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RESEARCH ARTICLE

Does genetic diversity of restored sites differ from natural sites? A comparison of *Vallisneria americana* (Hydrocharitaceae) populations within the Chesapeake Bay

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Abstract The goal of ecological restoration is to re-establish self-sustaining ecosystems that will resist future perturbation without additional human input. We focus here on the re-establishment of submersed aquatic macrophyte beds in the restoration of the Chesapeake Bay estuary. Degraded environmental conditions are often to blame for poor bed establishment, but genetic factors could also be contributing to low survival. We quantified the effect of restoration practices on genetic diversity in the submersed aquatic plant species *Vallisneria americana* Michx. (Hydrocharitaceae) in the Chesapeake Bay. In 2007, we collected 440 shoots from 8 restored/natural site pairs and 4 restoration stock repositories, and genotyped those individuals at 10 microsatellite loci. Restoration practices do not appear to negatively impact genetic diversity, and basic measures of genetic diversity within restored sites overlap with natural sites. However, small population size of restored sites, significant inbreeding coefficients within 3 sites, and low overlap of allele composition among sites provide cause for concern. These problems are relatively minor, and we propose several corrections that would alleviate them altogether. Managers should be encouraged by our findings as well as the current state of the genetic diversity within *V. americana* restoration efforts.

Keywords Submersed aquatic vegetation · SAV · Genotypic diversity · Restoration practice · Effective population size

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Introduction

The ultimate goal of ecological restoration is re-establishing self-sustaining ecosystems that will be resilient to future perturbation without additional human input (Procaccini and Piazzi 2001; Rice and Emery 2003; Ramp et al. 2006; Broadhurst et al. 2008; Liu et al. 2008). In practice, restoration can range from simply creating suitable physical conditions to allow natural colonization; planting pioneer species that will facilitate succession and eventual growth of target species; supplementing one or few species within a relatively intact ecosystem; to constructing diverse communities on denuded sites (Montalvo et al. 1997). Spatial scales of efforts range from small, local projects (e.g., <10 ha) to plantings that cover broad geographic areas (e.g. >100 ha; Broadhurst et al. 2008). Regardless of the scale of a project, restored populations must persist in dynamic settings in the short term (Jordan et al. 1988) and also retain the capacity to undergo adaptive evolutionary change in the long term (Montalvo et al. 1997; Rice and Emery 2003) to be considered successful. Genetic diversity of planting materials is a key consideration for restoration success for both time frames.

Unfortunately, genetic diversity is often not explicitly measured or considered in restoration and, owing to logistical constraints, restored populations are frequently founded

with a limited number of individuals that may represent only a portion of the genetic diversity present in natural populations. Small numbers of founding individuals can have two main genetic consequences. First, reduction in effective population size (Frankham 1995, 1996) can directly impact fitness due to increased inbreeding (Dudash 1990; Gigord et al. 1998; Keller and Waller 2002). Second, reduced effective population sizes can diminish allelic diversity and thus long-term evolutionary potential through increased rates of genetic drift (Whitlock 2000; Hartl and Clark 2007). Low levels of diversity can also arise from initial selection of few genotypes. Whether it results from initial selection of planting stock or losses over time, low genetic diversity can limit potential for resilience of populations under environmental stressors such as grazing (Hughes and Stachowicz 2004), heat shock (Reusch et al. 2005), or nitrogen loading (Tomas et al. 2011). In contrast, increased diversity resulting from restoration techniques can provide short-term benefits that can increase transplantation success (Procaccini and Piazzi 2001). There is also evidence to suggest that genetic diversity affects the structure of communities (Wimp et al. 2005; Ellers et al. 2011; Rowntree et al. 2011) and ecosystem functioning (Tomas et al. 2011) and may therefore be important in the provision of ecosystem services.

Such diversity can come from within one or a few local sites or can come from combining individuals from a site from a broader geographic area. Source populations are critical in restoration because selecting individuals from a limited number of sites can lead to the use of individuals adapted to environments that differ from environmental conditions at the restoration sites can negatively affect restoration efforts (Fenster and Galloway 2000; Montalvo and Ellstrand 2000; Montalvo and Ellstrand 2001; Hufford and Mazer 2003).

Thus, genetic diversity is critical to restoration success, and yet restoration practices themselves can negatively affect diversity. These potential consequences led us to quantify the effect of restoration practice on genetic diversity in the submersed aquatic plant species *Vallisneria americana* Michx. (Hydrocharitaceae). Submersed aquatic vegetation (SAV) communities are among the most threatened on earth (Short and Wyllie-Echeverria 1996). SAV declines have been well documented in the Chesapeake Bay (Dennison et al. 1993), one of the largest estuaries in the world. Dramatic reductions in *V. americana* cover and extent in the northern freshwater reaches of the Chesapeake Bay and its tributaries in the 1970s and 1980s (Kemp et al. 1983) led to targeted efforts to restore this taxon to denuded areas. These efforts have resulted in low establishment rates that are not unique to *V. americana;* most seagrass species have experienced net loss of habitat even with restoration efforts, and worldwide success of seagrass transplantation as judged by persistence and bottom coverage is roughly 30% (Fonseca et al. 1998).

Inappropriate site conditions coupled with continued poor water quality have likely contributed to low establishment rates in many restoration plantings (van Katwijk et al. 2009). However, it is also possible that genetic factors are contributing if diversity of planted individuals is lacking or does not represent the genetic diversity found within natural populations. Unfortunately we cannot know the nature of the diversity that was in failed sites in which plants no longer exist. We can only evaluate the diversity in naturally occurring sites and compare them with extant restored populations that vary in age and source.

