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# A genetic test for recruitment enhancement in Chesapeake Bay oysters, *Crassostrea virginica*, after population supplementation with a disease tolerant strain

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**Abstract**

Many of the methods currently employed to restore Chesapeake Bay populations of the eastern oyster, *Crassostrea virginica*, assume closed recruitment in certain sub-estuaries despite planktonic larval durations of 2–3 weeks. In addition, to combat parasitic disease, artificially selected disease tolerant oyster strains are being used for population supplementation. It has been impossible to fully evaluate these unconventional tactics because offspring from wild and selected broodstock are phenotypically indistinguishable. This study provides the first direct measurement of oyster recruitment enhancement by using genetic assignment tests to discriminate locally produced progeny of a selected oyster strain from progeny of wild parents. Artificially selected oysters (DEBY strain) were planted on a single reef in each of two Chesapeake Bay tributaries in 2002, but only in the Great Wicomico River (GWR) were they large enough to potentially reproduce the same year. Assignment tests based on eight microsatellite loci and mitochondrial DNA markers were applied to 1579 juvenile oysters collected throughout the GWR during the summer of 2002. Only one juvenile oyster was positively identified as an offspring of the 0.75 million DEBY oysters that were planted in the GWR, but 153 individuals (9.7%) had DEBY ×wild F1 multilocus genotypes. Because oyster recruitment was high across the region in 2002, the proportionately low enhancement measured in the GWR would not otherwise have been recognized. Possible causes for low enhancement success are discussed, each bearing on untested assumptions underlying the restoration methods, and all arguing for more intensive evaluation of each component of the restoration strategy.

## Introduction

Determining the magnitude of demographic connectivity among marine populations is of fundamental importance for effective fisheries management, conservation of small populations, population restoration and the design of marine protected areas (MPA). Unfortunately, dispersal

distances and the geographic scale of recruitment have rarely been measured in marine species on the ecological time scales relevant to conservation and management (Thorrold et al. 2002). Most species-specific estimates of gene flow have been made on an evolutionary time scale, using genetic measures of population differentiation to infer a long-term average rate (Neigel, 1997; Kinlan and Gaines, 2003). Connectivity over the short-term is not predictable from most evolutionary approaches because they are based on simplified migration models, assume equilibrium between migration and genetic drift, and are imprecise in situations with moderate to high gene flow (Waples 1998; Whitlock and McCauley, 1999). Nonetheless, a strong association between average dispersal distance and duration of larval residence in the plankton (Shanks et al. 2003; Siegel et al. 2003) suggests that features of life history might serve to predict realized gene flow. However, differences in larval behavior among species and heterogeneity of dispersal distances within species ranges make it imprudent to extrapolate from this broad association to specific populations (Hare and Avise, 1996; Hilbish, 1996; Baker and Mann, 2003; Rose et al. in press).

The phenotypic tagging and later recapture needed to directly measure short-term population connectivity is extremely difficult to apply on a useful scale in marine species with high fecundity and high juvenile mortality (reviewed in Thorrold et al. 2002), although there are notable recent successes (Jones et al. 1999). Perhaps the most promising application of mark and recapture to marine systems involves the analysis of natural 'tags' based on genetic variation (Hansen et al. 2001; Milbury et al. 2004), environmental markers (Swearer et al. 1999; Thorrold et al. 2001), or a combination of the two. Methods also have recently improved for estimating the proportion of first-generation migrants based on the analysis of natural population differences using highly polymorphic genetic markers (Pritchard et al. 2000; Wilson and Rannala 2003; Paetkau et al. 2004). The practical utility of these latter methods in natural populations, however, requires three things: (1) representative samples of all relevant source populations, (2) sufficient numbers of informative genetic markers, and (3) sufficient genetic heterogeneity among potential source populations to permit their discrimination (Hansen et al. 2001). The third requirement presents a conundrum in many situations because even low levels of persistent migration among populations will homogenize genetic variation, eliminating our ability to detect and measure gene flow on an ecological time scale relevant for management (Palumbi, 1996; Bohonak, 1999; Manel et al. 2005). There are several potential avenues for working around this conundrum to measure population connectivity in real time, including the analysis of non-equilibrium perturbations imposed by management procedures (applied here) or the use of high resolution data to detect genetic patterns that decay slowly after a migration event (e.g., linkage disequilibrium, Estoup et al. 2000).

In the Chesapeake Bay, the eastern oyster (*Crassostrea virginica* Gmelin) has declined in abundance for more than a century due to overfishing, declining water quality, loss of reef habitat, and since the late 1950s, protozoan parasitic diseases (Burreson and Ragone Calvo, 1996; Boesch et al. 2001; Jackson et al. 2001). Efforts to increase the abundance of eastern oysters in Maryland and Virginia have included the construction of artificial reef habitat using oyster shell, designation of oyster 'reserves' or 'sanctuaries', and seeding of reefs with hatchery-propagated juveniles or large adults purchased from fishermen. Results have been encouraging in some cases as evidenced by locally elevated recruitment in sub-estuaries where broodstock were planted at high density (Southworth and Mann, 1998; Brumbaugh et al. 2000). For example, in 1997 approximately $1.14 \times 10^6$ wild oysters with a mean shell total length of $\sim$95 mm were planted in the Great Wicomico River at a density of 300 $m^{-2}$. This same year the recruitment of juveniles in that river increased many times above the previous five year average (Southworth and Mann, 1998). This correlation provided convincing evidence for enhancement of recruitment because Southworth and Mann (1998) provided corroborative data on broodstock fecundity, larval abundance and water circulation. Furthermore, nearby tributaries without supplemental broodstock showed no spike in oyster recruitment during 1997 (Southworth et al. 2004), so the Great Wicomico recruitment could not be explained by a regional change in environmental conditions.

