on the lower axis* indicate geographical site locations (1, Barnegat Bay; 2, Ham Is.; 3, Sedge Is.; 4, Shelter; 5, Oyster Crk; 6, Manahawkin; 7, Marsh Elder; 8, Harvey Cedar; **9**, **Cedar Crk S. 2001; 10**, **Cedar Crk S. 2002; 11**, **Sedge 2001; 12**, **Sedge 2008; 13**, **Mordecai**; 14, Alaska outgroup). *Bold groups* are those which are restored. **b** Mean posterior probabilities of ten runs with SD *error bars* for each K, $K = 1$ to $K = 14$. **c** ΔK plotted for populations $K = 3$ to $K = 14$. $K = 11$ had the highest ΔK versus K peak height, indicating the most likely number of populations

Table 4 Bottlenecks and effective population size (N_e)

Populations	Bottleneck (TPM)	Bottleneck (IAM)	Effective population size (N_e)	95 % confidence interval
Cedar Creek South 2001	0.468	0.812	52.9	30.4–140.9
Cedar Creek South 2002	0.468	0.812	116.6	57.9–1,018.2
Sedge Island 2001	**0.023**	0.296	560.9	82.5–infinity
Mordecai Island 2001	0.296	1.000	144.4	65.9–infinity
Sedge Island 2008	**0.023**	0.375	235.8	105.5–infinity

The probability of population bottlenecks was determined using the two-tailed Wilcoxon test and the TPM or IAM (values below the α value of 0.025 (*bold*) support the occurrence of recent bottlenecks). Values of "infinity" indicate a value that was too large to calculate. Multiply sampled ramets have been excluded from these calculations. *Bold* value(s) indicates a putative bottleneck

Island 2001 had the highest effective population size ($N_e = 560.9$). If an infinity value was obtained for a confidence interval, all that can be concluded is that the value is too high to be calculated. Nelson and Soule (1987) suggested that a common rule of thumb to judge an effective population size is that 50 individuals is sufficient for short-term conservation of heterozygosity, but 500 are required for more long-term considerations of adaptability. Employing this metric, all the restored populations have N_e values above 50 and will be healthy in the short-term; however, only Sedge Island 2001, with an N_e of over 500, will likely survive in the long-term without genetic exchange with other populations.

Discussion

Genetic diversity of restored populations

Our research assessed the genetic quality of restored *Z. marina* populations within Barnegat Bay, New Jersey. The health of natural populations within this system varies

with respect to environmental stressors such as temperature elevations, macroalgal blooms (Bologna et al. 2001), recurrent brown-tides (Gastrich et al. 2004), and construction activities (Bologna and Sinnema 2006). It is also impacted by stochastic events like mussel settlement impacting water clarity (Bologna et al. 2005), but the reality is that New Jersey and most urbanized coastal areas are under significant eutrophication (Kennish 2002; Kennish et al. 2010). Natural populations in Barnegat Bay have been found to be inbred, often bottlenecked, and not genetically diverse (Campanella et al. 2010a).

Despite the hybridized nature of the restored populations, they still lack genetic diversity (Table 2). The clearest indication of this lack of diversity is a dearth of observed versus expected heterozygotes in the restored populations. However, despite this lack of genetic diversity, all the restored populations are still genetically healthier than the native donor populations. The mean difference between H_O and H_E in the natural Barnegat populations was $\Delta = 0.54$ (Campanella et al. 2010a), while the mean difference in the restored populations was $\Delta = 0.26$ (Table 2). This result supports the hypothesis that the mixed, restored populations, after multiple generations in Barnegat Bay, are more genetically diverse than the founder populations. Additional support for the enhanced genetic health of these populations is a reduced level of inbreeding (Table 2). While there is clearly inbreeding present and most of the F_{IS} values of the restoree loci are positive, the mean F_{IS} values are still lower than those of the natural populations, indicating reduced inbreeding (Table 2). This differs from Reynolds et al. (2012) who showed high levels of F_{IS} values for both donor and restored *Z. marina* populations, but their work related to seed restored sites only. Our results appear to concur with theirs and the combination of live-transplants supplemented with seeds may be a substantial step in restoring genetic health to New Jersey populations.

A third line of support for greater genetic health comes from the clonal diversity (C) values of the restored populations. By comparing the mean clonal diversity of the wild populations (0.80) (Campanella et al. 2010a) to that of the restorees (0.97) (Table 1), we find fewer clones and a greater degree of sexual reproduction occurring in the newer populations. The ratio of genets to ramets has been used in the literature to examine genetic diversity in clonal populations (Ellstrand and Roose 1987).

It appears that our "blind" hybridization of *Z. marina* ecotypes has resulted in genetically healthier restored populations. Obviously, these populations are not in optimal genetic states, with homozygote excess and continued inbreeding, but they are "healthier" than the natural populations from which they were founded. These results support the hypothesis that hybridized beds of *Z. marina* are more likely to survive long-term planting.

Bottlenecks and gene flow

Using the TPM model, the Sedge Island 2001 and 2008 sites showed evidence of bottlenecks (Table 4). It is possible that the bottlenecks arose from founder effects stemming from the three parental ecotypes (Marsh Elder, Ham Island, and Shelter Island) of the Sedge Island sites, since all these ecotypes had previously demonstrated strong bottlenecks (Campanella et al. 2010a). In fact, Marsh Elder formerly demonstrated the worst bottlenecks in the bay populations (Campanella et al. 2010a). What is a bit puzzling about this result is that the 2008 restoration event included additional seeds collected from numerous regions of the bay and coalesced into batch cultures for delivery on the site. It is possible that seed germination and spread within the population is limited, but the site showed high effective population size (Table 4).