There is extensive evidence of the ecological consequences of genetic diversity in SAV restoration efforts. For example, Williams and Davis (1996) noted that genetic diversity of transplanted *Zostera marina* L. beds was reduced relative to natural beds. Decreased genetic diversity in transplanted beds was associated with lower population growth and individual fitness (Williams 2001). In *Posidonia oceanica* Delile, genetic polymorphism in restoration stock was positively correlated with rhizome length, number of ramets per genet, and survival rate (Procaccini and Piazzi 2001). Similarly, genotypic diversity of *P. oceanica* within populations was positively correlated with shoot density (Zaviezo et al. 2006). A similar pattern was noted in *Z. marina*; however, a positive relationship between genotypic diversity and shoot density existed only in winter, potentially indicating enhanced tolerance to abiotic and biotic stressors associated with overwintering (Hughes and Stachowicz 2009). Variation in growth rates, production of secondary compounds, and structural characteristics (Tomas et al. 2011), may have contributed to the reasons that increased genotypic diversity of *Z. marina* enhanced population recovery and persistence (Hughes and Stachowicz 2004; Reusch et al. 2005; Hughes and Stachowicz 2011) and yet compared with monocultures, and in absence of disturbance, polycultures of *Z. marina* had decreased yield (Hughes and Stachowicz 2011).

We determined the degree to which genetic diversity within restored sites is representative of natural sites. Restored sites within the same tributaries may deviate from paired natural sites when non-local restoration stock or few local genotypes were used to re-establish a population. We compared levels of genotypic diversity and allelic diversity, as well as allelic composition among natural/restored pairs of *V. americana* populations in the Chesapeake Bay and in stock repositories that have been used for restoration activities. Additionally, we compared effective population size estimates of restored versus natural populations. Together, these comparisons allowed us to evaluate the state of natural populations and how restoration practices are affecting genetic diversity in restored populations and nursery stock.

Methods

Sampling locations

In 2007, we sampled from eight sets of paired natural and restored sites of *Vallisneria americana* located in tidal and non-tidal reaches of Chesapeake Bay tributaries (Fig. 1). Restoration efforts, including failed attempts, are not documented. We therefore identified restored sites with extant populations and paired natural sites with the help of managers and scientists working within the region. Site pairs were typically located within the same tributary between 165 and 5 km of each other (Table 1). Owing to scarcity of sites, the set in Virginia (HL/TAR) was paired across two tributaries (Fig. 1). The 8 restored sites differed in age; the oldest site was planted in 1985 and the youngest site was planted only weeks prior to sampling. Restoration efforts varied in techniques and source material (Table 2). Rooted plants obtained from areas surrounding the plantings were often used as source material (Table 2). From each of the 8 natural locations, we collected up to 30 shoots, each approximately 5–10 m apart. Our goal in sampling was to estimate the genotypic and allelic diversity at sites, not to document or compare the spatial distribution of diversity within sites. Therefore, the spatial scale of sampling differed within and among restored and natural sites to account for differences in population size and extent, where the distance between samples depended on the distribution of plants in each site. Latitude and longitude were recorded for each sampled shoot using global positioning system technology.

Sampling within restored sites was limited by the size of the plantings and if the planting had expanded. In the older restoration sites without herbivore exclosures (i.e., CPR, FBR, ENR, SCR), we sampled at 5–10 m increments as these sites had expanded to areas $\sim 150\ m^2$ and contained >500 stems. The WC site, although open, was of similar size to an enclosed site. Sites with herbivore enclosures (i.e., LOC, GC, TAR) were typically small $\sim 1.5\ m \times 3\ m$ in area and had <100 stems. We collected fewer shoots from these smaller restored sites to limit impacts to the new plantings. Although only a small amount of tissue is needed for genotyping, poor visibility prevented seeing plants to sample and simply accessing enclosed restored sites caused extensive dislodging of plants and we chose to minimize our access time. In these circumstances, we made a concerted effort to collect representative samples while not causing unnecessary damage. Despite the smaller sample sizes, samples from restored sites likely represent a larger proportion of the total number of shoots at a site than do samples from natural populations. Genotypic diversity of dense or extensive natural sites may be comparatively underestimated.

In addition to sites located within the estuary, three local restoration stock repositories were sampled: a propagation facility at Anne Arundel Community College in Maryland, the USDA Native Plant Materials Center in Beltsville, Maryland, and a nursery facility in Baltimore County, Maryland. We also sampled from a nursery in Wisconsin to compare local nursery stock with nursery stock that is shipped throughout the U.S.A. Sample sizes from repositories were limited by the amount of material provided by each center. For example, tissue is cultured in jars at the AACC, and to avoid contamination of jars due to sampling we were provided four jars from the repository. Field-collected shoot tissue was placed on ice within 1 h of collection, and immediately transported to the University of Maryland College Park, where the material was frozen at −80°C until DNA extraction.

DNA extraction and genotyping

Genomic DNA was isolated and purified using methods outlined in Burnett et al. (2009). We genotyped 10 microsatellite loci from each shoot using robust primers with specific amplification that we developed for the species (Burnett et al. 2009). Polymerase chain reactions (PCR) were performed as in Burnett et al. (2009), with the exception of the locus Vaam_AAG004, for which we added dimethyl sulfoxide and Q-Solution (QIAGEN) to reactions to optimize specificity. PCR products were separated, measured, and peaks analyzed using identical methods and quality control procedures, which included repeated analyses to ensure high reproducibility of PCR reactions, as detailed in Lloyd et al. (2011). Our final dataset contained 0.5% missing data.

Genotypic diversity

We detected clones within and across sites by identifying identical multilocus genotypes using the program GenClone v2.0 (Arnaud-Haond and Belkhir 2007). Because mutation and scoring errors can lead to assigning different genotypes to individuals that actually represent clones, we used GenClone to identify cases in which there was only a one-allele difference among genotypes. We examined these cases by hand to confirm scoring, and, when warranted, modified clonal assignments, which resulted in changing assignment of 16 genotypes. Within sites, the proportion of unique genotypes was calculated as $(G - 1)/(N - 1)$, where G is the number of unique genotypes and N is the total number of shoots sampled (Pleasants and Wendel 1989; Arnaud-Haond et al. 2007). Each genet was represented by only one shoot in subsequent analyses. We also identified clones that were shared across sites by repeating the GenClone analysis after pooling all samples.