Evidence of successful enhancement in the Great Wicomico River helped shape the restoration strategies subsequently used in Chesapeake

Bay (Luckenbach et al. 1999; Mann, 2000; U.S. Army Corps of Engineers, 2003). The important feedback provided by restoration monitoring is only helpful in the long run, however, if it is based on methods that can reliably measure failure as well as success. Also, the methods of greatest value will be those that provide spatial and temporal resolution on the degree of enhancement, especially if regional restoration plans hinge on the details of local enhancement in 'nursery' tributaries (U.S. Army Corps of Engineers, 2003; also see below). Interestingly, only two out of six sites in the Great Wicomico River showed elevated recruitment in 1997, and these were both upriver from the broodstock planting (Southworth and Mann, 1998). Two lessons are pertinent from Southworth and Mann (1998); (1) recruitment enhancement can be patchy within Chesapeake tributaries, and (2) their methods would provide equivocal evidence for local enhancement in a year when regional recruitment was high because both processes produce similar increases in recruitment (Southworth et al. 2004). The only way to remove this uncertainty and directly measure the magnitude and spatial pattern of enhancement is to distinguish spat (juvenile oysters) derived from restoration broodstock versus non-restoration ('wild') oysters.

Revised oyster restoration strategies in Virginia grew out of a perception that protozoan disease pressure is the primary obstacle to restoration. Eastern oysters bred for tolerance to infection by both *Perkinsus marinus* (Dermo disease) and *Haplosporidium nelsoni* (MSX disease), originally developed for use in aquaculture (Ragone Calvo et al. 2003), have been used for restoration seeding of oyster reefs since 1999 (Brumbaugh et al. 2000). If disease tolerant oyster strains can survive and reproduce for longer than natural hatchery-raised seed, and certain sub-estuaries are more conducive to larval retention, then these locations may potentially serve as persistent natural 'incubators' for local recruitment of disease tolerant progeny. In this vision of 'terraforming the Bay' (Allen et al. 2003), regional population enhancement could be achieved by dredging the disease tolerant spat in 'incubator' sub-estuaries and transplanting them to other areas of priority. A further advantage proposed for this strategy is the potential for large scale inoculation of wild populations with alleles underlying disease tolerance (Allen et al. 2003). This untested strategy has been adopted by the Army Corps of Engineers for Virginia waters with primary efforts initially focused on the Great Wicomico River as a retentive incubator (U.S. Army Corps of Engineers, 2003).

Thus, current oyster restoration efforts in Chesapeake Bay rest on four unconfirmed assumptions: (1) that disease tolerant oyster strains will have sufficient overall fitness in the wild to make a substantial contribution to recruitment compared to wild broodstock; (2) that recruitment is predominantly local in the Chesapeake sub-estuaries being used as incubators; (3) that the disease tolerance of artificially selected oyster strains is not compromised by interbreeding with wild oysters; and (4) that the mixing of artificially selected and wild stocks results in genetically healthy populations with sufficient variation for long-term viability. This study focused on testing assumptions 1 and 2 and provides preliminary results bearing on assumption 4.

The first assumption has previously been addressed by showing that growth rate and survivorship under disease challenge of DEBY-strain oysters was equivalent or higher than wild controls (Ragone Calvo et al. 2003). Other components of fitness such as fecundity have not been measured in DEBY oysters, so there could be fitness-related traits that suffered during selection for disease tolerance. Also, strong predation is expected on some hatchery-bred oysters relative to wild (see Discussion), so enhancement success may be a function of predation strength more than magnitude of the planting, fecundity or disease tolerance.

With respect to the second assumption, the magnitude and consistency of larval retention has never been measured directly. However, several indirect lines of evidence collectively make a strong case for local oyster recruitment in Chesapeake Bay in general and in some tributaries in particular. First, Bay-wide analysis of population structure using microsatellite markers showed a significant pattern of isolation by distance, i.e., genetic divergence increasing with aquatic distance, at the scale of the entire Chesapeake Bay (Rose et al. in press). Second, several studies have demonstrated the potential for larval retention based on hydrodynamics, larval behavior, or both (reviewed in Kennedy, 1996). Third, previous studies in the Great Wicomico River (Southworth and Mann, 1998) found an association between

retentive drifter movements, larval abundance, and spat recruitment that were interpreted as evidence for local recruitment. These lines of evidence taken together suggest that larval retention mechanisms act with enough regularity (at a yet undefined spatial scale) to produce isolation by distance over an evolutionary time scale.

A side-effect of artificial selection for disease tolerance has been a strong shift in allele frequencies at neutral marker loci relative to 'wild' Chesapeake oysters and a narrowing of molecular genetic variation. Thus, restoration plantings of these selected strains provides an opportunity to genetically test for local recruitment based on mark and recapture, with the genome of every offspring from the selected-strain broodstock indelibly marked by inbreeding. We report here on an artificially selected *C. virginica* strain planted in 2002 in two Chesapeake tributaries where larval retention was assumed. Restoration plantings typically entail hatchery breeding of a small number of individuals (amplification), setting of the larvae on shell substrate, and growth during a nursery period before planting at high density on a reef. Random variability in allele frequencies between hatchery amplification spawns of the same selected line can affect the ability to genetically distinguish restoration plantings from wild oysters. Here we analyzed two separate amplification spawns of a single selected strain of oyster, planted in two distant sub-estuaries, to determine our power to genetically discriminate them from each other and from the wild populations into which they were planted. One of the two plantings in 2002 included oysters of reproductive age, so multilocus assignment tests were applied to juveniles collected in 2002 to test for local recruitment.

## Materials and methods

### *Oyster planting*

We selected two study sites, Little Choptank River, Maryland (LCR), and Great Wicomico River, Virginia (GWR), based on hydrodynamic evidence for 'trap-like' circulation and/or historical oyster recruitment patterns suggestive of local recruitment. Into these sites we deployed oysters from the DEBY strain of *C. virginica*, derived from four generations of selection at the Virginia Institute of Marine Science starting with oysters from Delaware Bay (Ragone Calvo et al. 2003). Plantings of this strain in Chesapeake Bay prior to 2002 were very limited and did not occur near the two study sites (R Brumbaugh and D Meritt, unpublished data).