At the same time, because these results are puzzling, it must be remembered that under the IAM test, neither of these populations evinced signs of bottlenecks. Some researchers have pointed out (Cristescu et al. 2010) that caution is recommended for studies of populations with an unknown history, especially when two tests performed by the BOTTLENECK program are inconsistent. Therefore, it may be difficult to conclude any absolutes about the presence of bottlenecks in the Sedge populations at this time. Interestingly, Mordecai Island, with the same founder ecotypes as the Sedge Island 2001 site, did not demonstrate any signs of bottlenecks, possibly because its primary extant founder is Shelter Island, which is a less historically bottlenecked population (Campanella et al. 2010a).

Additionally, we suspect that the Cedar Creek 2001 and 2002 restored populations showed no evidence of bottlenecks under either TPM or IAM because the founding Barnegat Bay Inlet population was never bottlenecked in the first place (Campanella et al. 2010a). This result supports the hypothesis that founding populations have a great deal of genetic influence on later restorees—even many generations later.

There seems to be some evidence for gene flow between the natural and restored populations. We see there are rare alleles in the restored populations that have their source in the natural populations (Table 3). Most of these rare alleles probably have a basis in out-crossing between natural and restored populations. These rare alleles were not likely to already be present in the restored populations when they were founded, because the allele frequencies have remained as low in most restored cases as in the natural populations. The exception to this observation appears to be CT17 (158) which is fixed at a high frequency in Cedar

Creek South 2001 (0.709) and Sedge Island 2001 (0.216). Based on these observations, Cedar Creek South 2002 appears to have the lowest levels of outcrossing since it has the fewest rare alleles.

Restoration and management implications

One of the most promising results to come from our study is the ability, using PCoA and cluster analysis, to track which parental founder(s) best survived after multiple generations. Each of the restored populations was compared in terms of genetic distance to the natural, extant Barnegat Bay populations, a distance matrix was generated, and then the matrix employed to create a PCoA. Each of the restorees appeared in a quadrant near a founding parental ecotype that predominated in its genome. The Mordecai Island and the Sedge Island populations from both 2001 and 2008 grouped closest to the Marsh Elder and Shelter Island ecotypes (Fig. 2), suggesting those founders best endured over time. The PCoA result was primarily supported in a cluster analysis (Fig. 3) employing the MCMC method to determine commonality.

The Cedar Creek populations, 2001 and 2002, were planted from a Barnegat Bay Inlet ecotype (Fig. 1). That donor population is now extinct and can no longer be found at the same location in the Barnegat Bay Inlet, due to dredging and changes in flow dynamics. It appears from both the PCoA (Fig. 2) and cluster analysis (Fig. 3) that either the extant Barnegat Bay Inlet population is not genetically similar to the lost donor population, or the Cedar Creek restorees have genetically drifted considerably from the Barnegat Bay Inlet Donors. There is no basis to determine which situation is the correct one, since the donor population is not alive to test, although the cluster analysis (Fig. 3) suggests that the Cedar Creek 2001 planting has some genetic similarity to Manahawkin Bay and Marsh Elder, clustering at ~ 1 %.

Although it is uncertain whether the most successful restorations arose from multiple ecotype donors, there is some support for that conclusion. We found that a majority of the seven microsatellite loci deviated from HWE in four of the planted populations (Table 2). These observations could have resulted from genetic drift, selection, population mixing, or inbreeding (Rousset and Raymond 1995). It is likely that inbreeding, at least in part, might account for the deviation from HWE for the restored populations that we detected.

Since most of the restored populations were not in HWE, we performed an additional equilibrium analysis of the natural *Z. marina* populations from Campanella et al. (2010b) and found *none* of those natural populations in HWE (data not shown). We found 10 total loci in HWE among the restored populations and *zero* in the natural populations. That result is an additional piece of evidence to support the hypothesis that our mixed, restored populations seem to be more diverse and genetically healthier than the natural populations.

The Sedge Island 2008 planting was the only population in the study that appears to be in HWE (Table 2). We hypothesize that HWE in the case of Sedge Island 2008 arose from a "forced outbreeding", because this population had multiple live and seed donors.

Furthermore, Sedge Island 2008 appears to be the most genetically healthy population (natural or restored) that we have observed in Barnegat Bay, based on its genetic diversity, HWE, "low" inbreeding frequency, and "high" clustering frequency (Tables 1, 2, 3, 4). This result supports the hypothesis that a combination of varied seed and live donors may lead to even greater potential genetic health, similar to the findings of Reynolds et al. (2012).

The Sedge Island 2008 planting may yet yield additional worthwhile genetic footprint information. If this site retains its donor signature in 7 years, it may be concluded that continued seeding efforts play a limited role in determining genetic diversity within *Z. marina* restorations, but lead to higher effective population sizes (e.g., Sedge Island 2001). However, if this site demonstrates not only higher effective population size (Table 4), but also an increased genetic diversity, the ability to easily track donor identity may be lost—although this might yield more diverse and robust restored populations. This result would be the ultimate goal, as this type of population would be more resilient to individual environmental stressors and lead to long-term survival.

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