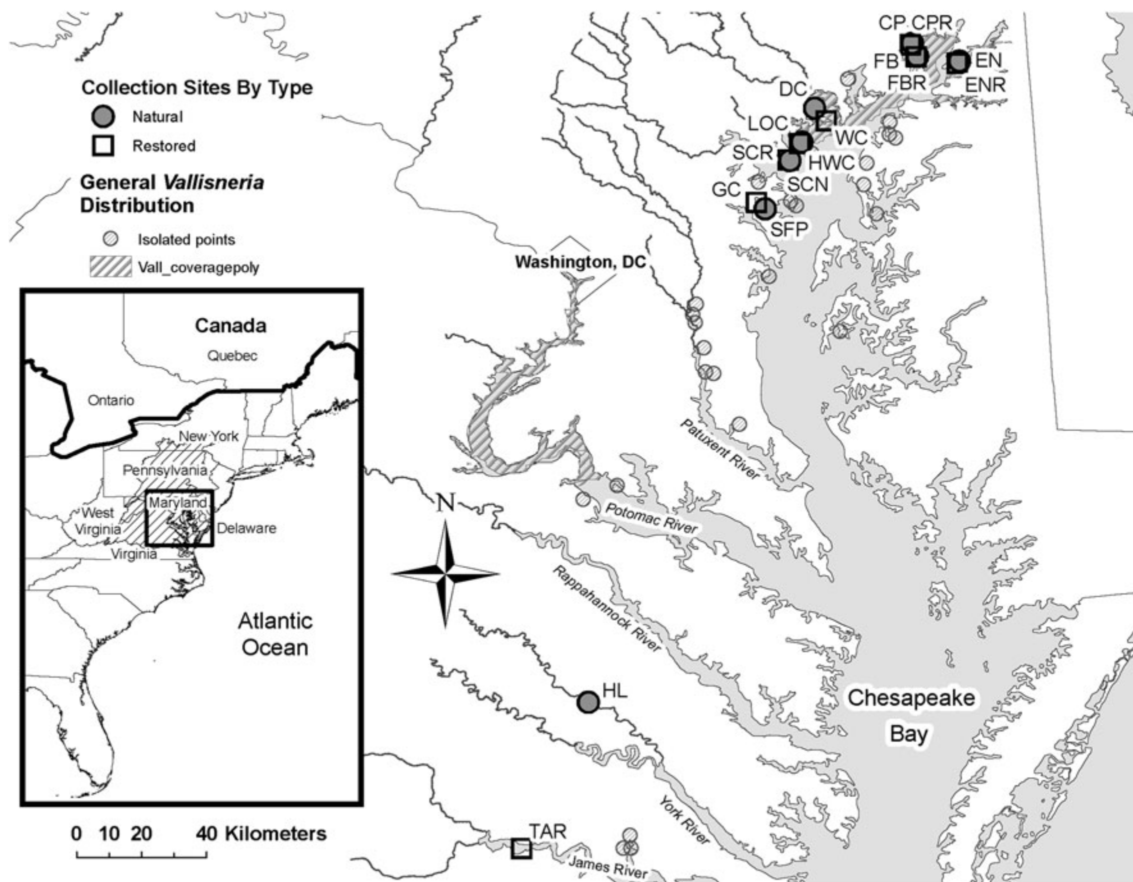

Fig. 1 Paired natural restored collection sites sampled in 2007

Measures of genetic diversity

We used GDA v1.1 (Lewis and Zaykin 2001) to calculate proportion of polymorphic loci (P), observed number of alleles (A), private alleles (A_p), unbiased expected heterozygosity (H_e), and observed heterozygosity (H_o) within sampling locations. We used rarefaction on pairs of restored and natural sites to account for different sample sizes (HP-Rare v1.0; Kalinowski 2004, 2005b). We tested for differences in genetic measures among all natural, restored and stock sites using Kruskal–Wallis tests in R v2.12.1 (R Development Core Team 2010). Differences in all genetic measures among the paired sites were examined using Mann–Whitney tests in R. Simple linear regression was used to test the relationship between genotypic diversity and all basic statistics (P, A, A_p, H_e, and H_o) in R to test for the influence of genotypic diversity on the basic statistics. Finally, the relationship between restoration practices (i.e., age of sites, source of plants, type of plants, # plantings per site; Table 2) and measures of genetic diversity were assessed using Kruskal–Wallis tests, Mann–Whitney tests or simple linear regression in R (R Development Core Team 2010). We accounted for multiple comparisons in tests that determined differences among individual site pairs. The format of the response variable determined the type of test used.

Departure from Hardy–Weinberg equilibrium

Wright's F_{is} was calculated for each site using the estimator f (Weir and Cockerham 1984) in GDA to test for population level deviations from Hardy–Weinberg equilibrium. f is the correlation of genes within individuals relative to each site (Weir and Cockerham 1984). We used confidence limits around each estimate generated by 1000 bootstraps in GDA to assess significance of f, which indicates departure from random mating.

We examined each site and the three repositories that had two or more genetically distinct individuals for presence of a recent genetic bottleneck using a test for heterozygote excess in the program Bottleneck v1.2.02 (Cornuet and Luikart 1996). Bottleneck computes heterozygote excess as the difference between expected heterozygosity (H_e) and heterozygosity expected at equilibrium (H_{eq}) for each site from the number of alleles given the sample size (Cornuet and Luikart 1996). We tested significance of the difference

Table 1 Measures of clonal and genetic diversity in populations of Vallisneria americana sampled from the Chesapeake Bay