For the LCR planting, we set larvae produced from a mass spawn of DEBY broodstock on clean oyster shell in nylon mesh bags at the Horn Point hatchery (University of Maryland Center for Environmental Studies) and grew them to approximately 10 mm total shell length in nursery waters, before planting approximately one million spat-on-shell directly onto a subtidal shell bed within a private lease (Figure 1a) during July 2002. In Virginia, DEBY larvae were set on shell fragments ('cultchless') in a commercial hatchery during 2001 and grown through the winter in floating screen boxes. Between June 14 and July 10, 2002, an estimated 785,700 of these DEBY oysters were planted in eight batches within a harvest sanctuary of the GWR (Shell Bar Reef; Figure 1b). At the time of planting, shell length averaged 64.1 mm (SD among 8 batch means = 1.8 mm). In both rivers the plantings were dispersed over the top of a single subtidal mound reef. We expected reproduction of DEBY oysters during summer 2002 in the GWR but not the LCR based on average size at reproduction (Thompson et al. 1996).

### *Sampling*

DEBY reference samples for the LCR consisted of 100 individuals collected from the restoration reef one month after planting. The DEBY reference sample for the GWR planting consisted of 82 individuals randomly subsampled at the time of planting. Adult Chesapeake Bay oysters for use as 'wild' reference samples were collected by dispersed dredging away from known restoration or commercial lease sites in both the LCR (April 2002) and GWR (May and September 2002).

To monitor spatfall, we deployed spat collectors at six or seven sites in each tributary (Figure 1) from July through October, cycling in fresh oyster shell substrate for settlement every two weeks. In the LCR, spat collectors consisted of 2–4 extruded plastic mesh bags filled with clean oyster shell (~1/3 bushel each) hung from a piling at 0.3–1.0 m below mean low water. In the GWR, spat collectors were wire mesh cages filled with clean oyster shell (0.02 $m^3$ each) suspended from

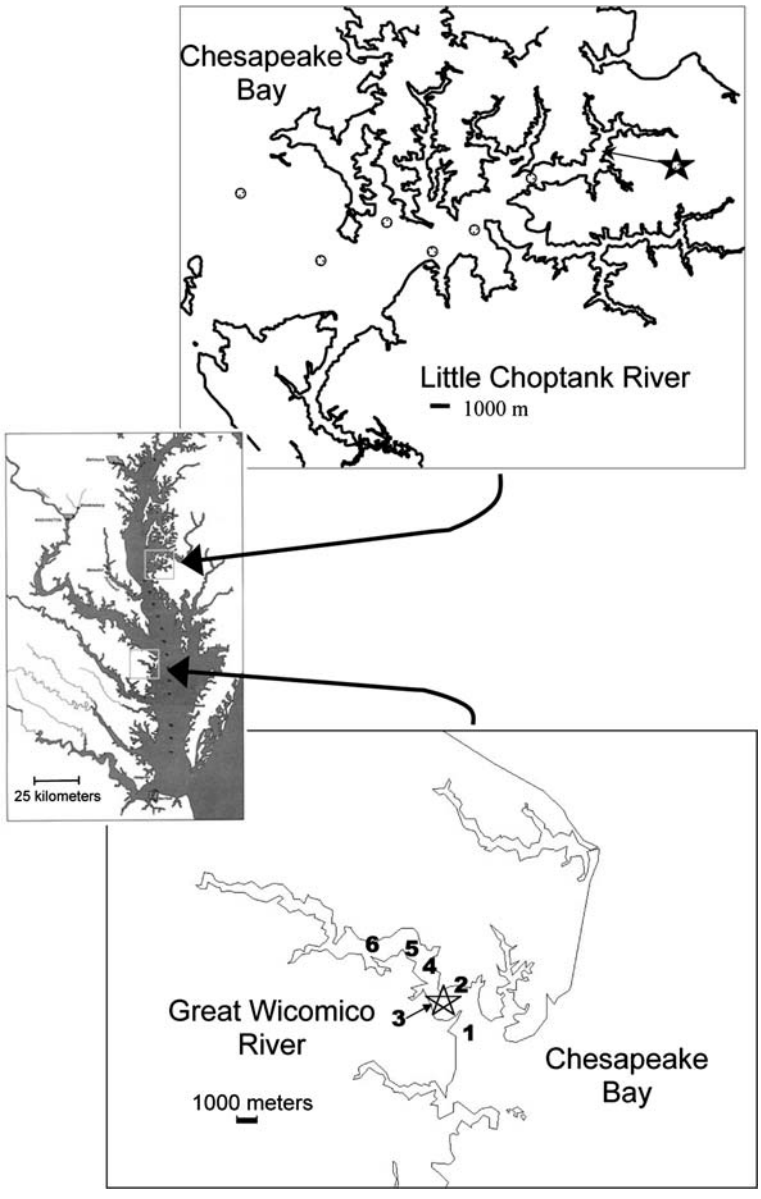


*Figure 1.* Maps of study sites in the Little Choptank River (LCR), and Great Wicomico River (GWR), with their spatial proximity shown with boxes on a map of Chesapeake Bay. Spat collection sites are shown as circles in the LCR and are numbered in the GWR, with one collection site in each river coincident with the DEBY broodstock planting site (star).

docks or resting subtidally on oyster reef substrate. Sediment was washed from shells before examination by eye for spat. Spat were preserved whole in 90–95% ethanol.

*Genotyping*

We extracted DNA from all or part of the soft tissue from individual oysters using either a FastPrep FP120 robot (BIO 101, Vista, CA; Virginia samples) or QIAGEN DNeasy kits (Maryland samples) using the animal tissue protocol and eluting in the vendor's AE buffer. Spectrophotometric readings at 260 nm were used to quantify and standardize DNA concentration at 50 ng/$\mu$l.

We assayed mitochondrial DNA haplotypes using a combination of two or three restriction fragment length polymorphisms (RFLP) at

cytochrome oxidase (CO) I, COIII or NADH dehydrogenase 4 (ND4) genes. These RFLPs all have very different frequencies in wild and DEBY oysters. Polymerase chain reaction (PCR) amplification of COI and COIII gene portions followed Milbury (2003) with *Hae*III and *Hin*fI digestion, respectively, to score RFLPs. The ND4 RFLP was assayed as described in Hare and Avise (1996). Digestion profiles were scored after electrophoresis in agarose gels with ethidium bromide staining. Two RFLPs were usually sufficient to distinguish between two prevalent haplotypes. We assayed ND4 to determine mtDNA haplotype by majority rule when COI and COIII RFLPs disagreed.

In addition, after optimization of our PCR procedures, we genotyped each individual in both the wild and DEBY reference samples at ten microsatellite loci using 7.5 $\mu$l PCR reaction volumes, 0.3 U *Taq* polymerase (Invitrogen), and 0.2 $\mu$M final concentration of forward and reverse primers, but with the forward primer a mixture of fluorescently labeled and unlabeled primer. PCR optimization procedures included extensive testing of high and low-stringency amplification conditions on apparent homozygotes and heterozygotes to test for allele drop-out and amplification of paralogous alleles. Overall genotyping efficiency was improved by amplifying most loci individually and co-loading no more than two fluorescently labeled PCR products with an internal size marker (ROX 500, Applied Biosystems) in a single capillary of an Applied Biosystems 3100 genetic analyzer. Two loci either lacked sufficient variation to be informative (Cvi-2k14, data not shown) or showed non-Mendelian results (e.g., three alleles within an individual for Cvi-1g8, Reece et al. 2004). A full description of these loci and their primers is given in Brown et al. (2000) and Reece et al. (2004). Optimized PCR conditions for the eight microsatellite loci used in this study are given in Rose et al. (in press).

We binned alleles into length classes by eye based on the allele length frequency distributions from several hundred individuals. Genescan ver. 3.7 and Genotyper ver. 2.5 (Applied Biosystems) software were used for quality control and automated genotyping. If initial results for any locus in any individual showed either no signal or if an apparently homozygous genotype had signal amplitude <500 relative fluorescent units, we used additional *Taq* enzyme to reamplify that locus in that sample. If results did not change we scored the genotype as null in the first case or homozygous in the second case if signal amplitude was >100 relative fluorescent units.

*Data analysis*

Gene diversity was calculated as in Nei (1987, eq. 7.39, p. 164). Because the number of alleles is highly dependent on sample size, we also compared estimates of allelic richness among populations (Goudet, 2001). To test for significant deviations from Hardy–Weinberg equilibrium (HWE) within and between populations we used permutation tests with Weir and Cockerham's (1984) *F*-statistics. For within-population tests we permuted alleles among individuals within samples. Population differentiation was tested by permuting genotypes among samples. We tested for linkage disequilibrium (LD) by permuting genotypes within loci and samples. All of these calculations and tests were done with FSTAT ver. 2.9.3 (Goudet, 2001). Significance was adjusted to a table-wide alpha of 0.05 using a strict Bonferroni correction.

We performed assignment tests following Cornuet et al. (1999) using GeneClass2 ver. 2.0.d (Piry et al. 2004). This program calculates the log-likelihood for the assignment of each multilocus genotype tested against each reference sample (representing potential source populations). The relative likelihood of assignment of an 'unknown' individual to wild and DEBY oyster source populations was evaluated based on a log-likelihood difference statistic, $\Lambda = [-\log 10\mathrm{L}(\text{wild source})] - [-\log 10\mathrm{L}(\text{DEBY source})]$. This statistic has positive values for genotypes similar to DEBY and negative values for genotypes similar to Chesapeake Bay wild oysters. A $\Lambda$ value of zero indicates equal support for assignment in the two potential sources, whereas values of 1, 2 or 3 (positive or negative) indicate that assignment is 10, 100 and 1000 times more likely to one population relative to the other, respectively. The criterion used for computation of the assignment log-likelihoods was either Bayesian (Rannala and Mountain, 1997), or for the purposes of accuracy comparisons, genetic distances were used (Cornuet et al. 1999). In the first case this means that the likelihood of a genotype in a population depends on the allele frequencies estimated for that population under an assumption of Hardy–Weinberg equilibrium (Paetkau et al. 2004). To ameliorate the potential for sampling error, the Bayesian procedure

estimates allele frequencies from a Dirichlet prior distribution that narrows the possible allele frequencies when there is a larger sample. The genetic distance criterion, in contrast, is based on a measure of allele sharing between individuals, averaged across all the pairwise comparisons between a test subject and individuals in a reference sample. No assumption of Hardy–Weinberg equilibrium is required to calculate the genetic distances. Assignment of each individual is made to the population with which it has the smallest average genetic distance.

Assignment accuracy was measured using leave-one-out reassignment tests (Paetkau et al. 1998; Hansen et al. 2001) in which each individual in turn is removed from a reference sample and treated as an unknown in assignment tests to all potential source populations. Re-assignments were based on reference sample allele frequencies calculated after removal of each individual to avoid upward bias of assignment success. Accuracy was calculated as the proportion of mock unknowns that were correctly assigned, out of all assignments attempted. Because assignment accuracy can be asymmetrical among reference populations (Davies et al. 1999), we calculated it for each reference population separately.

Assignment accuracy depends on the stringency of assignment criterion used. When reference samples (known source) include individuals that are misassigned at one level of stringency (e.g., low assignment power results in positive $\Lambda$ when it is expected to be negative), it prompts application of a higher stringency level for evaluation of unknowns to minimize false positives. In other words, when the $\Lambda$ distributions overlap for leave-one-out results from two reference samples, then an assignment criterion of zero will produce misassignments. Using a more stringent assignment cut off of $|\Lambda| > 1$, 2 or 3, as is commonly done (Roques et al. 1999; Campbell et al. 2003), usually reduces the proportion of individuals that can be assigned while also reducing incorrect assignments (Campbell et al. 2003). We have reported the distribution of $\Lambda$ values so that the stringency of acceptable assignment is at the discretion of the reader.