Sample location	Code	Long.	Lat.	Dist.	N	G	$\frac{(1-G)}{(1-N)}$	A	A_p	P	H_o	H_e	f
Conford Point[a]	CP	−76.098	39.528		29	26	0.89	5.2	2	1	0.54	0.59	0.089
Conford Point[b]	CPR	−76.100	39.525	0.49	30	17	0.55	4.5	0	0.9	0.64	0.59	**−0.083**
Elk Neck[a]	EN	−75.968	39.480		30	23	0.76	5.5	2	0.9	0.63	0.60	**−0.057**
Elk Neck[b]	ENR	−75.969	39.475	0.63	30	12	0.38	4.2	0	0.9	0.63	0.56	**−0.113**
Fishing Battery[a]	FB	−76.083	39.493		30	26	0.86	4.8	0	0.9	0.63	0.60	−0.044
Fishing Battery[b]	FBR	−76.084	39.492	0.16	30	20	0.66	4.6	1	0.9	0.61	0.58	−0.059
Dundee Creek[a]	DC	−76.363	39.341		30	30	1.00	5.5	1	1	0.58	0.61	0.052
Weir Cove[b]	WC	−76.333	39.314	5.00	15	13	0.86	4.7	2	1	0.49	0.61	**0.199**
Hawks Cove[a]	HWC	−76.404	39.254		29	27	0.93	5.8	2	1	0.67	0.66	−0.014
Long Cove[b]	LOC	−76.408	39.254	0.47	15	13	0.86	4.7	0	1	0.57	0.65	**0.126**
Shallow Creek[a]	SCN	−76.437	39.205		30	6	0.17	3.2	0	0.9	0.52	0.58	0.127
Shallow Creek[b]	SCR	−76.438	39.206	0.20	15	15	1.00	4.4	0	1	0.63	0.61	−0.032
South Ferry Point[a]	SFP	−76.505	39.071		15	5	0.29	3.8	0	0.9	0.60	0.63	0.055
Grachur Camp[b]	GC	−76.525	39.088	2.55	3	2	0.50	2.3	0	0.9	0.55	0.62	0.154
Horse Landing[a]	HL	−76.993	37.706		30	3	0.07	2.3	0	0.6	0.60	0.45	**−0.469**
Tar Bay[b]	TAR	−77.190	37.307	47.76	10	3	0.22	2.4	0	0.6	0.37	0.46	**0.241**
	Avg.				23.19	15.06	0.62	4.24	0.63	0.90	0.58	0.59	0.011
	SD				9.27	9.52	0.32	1.15	0.89	0.13	0.07	0.06	0.166
Restoration Repositories													
Wisconsin Nursery	WISC	N/A	N/A	N/A	5	3	0.50	2.4	0	0.7	0.40	0.47	0.172
Anne Arundel Com. College	AACC	N/A	N/A	N/A	4	1	0.00	1.9	1	0.9	0.90	0.90	N/A
Kollar Nursery	FARM	N/A	N/A	N/A	30	28	0.93	4.4	0	0.9	0.60	0.60	−0.004
USDA Plant Material Center	USDA	N/A	N/A	N/A	30	9	0.28	4.2	2	1	0.67	0.65	−0.031
	Avg.				17.25	10.25	0.43	3.23	0.75	0.88	0.64	0.65	0.034
	SD				14.73	12.31	0.39	1.26	0.96	0.13	0.21	0.18	0.093

Long longitude, *Lat* latitude, *Dist* distance among paired sites (km); *N* number of sampled ramets, *G* unique genets, *genotypic diversity* = 1 − *G*/1 − *N*; *A* average number of alleles, *Ap* number of private alleles, *P* proportion polymorphic loci, *Ho* observed heterozygosity, *He* expected heterozygosity, *f* correlation of alleles within individuals within populations. *Bold* $P < 0.05$

[a] Natural sites

[b] Restored sites

between H_e and H_{eq} using a one-tailed Wilcoxon signed-rank test under a two-phase mutation model. This model provides results intermediate between an infinite allele model and a stepwise mutation model and is considered to be most appropriate for microsatellites (Di Rienzo et al. 1994).

Shared allelic identity among natural and restored pairs

We used principal components analysis (PCA) to assess similarity of allelic composition among genets sampled from natural versus restored sites. We implemented one individual-based PCA using the variance–covariance matrix for all unique genets sampled in Genodive v2.0b17 (Meirmans and Van Tienderen 2004). We then calculated percent overlap in the distribution of PCA scores of individuals in individual natural-restored population pairs along the first and second PCA axes. For restoration stock repositories, we compared the composition to all sampled natural diversity in the Chesapeake Bay. The degree of overlap at the site level provides insight into how well allelic diversity in restored sites represents local natural diversity. The degree of deviation from 100% overlap along either axis indicates the degree to which allelic composition differs among population pairs within the context of the total diversity in the sampled sites (Fig. 2).

Effective population size

We estimated effective population size (N_e) using LDNe v1.31 (Waples and Do 2008) with $P_{crit} = 0.05$. LDNe utilizes the Burrows method to calculate linkage disequilibrium, which is subsequently used to calculate N_e from a single population sample (Waples 2006).

Table 2 Location and associated details for restoration planting sites. All sites were planted with rooted shoots or tubers, the origin and source of which varied

Abbreviated name	Year(s) planted	Region whole plants obtained	Local source material	Type of whole plants used	Repeated planting
CPR/ENR/FBR	1985–1990	Confluence of the Susquehanna River	Yes	Fresh harvest from natural sites	Yes
WC	1997	Mouth of Dundee Creek (Gunpowder River), AACC, USDA	Yes and No	Repository and fresh harvest from natural sites	No
LOC	2006	Susquehanna Flats, Gunpowder River	No	Grown from seed harvested from natural sites	No
SCR	1999–2001, 2003	AACC, USDA	No	Repository	Yes
GC	2007	Susquehanna Flats	No	Grown from seed harvested from natural sites	No
TAR	1999[a], 2004, 2006, 2008[b]	Repository stocked from the Potomac River	No	Repository stocked with seed and entire plants harvested from natural sites	Yes