One assumption of assignment tests is that reference samples are representative of potential source populations. When assignment tests are conducted using markers with a large number of alleles at low frequency (e.g., microsatellites), in populations with high genetic diversity, it is possible for sampling error to generate low accuracy or biased assignments (despite the application of Bayesian priors; Cornuet et al. 1999; Paetkau et al. 2004). Leave-one-out accuracy measurements will not reveal this limitation, so we extended the procedure to leave-*n*-out assignment tests to assess sensitivity of assignment accuracy to the reference sample size. The sample of DEBYs from LCR (100 individuals) was larger than that from GWR (82), so we used the former for these tests. In each of ten replicates, $n$ DEBY individuals were randomly chosen and their multilocus genotypes removed to a separate file for analysis as unknowns. The unknowns were compared against reference samples consisting of the remaining DEBY individuals from LCR and the combined wild reference. For example, 75 random DEBY individuals were moved to a new file and treated as unknowns for testing against the remaining 25 DEBYs and the entire wild reference sample. This was done ten times for $n = 90$ and 75, corresponding to DEBY reference sample sizes of 10 and 25 individuals. Average accuracy (with $\Lambda > 0$ stringency) was compared to leave-one-out results (reference sample size 99).

Using the leave-one-out procedure in each of two reference samples also provides a measure of assignment confidence based on the degree of overlap between $\Lambda$ distributions for the two reference samples. These distributions could be used to calculate an exclusion probability, the probability that an individual *does not* originate from a particular source population based on whether the test subject's $\Lambda$ value is more extreme than 95% of the reference individuals (a one-sided test for each reference population). Assignment to one source population (by the criteria above) and exclusion from all others would provide a more conservative criterion than assignment alone. However, even a large sample from a reference population contains a miniscule proportion of the possible multilocus genotypes for a given set of allele frequencies, so exclusion probabilities calculated from empirical $\Lambda$ distributions will be biased downward (too liberal). A more robust exclusion probability was calculated for individual oyster spat assigned to the DEBY source population by simulating 10,000 multilocus genotypes expected from the allele frequencies in the DEBY reference sample, assuming random mating and linkage equilibrium. Assignment scores

were then calculated for each of the simulated genotypes against the DEBY reference sample, generating an assignment criterion ($\Lambda$) distribution against which the oyster spat assignment scores could be compared (Cornuet et al. 1999). We used this method, implemented in GeneClass2, to estimate exclusion probabilities.

We assessed the impact of deviations from HWE on assignment accuracy by comparing results using raw and permuted data. Alleles were permuted within samples for each locus using GENETIX 4.04 (Belkhir et al. 2001). By default, GENETIX permutes everything except null homozygous genotypes, so the permuted data had the same amount and pattern of missing data (null homozygotes). Permutation therefore eliminated deviations from HWE within populations without changing gene diversity or population allele frequencies. Multilocus genotypes, the unit of analysis in assignment tests, were scrambled within populations by this procedure but remained representative of those expected from random mating. Genotypes were also permuted among individuals within samples to assess the impact of linkage disequilibrium on assignment accuracy.

Some spat were genotyped for only a subset of the microsatellite loci. We used the critical population procedure in the WHICHLOCI program (Banks et al. 2003) to rank order the loci in terms of assignment accuracy to the DEBY population and preferentially assayed more informative loci.

Assignment methods were also used to test whether the multilocus genotype of each oyster spat was consistent with expectations for F1 progeny of a DEBY $\times$wild cross. These tests used only microsatellite loci and were made with the Bayesian procedures implemented in IMMANC5 (Rannala and Mountain, 1997) by comparing the DEBY reference sample against the (predominantly wild) GWR spat sample. The alpha level for significance was set at 0.05 and the simulation used for testing significance was replicated 1000 times.

## Results

### *Genetic diversity and differentiation of potential source populations*

In the LCR we did not expect the DEBY oysters planted in 2002 to reproduce that year because their shell length averaged less than 5 cm. Thus, 164 newly settled spat collected in the LCR during 2002 were evaluated as a wild reference sample along with adults dredged from the LCR ($n$ = 100) and GWR (90). The microsatellite loci were highly variable in these reference samples, with the total number of alleles per locus ranging from seven to 36 and gene diversity per locus (heterozygosity) ranging from 0.61 to 0.95 (Table 1). Deviations from HWE within samples were common and always caused by heterozygote deficiencies, sometimes quite extreme (e.g., $F_{IS}$ = 0.55 at 2i4 locus in GWR-DEBY). However, two loci showed no deviations (Cvi2g14, Cvi2i23) and two others showed fewer and more moderate deviations (Cvi2j24, 1g3) from HWE. There was some indication that null alleles contributed to the heterozygote deficits. When four or more loci failed to amplify from an individual we interpreted this as a result of poor genomic DNA and removed the individual from the data set. In the remaining data from reference individuals (Table 1), the proportion of individuals that had zero, one, two or three null homozygous genotypes (out of eight) was 77.5, 19, 3, and 0.5%. Comparing each reference sample-by-locus, the magnitude of $F_{IS}$ for a locus showed a significant positive relationship with the proportion of individuals null for that locus (ANOVA with 39 df, $P = 0.015$).

DEBY oysters had lower genetic diversity compared with Chesapeake Bay wild oysters. The combined DEBY samples had lower allelic richness (one-tailed sign test, $P < 0.05$) and a trend toward lower gene diversity (two-tailed sign test $P = 0.07$) than the combined wild populations. Although there was no difference in the magnitude of $F_{IS}$ in DEBY versus wild oysters (Table 1), significant LD was only found in the DEBY oysters. In the combined wild sample there was no evidence for LD among the microsatellite loci after Bonferroni correction ($N$ = 373, adjusted alpha = 0.00036, all pairwise $P > 0.0032$). In contrast, there were nine and eleven pairwise locus comparisons with significant LD in the LCR and GWR DEBYs, respectively (some of them marginally so; all $P \leq 0.00036$, the adjusted alpha). Eight of these pairwise locus comparisons involving Cvi2g14, Cvi2i23 and Cvi2i4, were significant in both DEBY samples.

The oysters used here to represent wild populations were also included in a study that found

*Table 1.* Diversity statistics by locus for DEBY and wild reference samples

| | *n* | | Cvi-2g14 | Cvi-2i23 | Cvi-2i4 | Cvi-2j24 | Cvi-12 | Cvi-9 | Cvi-i24 | Cvi-1g3 | Average |
|---|---|---|---|---|---|---|---|---|---|---|---|
| DEBY | | | | | | | | | | | |
| LCR | 100 | Number of alleles | 12 | 10 | 11 | 7 | 13 | 10 | 11 | 4 | 9.75 |
| | | Gene diversity | 0.838 | 0.814 | 0.772 | 0.802 | 0.816 | 0.832 | 0.815 | 0.679 | 0.80 |
| | | Fis | 0.07 | 0.045 | **0.491** | 0.143 | **0.527** | **0.315** | **0.303** | 0.06 | 0.24 |
| | | % Null | 0.000 | 0.010 | 0.160 | 0.040 | 0.120 | 0.070 | 0.050 | 0.060 | 0.064 |
| GWR | 82 | No. alleles | 12 | 11 | 11 | 8 | 8 | 9 | 10 | 5 | 9.25 |
| | | Gene diversity | 0.849 | 0.821 | 0.842 | 0.784 | 0.802 | 0.775 | 0.795 | 0.652 | 0.79 |
| | | Fis | −0.104 | −0.07 | **0.554** | −0.065 | **0.261** | **0.427** | **0.307** | **0.272** | 0.20 |
| | | % Null | 0.024 | 0.000 | 0.024 | 0.037 | 0.073 | 0.012 | 0.049 | 0.024 | 0.030 |
| Total DEBY | | No. alleles | 15 | 13 | 15 | 8 | 14 | 11 | 13 | 5 | 11.75 |
| | | Gene diversity | 0.854 | 0.828 | 0.826 | 0.809 | 0.830 | 0.820 | 0.825 | 0.681 | 0.809 |
| Wild ref | | | | | | | | | | | |
| GWR adult | 91 | Number of alleles | 29 | 28 | 21 | 10 | 23 | 14 | 16 | 7 | 18.50 |
| | | Gene diversity | 0.949 | 0.899 | 0.928 | 0.861 | 0.886 | 0.897 | 0.875 | 0.635 | 0.87 |
| | | Fis | 0.102 | 0.01 | 0.093 | 0.116 | **0.16** | 0.073 | **0.383** | 0.133 | 0.13 |
| | | % Null | 0.033 | 0.000 | 0.099 | 0.033 | 0.011 | 0.088 | 0.044 | 0.022 | 0.041 |
| LCR spat | 164 | Number of alleles | 29 | 28 | 24 | 14 | 20 | 18 | 17 | 7 | 19.63 |
| | | Gene diversity | 0.95 | 0.897 | 0.919 | 0.878 | 0.869 | 0.908 | 0.867 | 0.613 | 0.86 |
| | | Fis | 0.018 | 0.024 | **0.219** | **0.297** | **0.313** | **0.297** | **0.468** | **0.248** | 0.24 |
| | | % Null | 0.000 | 0.018 | 0.006 | 0.012 | 0.000 | 0.006 | 0.049 | 0.061 | 0.019 |
| LCR adult | 118 | Number of alleles | 27 | 28 | 21 | 16 | 16 | 17 | 18 | 7 | 18.75 |
| | | Gene diversity | 0.948 | 0.88 | 0.927 | 0.874 | 0.865 | 0.907 | 0.898 | 0.654 | 0.87 |
| | | Fis | 0.035 | 0.067 | **0.104** | **0.273** | **0.213** | **0.405** | **0.389** | 0.175 | 0.21 |
| | | % Null | 0.000 | 0.008 | 0.051 | 0.000 | 0.017 | 0.042 | 0.042 | 0.025 | 0.023 |
| Total wild | | Number of alleles | 31 | 36 | 27 | 18 | 25 | 19 | 23 | 7 | 23.25 |
| | | Gene diversity | 0.950 | 0.891 | 0.924 | 0.872 | 0.871 | 0.905 | 0.880 | 0.630 | 0.865 |
| Overall | | % Null average | 0.011 | 0.007 | 0.068 | 0.024 | 0.044 | 0.044 | 0.047 | 0.039 | 0.036 |

Bold $F_{IS}$ values are significantly different from zero ($P \leq 0.05$). Proportion of homozygous null genotypes = '% null'.

low levels of genetic differentiation structured in a pattern of isolation by distance across Chesapeake Bay (Rose et al. in press). Here, no significant microsatellite differentiation, as measured by $F_{ST}$, was detected among the wild adults from GWR and LCR, or between wild adults and LCR spat. Also, preliminary assignment tests treating the LCR spat as unknowns did not identify any DEBY-like spat, as expected. Therefore, oyster spat from the LCR were combined with wild adults from LCR and GWR to make a combined wild reference sample.

Microsatellite allele frequencies were significantly differentiated between the pooled wild reference sample and each DEBY sample ($F_{ST} = 0.053$ and 0.062 averaged across loci for GWR and LCR, respectively, with $P < 0.0001$ for both). The DEBY samples were also significantly different from each other ($F_{ST} = 0.038$; $P < 0.0001$).

Collapsing all mtDNA variation into two haplotypes, frequencies were significantly differentiated ($P \leq 0.0002$) between the wild reference sample and each DEBY sample ($F_{ST} = 0.82$ for LCR, 0.67 for GWR) as well as between the LCR and GWR DEBY samples produced from separate hatchery spawnings ($F_{ST} = 0.093$, $P \leq 0.002$). The most common haplotype in the wild had frequencies of 0.99, 0.55 and 0.31 in the wild, GWR DEBY and LCR DEBY samples, respectively.

### *Assignment tests, checking assumptions and measuring accuracy*

For accurate assignment tests, the reference sample must be representative of genetic diversity in the potential source populations. It is not obvious, however, what size reference sample is sufficient for

a given level of microsatellite diversity. Leave-*n*-out analysis with LCR DEBY data showed little loss of accuracy for DEBY assignments when reference sample sizes were reduced from 99 to 25 (Figure 2). Because Bayesian assignment tests assume Hardy–Weinberg genotype frequencies, but no such assumption is necessary for assignments based on genetic distances (Cornuet et al. 1999), we also used leave-*n*-out procedures to test which method is more accurate given the observed deviations from HWE. The Bayesian assignment method had 94% accuracy, better than that achieved with Cavalli-Sforza and Edwards distance-based assignments at all reference sample sizes (Figure 2). Accuracy of leave-one-out assignment for wild oysters was 99%.