[a] Plant material removed by herbivory

[b] Post-sampling

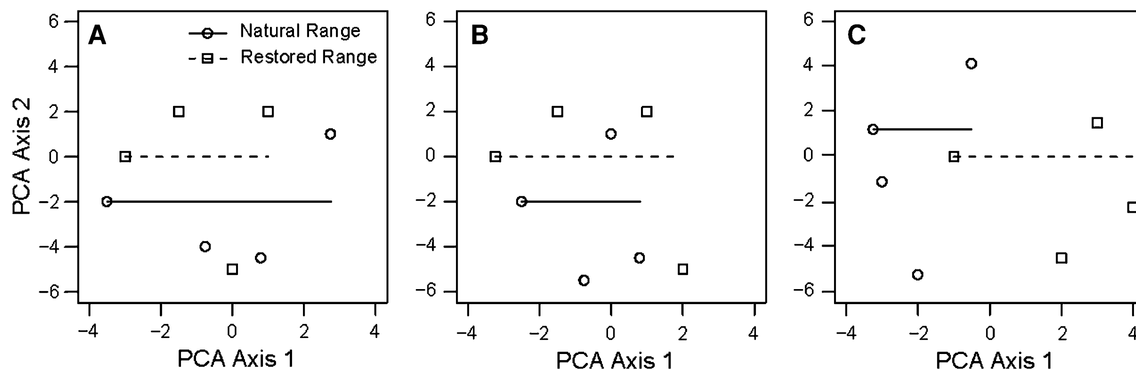

Fig. 2 Caricature of PCA arrangements showing: **a** allelic composition within natural sites larger (100% overlap) than within restored sites (<100% overlap), **b** allelic composition within restored sites greater (100% overlap) than within natural sites (<100% overlap), **c** asymmetric allelic composition among natural and restored sites (both <100% overlap). *Round* and *square* symbols represent 8 theoretical genets from one natural/restored site pair (4 genet per site). *Lines* show the range of values along one of two PCA axes. Each axis is examined separately

Results

Genotypic diversity

We sampled 223 shoots from 8 natural and 148 shoots from 8 restored sites within the Chesapeake Bay (Table 1). On average, we sampled fewer individuals from each restored site ($\bar{x} = 18$ shoots) than from natural sites ($\bar{x} = 28$ shoots) due to the small size of many restoration plantings. A total of 69 shoots were sampled from the stock repositories, with an average of 17 shoots per repository.

Of the shoots sampled within the Chesapeake Bay, 241 were unique genets, and 41 of the shoots sampled from stock repositories were unique. Genotypic diversity as measured by $(G - 1)/(N - 1)$ ranged from 7 to 100% with an average of 62% (Table 1). The percentage of unique genets did not differ between natural versus restored sites ($\bar{x} = 62\%$ for both; $W = 45$; $p = 0.41$). However, within paired comparisons genotypic diversity was greater in natural sites than restored sites five out of the eight times (Table 1). In general, sites with the highest genotypic diversity were located in the northern Chesapeake Bay. The lowest genotypic diversity sites were the HL/TAR, SCN and SFP sites (Table 1). Samples taken from stock repositories typically supported fewer genotypes (43% unique genets), but they were not significantly less than either natural or restored sites ($H = 0.31$; $df = 2$; $p = 0.86$). The Wisconsin stock repository supported the highest genotypic diversity with 93% of sampled shoots belonging to different genets, and the 4 Anne Arundel Community College samples represented the same genet.

Specific genotypes were shared among paired sites within two sets. In the EN/ENR set, one genotype was sampled 4

Table 3 Private allele frequency for 13 alleles found across 8 sampled populations

Type	Code	Locus	Allele	Frequency
Natural	CP	AAGX030	165	0.038
	CP	M16	184	0.019
	EN	AAG004	400	0.043
	EN	AAG004	403	0.043
	DC	M49	195	0.033
	HWC	M49	198	0.019
	HWC	AAGX051	199	0.019
Restored	FBR	AAGX051	202	0.026
	WC	M13	286	0.038
	WC	M16	196	0.077
Stock	AACC	AAGX030	150	0.500
	USDA	AAGX071	248	0.056
	USDA	AAGX051	204	0.056

times in EN and 11 times in ENR; in the FB/FBR set, one genotype was sampled 1 time in FB and 9 times in FBR.

Measures of genetic diversity

The proportion of polymorphic loci (*P*) within genets sampled among sample locations averaged across populations was $\bar{x} = 0.90$ ($SD = 0.12$). On average, genets at natural and restored sites did not differ in polymorphic loci ($\bar{x}$ natural $p = 0.90$, $SD = 0.13$; $\bar{x}$ restored $p = 0.90$, $SD = 0.13$; $W = 40.5$; $p = 0.68$). Within restoration stock repositories, the average proportion of polymorphic loci for sampled genets was $p = 0.88$ ($SD = 0.13$), which was not different from either natural or restored sites ($H = 0.21$; $df = 2$; $p = 0.90$). There was no difference in the proportion of polymorphic loci between any of the 8 pairwise sets.

The average number of alleles per locus (*A*) across all sampled genets and loci was 8.10 ($SD = 3.25$). The average number of alleles per locus within individual sites was 4.24 ($SD = 1.15$). Genotypic diversity and uncorrected allelic diversity were strongly correlated ($y = -15.4231 + 7.3077x$; $R^2 = 0.77$; $p < 0.001$). Natural and restored sites supported genets with similar numbers of alleles per locus (natural $A = 4.51$, $SD = 1.26$; restored $A = 3.98$, $SD = 1.02$) before ($W = 54$; $p = 0.09$) and after ($W = 48$; $p = 0.27$) using rarefaction. Restoration stock repositories supported fewer alleles than either natural or restored sites, $\bar{x} = 3.22$ ($SD = 1.26$; $H = 9.84$; $df = 2$; $p = 0.007$), and the difference remained following rarefaction ($H = 5.94$; $df = 2$; $p = 0.05$). Following correction for multiple comparisons and rarefaction, allelic richness did not differ among any of the paired sites.

Eight sampled sites supported at least one of 13 private alleles. Each sample type (natural, restored, stock repository) supported at least one rare allele. Relative frequency of the private alleles in all but one of the sites varied from 0.02 to 0.07 (Table 3). Allele 150 at the AAGX030 locus had a frequency of 0.50 because it was present in a heterozygous state in the single genotype of the AACC stock repository sample.