These results were consistent with previous simulations (Cornuet et al. 1999) and provided confidence that for DEBY assignments, our sample sizes were sufficient and that Bayesian assignment procedures provided the highest accuracy despite deviations from assumptions. However, one of the oyster reference samples deviated from both the Hardy–Weinberg and linkage equilibrium assumptions, so we attempted to discern which factor caused reduced accuracy. When alleles were permuted within samples to remove Hardy–Weinberg and linkage disequilibrium (while maintaining differentiation between samples), accuracy of all assignment methods improved to 99–100% (Figure 2). The same improvement in accuracy was generated by permuting genotypes within samples, instead of alleles, to remove linkage disequilibrium among loci while maintaining heterozygote deficits within loci (results not shown).

We examined the log-likelihood $\Lambda$ distributions for all individuals of known source to further quantify accuracy. Results of Bayesian leave-one-out assignments for GWR and LCR DEBY oysters are shown in Figure 3a and b relative to the distribution for the combined wild reference sample. The 94% accuracy for self-assignment of LCR DEBY samples reflected $\Lambda$ distributions with little overlap except for DEBY outliers with high probabilities of deriving from wild parents (Figure 3a). This could indicate that DEBY oysters planted in the LCR became contaminated with wild oysters in the hatchery during breeding or, alternatively, wild oysters settled on the DEBY spat-on-shell before the DEBYs were sampled. The DEBY oysters planted in the GWR had a narrower distribution of $\Lambda$ scores than did LCR DEBYs (compare Figure 3 a and b), but overlap between DEBY and wild $\Lambda$ distributions resulted in 96% self-assignment accuracy for GWR DEBYs (Figure 3b). The wild reference oysters had a self-assignment accuracy of 99% when compared with GWR DEBYs and $\Lambda$ scores were as high as 1.76, indicating that values greater than this (stringency of $\Lambda > 2$) are necessary for confident assignment of unknowns as DEBY progeny (Figure 3b).

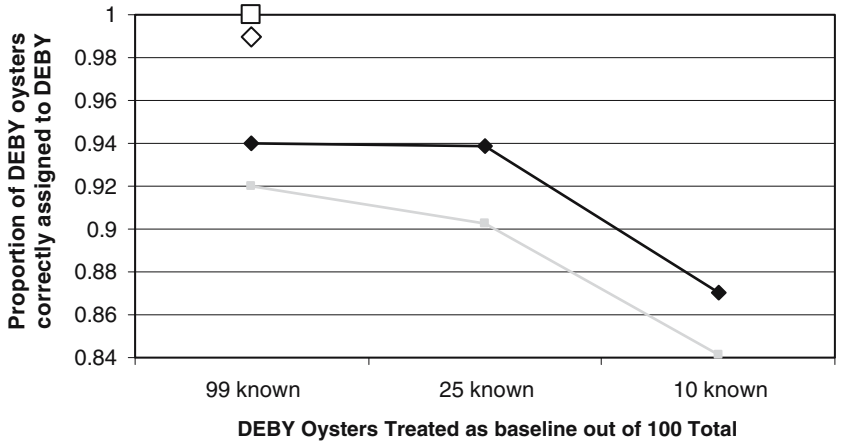


*Figure 2.* Leave-*n*-out accuracy analysis using LCR DEBY reference sample split into 'known' and mock 'unknown' fractions. Lines show results for unpermuted data using Bayesian estimates of allele frequencies (black with diamonds) or Cavalli-Sforza and Edwards genetic distances (gray with squares). Open symbols show results for leave-1-out assignment tests after permuting alleles to eliminate deviations from Hardy–Weinberg and linkage equilibrium.

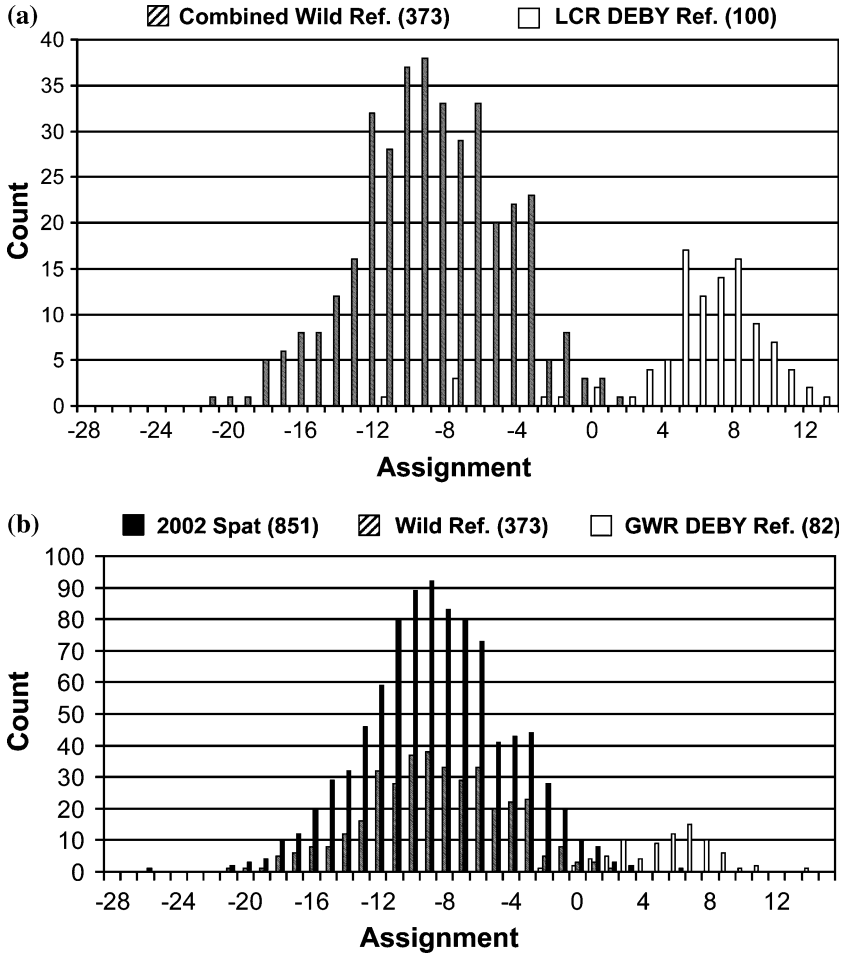


*Figure 3*. Assignment log-likelihood Λ distributions for LCR DEBY reference sample relative to the wild reference sample (a) and GWR DEBY reference compared to the same (b). Assignment Λ scores for 2002 GWR spat are also shown in b. The reference distributions in slashed and white columns are based on a leave-one-out procedure using individuals of known source whereas GWR spat, shown with black columns in (b), were all treated as unknowns in assignment tests against the two reference samples. Positive scores indicate that a multilocus genotype is more likely to derive from the DEBY source population, negative scores are more likely with a wild source. Every unit away from zero corresponds to an order of magnitude higher assignment likelihood for one source population relative to the other.