Average observed heterozygosity of genets within all sample sites was 0.58 ($SD = 0.07$), and did not differ between all natural and restored sites combined ($\bar{x}$ natural $H_o = 0.59$, $SD = 0.05$; $\bar{x}$ restored $H_o = 0.56$, $SD = 0.09$; $W = 45$; $p > 0.41$). There was also no difference in observed heterozygosity between any of the 8 pairwise sets of natural-restored sites. Average observed heterozygosity of genets sampled from restoration stock repositories ($H_o = 0.64$; $SD = 0.21$) did not differ from natural or restored sites ($H = 3.60$; $df = 2$; $p = 0.16$).

Departure from Hardy–Weinberg equilibrium

Four loci departed significantly from Hardy–Weinberg equilibrium (Table 4). Three restored sites showed heterozygote deficit (Table 1): WC ($f = 0.20$; 0.04–0.26), LOC ($f = 0.13$; 0.04–0.21), and TAR ($f = 0.24$; 0.03–0.44). The AACC sample had only 1 unique genotype; therefore, *f* could not be estimated. Four sites showed signs of heterozygote excess: EN ($f = -0.06$; −0.13 to −0.03), HL ($f = -0.47$; −1.00 to −0.47), CPR ($f = -0.08$; −0.22 to −0.01), and ENR ($f = -0.11$; −0.27 to −0.04).

Based on analysis with the program Bottleneck (Cornuet and Luikart 1996), 4 of the 18 sites we could analyze (LOC, $p = 0.007$; HL, $p = 0.008$; FARM, $p = 0.001$; USDA, $p = 0.007$) showed evidence that H_e (expected heterozygosity) significantly exceeds H_{eq} (heterozygosity expected at equilibrium) indicating potential of a recent bottleneck.

Restoration practices related to genetic diversity

Of all restoration practices and genetic diversity measures, only the correlation between age of restoration sites and inbreeding coefficient was significant ($y = 0.21–0.013x$; $R^2 = 0.67$; $p = 0.008$; Fig. 3).

Shared allelic identity among natural and restored pairs

The first axis explained only 10.14% of the variation in allele frequencies, and the second axis explained only 6.31% of the variance. The small amount of variance explained by these axes indicates that variance in frequencies of different alleles cannot be easily collapsed into a smaller number of axes. Although the total variance is low, the amount represented on each of the first two axes captures the largest concentration of variation due to allele composition. Percent overlap within individual pairs of natural/restored sites along the first and

Table 4 Genetic diversity of individual loci over all samples

Locus	A	H_o	H_e	Percent missing data	f
AAGX071	10	0.74	0.78	0.355	0.055
AAGX051	13	0.80	0.87	2.482	**0.073**
AAGX012	6	0.61	0.62	0.000	0.024
ATG002	8	0.73	0.77	0.000	0.047
AAGX030	5	0.58	0.56	0.000	−0.043
M49	12	0.63	0.74	0.000	**0.138**
M13	10	0.67	0.80	1.773	**0.156**
AAG002	4	0.54	0.54	0.000	−0.007
M16	4	0.11	0.12	0.000	**0.116**
AAG004	9	0.59	0.63	1.064	0.065
Average	8.1	0.60	0.64	0.567	0.064
SD	3.25	0.19	0.21	0.903	0.063

A total number of alleles, H_o observed heterozygosity, H_e expected heterozygosity, f correlation of alleles within individuals within all samples. *Bold* $p < 0.05$

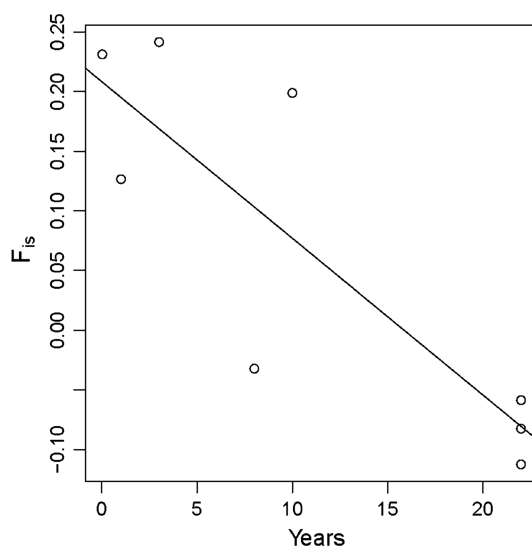

Fig. 3 Inbreeding coefficient (F_{is}) of restoration sites against age of restoration site in years

second PCA axes provides a measure of similarity in allelic composition between genets planted within a restored site and genets growing in natural sites. We observed three different patterns of overlap among restored and natural sites (Fig. 2) (A) The range of allelic composition within natural sites was larger than the range within restored sites (Fig. 2a; Table 5 (B) The range in allelic composition within restored sites was greater than the range within natural sites (Fig. 2b; Table 5). (C) Overlap of allelic composition among natural and restored sites was asymmetric, where both natural and restored sites had allelic composition that fell outside the range of the other site (Fig. 2c; Table 5).

When compared to all sites sampled from natural locations within the Chesapeake Bay, genets from the WISC stock repository were limited to a small portion of multivariate space (4.6% along axis 1, and 21.78% along axis 2). Genets from the USDA stock repository occupied 55% of the first axis and 64% of PCA axis 2. The single AACC genet fell within the range of allelic diversity sampled from the Chesapeake Bay, but many genets sampled from the FARM stock repository fell outside of the multivariate space occupied by genets we sampled Bay-wide.

Effective population size

Effective population size (N_e) in 8 sites (3 natural, 3 restored, and 2 stock repositories) ranged between 1.9 and 41.0 genets (Table 6). In the remaining 12 sites, estimates of N_e were indistinguishable from infinity, which occurs when linkage disequilibrium is less than sampling error (Waples 1991).

Discussion

Our results suggest that natural populations of *V. americana* in the Chesapeake Bay are genetically diverse (see also Lloyd et al. 2011) and that restoration practices are generally successful in re-establishing populations that are as genetically diverse as natural populations, especially when calibrated for the number of individuals sampled. All basic measures of genetic diversity (i.e., number of alleles, heterozygosity, proportion of polymorphic loci, number of genets) within restored sites mirror the levels of genetic diversity contained within their natural paired sites. N_e overlaps substantially among two paired natural/restored sites in which it could be measured. The detection of shared genotypes at two of the eight-paired sites shows that managers are at least in some cases either actively using local genets from adjacent sites, or that the restored sites have become integrated with their natural counterparts through vegetative expansion. The local nature of planting

Table 5 Percent overlap of paired natural-restored sites on the first and second PCA axes

	PCA axis 1 restored overlap with natural	PCA axis 1 natural overlap with restored	Scenario type	PCA axis 2 restored overlap with natural	PCA axis 2 natural overlap with restored	Scenario type
CP/CPR	100.00	70.71	A	93.36	99.93	C
EN/ENR	100.00	73.41	A	16.58	26.24	C
FB/FBR	81.67	100.00	B	100.00	79.05	A
DC/WC	96.68	85.86	C	67.93	90.84	C
HWC/LOC	92.41	88.32	C	67.37	100.00	B
SCN/SCR	95.27	82.27	C	45.74	100.00	B
SFP/GC	98.38	79.16	C	92.99	71.44	C
HL/TAR	100.00	50.84	A	7.48	100.00	B
WISC	100.00	4.60		100.00	21.78	
FARM	100.00	55.31		64.47	50.45	
USDA	100.00	55.31		100.00	53.59	

Scenario type corresponds to diagrams in Fig. 2; each axis was examined separately. Percent overlap for stock repositories was calculated based on all populations. Presence of only one genet within AACC precluded inclusion of this repository in this analysis

Table 6 Mean effective population size estimates and upper and lower 95% confidence intervals of sample sites

Abbreviated name	N_e	Lower 95%	Upper 95%
CP	34.50	18.20	109.60
CPR	29.70	12.70	595.90
FB	41.00	20.40	183.50
FBR	27.80	13.80	108.20
LOC	15.10	6.60	69.00
SCN	1.90	1.00	13.10
FARM	11.00	11.00	17.20
USDA	10.70	10.70	197.90

CP/CPR and FB/FBR are paired sites. FARM and USDA are repositories

stock is also confirmed by substantial overlap in allele composition between paired natural and restored sample sites that indicates that genetic material mostly does not originate from sources foreign to surrounding natural genets. Although the outlook is generally very positive, we found evidence for use of non-local stock in three sites and also identified signs that some restored sites might be planted with genetic stock from multiple populations based on differences in allele composition and departure from Hardy–Weinburg equilibrium. We also found reduced effective population size at eight sites that cause concern for future loss of diversity.

Genotypic diversity

Although genotypic diversity of *V. americana* in restored sites did not differ statistically from natural sites, five of the eight restored sites supported fewer genotypes than the paired natural sites (Table 1). Reduced genotypic diversity in *Zostera marina* has been associated with decreased shoot density (Ehlers et al. 2008), decreased resistance to heat shock (Reusch et al. 2005), and decreased resistance to grazing (Hughes and Stachowicz 2004). It has been associated with increased selfing (Reusch 2001), which can have subsequent effects on genetic diversity beyond initially low diversity. Prolonged periods of mating among close relatives within submersed aquatics can lead to substantial declines in reproductive fitness (Ruckelshaus 1995); however, the effect of inbreeding on fitness varies greatly among species (Hedrick and Kalinowski 2000). Because *V. americana* is dioecious, there is no risk of increased selfing. Rather, lower genotypic diversity decreases chances that both male and female plants will be present at a site, thereby reducing the potential for sexual reproduction. For example, the 6 genotypes documented from the SCN population are male, the SFP population is skewed towards female genets (4 female, 1 male; Engelhardt, pers. obs.) and the sample of 30 ramets from HL supported 1 known male and 1 female genotype. The low number of genets decreases opportunities for recombination at those sites and increased inbreeding will ensue as full and half sib offspring from a small number of parents reproduce (Hedrick and Kalinowski 2000).

Genotypic diversity affects basic measures of genetic diversity such as allelic diversity, heterozygosity, and Wright's fixation indices (Kalinowski 2005a; Pruett and Winker 2008). Consequently, the number of genotypes present in a population will necessarily affect analyses that rely on such basic genetic statistics. Our genetic diversity results could reflect low genotypic diversity in source populations. Moreover, the low genotypic diversity we

observed in three natural sites (i.e., SCN, SFP, and HL) presents a larger problem. If these sites were subsequently used for restoration stock material the resulting restored site would also have low genotypic diversity potentially creating the negative consequences that were discussed in the paragraph above.

Non-random mating

We detected departure from random mating based on significant heterozygote deficits in three (WC, LOC, TAR) of the eight restored sites (Table 1). Although the number of restored populations showing significant heterozygote deficit may be relatively small, they represent a large proportion of populations when compared to a Bay-wide sample, where only three of 27 natural populations showed significant heterozygote deficit (Lloyd et al. 2011). The deficits in the restored populations could be the result of true inbreeding or of mixing individuals from different gene pools during planting, in essence a restoration-induced Wahlund effect. The three sites with significant positive *f* values were planted with individuals from multiple donor populations (LOC and TAR) or from multiple repositories (WC; Table 2). Planting materials in the three sites were either plants germinated from seed exclusively or in addition to freshly harvested tissue.

Use of planting material derived from seeds germinated from a limited number of fruits, or sampled from one or a few clones could lead to planting a large number of full and half siblings. Subsequent mating among those individuals would increase the degree of inbreeding relative to source populations. If fruits are collected from different but spatially aggregated maternal genotypes, the same father or small set of fathers may have sired the seeds, which would also yield many full and half siblings. In fact, we saw that inbreeding coefficients of restoration sites significantly declined with the age of restored sites (Fig. 3), which supports the hypothesis that apparent inbreeding is due to mixing gene pools. The declines in the inbreeding coefficient with time could be the result of the establishment of Hardy–Weinberg equilibrium as individuals from the different gene pools mate and generate the expected number of heterozygotes. The majority of populations were not heterozygote deficient; thus, minimizing inbreeding is a relatively minor management concern. However, the issue could be completely avoided by increasing the spatial extent of sampling from within natural source populations thereby avoiding the use of many individuals from any single clone. Additionally, planting restoration sites in proximity to natural sites would facilitate gene flow among the sites and increase mixture among non-related individuals.

Genets sampled from restoration stock repositories were not out of Hardy–Weinberg equilibrium, which suggests that managers have been avoiding increased mating among close relatives. Alternatively, the original material grown in repositories may have be representative of a source population that were in Hardy–Weinberg equilibrium and those genotypes have been maintained following initial cultivation. There is evidence of population bottlenecks within both the FARM and USDA sites. This is a situation that gives cause for concern if the lack of genotypic diversity and apparent population bottlenecks are not due to sampling error but rather due to a true lack of diversity in FARM and USDA stocks. Periodically adding new genetic material from the wild to repositories is essential for alleviating the issues of low genotypic diversity and the effect of bottlenecks.

Effective population size

Guidelines for effective population sizes necessary to maintain genetic diversity range from $N_e \geq 50$ to prevent greater than 1% loss of heterozygosity per generation (Soule 1980; Franklin 1980), to $N_e \geq 500$ to prevent loss of alleles through genetic drift (Soule 1980), to upwards of N_e 1000–5000 (Lynch and Lande 1998) to maintain long-term evolutionary potential. Although there is still debate about which if any of these effective population sizes are necessary or sufficient for maintaining genetic diversity, the mean estimates of N_e in the 8 sites we could measure ($N_e = 1.90$–41) were well below all of the commonly accepted suggestions, and the upper limit of the 95% confidence interval exceeded 500 in only one site. Large deviations between N_e and census size are known for a number of marine organisms (Palstra and Ruzzante 2008) and can result from sampling across genetic neighborhoods in continuous populations (Neel et al. submitted). The small sizes we observed are potentially of concern, thus their cause needs to be better understood.

Reduced effective population size can rapidly increase rates of loss of genetic diversity (Ellstrand and Elam 1993), leading in general to increased inbreeding, decreased fitness, and decreased survivorship (Newman and Pilson 1997; Hedrick and Kalinowski 2000). Low population size or planting densities may suffer from decreased population growth rates (i.e., Allee effect), which can play a substantial role in the outcome of restoration plantings (Deredec and Courchamp 2007). Evidence of population bottlenecks within one restored site (LOC) reinforces that either planting sizes may be too small or initial plantings contained too few individuals to support long-term fitness and survivorship. We know from plant invasion literature that number of propagules and invasion events are critical determinants of persistence and expansion (e.g., propagule pressure hypothesis; Richardson and Pysek 2006; Zayed et al. 2007). Thus, the number of individuals planted and the number of planting events should impact the overall

success of a restoration effort as well. In animal restoration programs, increased reintroduction size and frequency are known to correlate with restoration success (Griffith et al. 1989; Hopper and Roush 1993). We recognize the practical constraints associated with restoration plantings. However, plantings in larger areas should be encouraged or, at the very least, a large number of individuals should be planted within each site through time. Collecting individuals from large areas within donor patches would also be beneficial.

Overlap of allelic composition

The large degree of overlap in allele composition among natural, restored, and stock repositories implies that managers are typically matching the allelic composition of adjacent natural sites. However, interpretation of these results is hampered by the low explanatory power of the first two PCA axes (Fig. 2). The limited variation explained is indicative of a high degree of shared alleles among individuals across sites. Regardless, each of the three scenarios of allele composition overlap we note (Fig. 2; Table 5) highlights a different type of genetic risk. When individuals for restoration represent a limited genetic pool, the allelic composition of the resulting restored site will represent a subset of the natural allelic composition, which can negatively impact both immediate plant growth and long-term individual plant fitness (Williams and Davis 1996; Williams 2001). The departures were minimal in this direction, which is a positive result and indicates low potential for genetic diversity being limited due to poor stock selection.

Conversely, genetic diversity of restored and restoration stock genets either did not overlap or extended beyond observed natural variation in three cases: SCR, ENR, and FARM. This potentially indicates mixing of sources or potential sample bias that is introduced when sampling a greater proportion of the population in sparse restoration sites. The FARM site in particular had greater range along PCA axis 2 than did any other site we sampled. This could be problematic if individuals from this repository were used for planting within the Chesapeake Bay. When allelic composition of restored sites does not overlap with natural diversity, populations can experience outbreeding depression (Fenster and Dudash 1994; Montalvo and Ellstrand 2001) or exhibit decreased fitness as the result of being maladapted to local conditions (Linhart and Grant 1996; Fenster and Galloway 2000; Montalvo and Ellstrand 2000). We observed negative inbreeding coefficients within both the EN and ENR sites and also within CPR, which indicates an excess of heterozygous individuals at these sites. An excess of heterozygous individuals can result from the recombination of genotypes from populations with different allelic composition. Given the geographic proximity of EN and CP to their restored counterparts, it is possible that the recombination of diverse gene pools is driving the observed negative inbreeding coefficients.

Further investigation is required to determine if either scenario (greater diversity or lack of overlap) affects fitness of restored or stock populations, but it does emphasize the need to avoid planting too few individuals or genotypes with limited or highly varied genetic diversity. An effort to avoid exchanging materials among regions should also be actively adopted to maintain similar patterns of allelic composition. We detected genetic isolation between the Northern and Central Bay, with the division line roughly between DC and FB (Lloyd et al. 2011), suggesting that movement of genetic materials across large geographic distances is limited. The majority of restoration projects have already been using local plant tissue for restoration.

Conclusions

The issues relating to small population size, increased inbreeding, and a lack overlap in allelic composition are not ubiquitous across *Vallisneria americana* restoration sites and stock repositories. With a few minor changes to propagation and planting protocols, as well as propagule collection techniques, we expect that the genetic diversity of restored populations will directly mirror naturally occurring genetic diversity within the Chesapeake Bay. However, simply mirroring naturally occurring genetic diversity may not be enough. The relationships between genetic diversity of *V. americana* and the resulting ecological functioning and ecosystem services are ripe for increased investigation. Such understanding will provide insight into the role of genetic diversity in returning seagrass beds to their prior ecological prominence in the Chesapeake Bay.

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