*Assignment testing of 2002 recruits*

A total of 1579 spat were collected in 2002 from the GWR (Table 2) and analyzed in two sets. First, 851 spat with 7 to 9 loci scored (mtDNA plus microsatellites) were subjected to assignment tests. The number of spat with 9, 8 and 7 loci was 424, 235, and 192. These missing data mostly resulted from a decision not to genotype the least informative microsatellite loci. However, missing mtDNA data from 37 individuals (2.3%) and null single-locus microsatellite genotypes from approximately 141 individuals (8.9%) probably indicated poor quality DNA or null mutations. Figure 3b shows the distribution of Λ scores for these spat relative to the reference distributions. The distribution for spat is nearly identical to that for Chesapeake wild oysters except it has a slightly longer tail of positive Λ scores. Fourteen spat have Λ scores that are positive, with the three highest values equal to 2.121, 2.517, and 5.167. This is equivalent to assignment likelihoods that are two to five orders of magnitude higher for DEBY versus wild oyster source populations. Simulation-based exclusion probabilities calculated for these DEBY-like individuals mostly had

*Table 2.* Analyzed spat collected on seven dates in 2002 from six sites in the Great Wicomico River, Virginia

| Date | Collection location | | | | | | Totals |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | |
| 22 July | 73 | 44 | 54 | 20 | 69 | 32 | 292 (25) |
| 2 August | 0 | 50 | 48 | 55 | 57 | 27 | 237 (10) |
| 15 August | 27 | 36 | 57 | 4 | 59 | 19 | 202 (9) |
| 30 August | 0 | 56 | 37 | 51 | 57 | 60 | 261 (6) |
| 13 September | 31 | 43 | 32 | 49 | 47 | 49 | 251 (65) |
| 27 September | 29 | 60 | 39 | 50 | 34 | 33 | 245 (15) |
| 10 October | 5 | 14 | 17 | 28 | 22 | 5 | 91 (23) |
| Totals | 165 | 303 | 284 | 257 | 345 | 225 | 1579 |

Numbered collection locations are shown in Figure 1. Number of spat identified as DEBY ×wild hybrids shown in parentheses.

moderate values for both reference samples, i.e., neither could be formally excluded. Only one individual, with the 5.167 assignment score, had an exclusion probability (probability of not belonging) that was more than 0.1 lower for the wild reference than for the DEBY reference ($P_{sim}$[DEBY] = 0.558, $P_{sim}$[wild] = 0.243). In this respect the empirical and simulation criteria agreed only for this single individual, collected September 27 at the collection site 6 km upstream from the planting.

A second set of 728 spat had a minimum of four and maximum of six of the most informative microsatellite loci scored (Cvi-2g14, Cvi-2i23, Cvi-2i4, Cvi-9) plus mtDNA in most cases. The number of individuals with six, five, and four loci were 107, 614, and 7, respectively. Accuracy of leave-one-out assignment of GWR DEBY oysters using only the four most commonly scored loci was 95%. No spat in this second set satisfied both the Λ-score and simulation assignment criteria. Scoring additional loci in the ten individuals with the highest positive Λ scores did not change their assignments.

If DEBY reproduction in the GWR had mostly consisted of crosses with wild oysters, the resulting F1 'hybrid' progeny would not be identified applying the above criteria. Therefore, in order to test for wild×DEBY crossing, we attempted assignment tests of all 1579 spat against expectations under this F1 hypothesis. Unfortunately, the power of these tests with the available data is insufficient to assign any one spat as an F1 hybrid with strong confidence given the level of divergence between reference groups (Rannala and Mountain, 1997). However, if random sampling error is the cause of false positives, then under the null hypothesis they should be randomly distributed among sampling periods, whereas a true signal of F1 hybrid recruitment should be heterogeneous in time because of synchronous spawning in the tightly aggregated DEBY plantings. A total of 153 spat (9.7%) had significant likelihood of being F1 hybrids (Table 2) and these individuals were temporally clustered compared with expectations based on the number of spat collected on each of seven sampling periods from July to October ($\chi^2$, $P \ll 0.001$). The only two collection dates containing substantially more hybrid spat than expected under the null hypothesis were September 13 (65 instead of 24) and October 10 (23 instead of 9). The distribution of these F1 progeny across sites was not significantly different from expectations based on sample sizes ($P = 0.09$).

## Discussion

Because natural oyster recruitment in Chesapeake Bay varies tremendously across sub-estuaries and years, it is extremely difficult to evaluate the effectiveness of enhancement efforts from the number and distribution of spatfall. We have used genetic differences between selectively bred, disease tolerant restoration broodstock and wild Chesapeake Bay oysters to directly measure the local recruitment attributable to a large restoration planting. One of 1579 juvenile oysters from the GWR was positively identified as DEBY progeny. This recruit was sampled in September, 2002, 6 km upriver from the DEBY broodstock planting. In addition, genotypes in 9.7% of the 2002 spat had

DEBY × wild F1 multilocus genotypes and these spat were statistically overrepresented in September and October samples. These are the first direct measurements of recruitment enhancement and dispersal distances for this species that we are aware of (but see Milbury et al. 2004). Recruitment upriver from the restoration planting is consistent with patterns of larval movement found in the GWR by Southworth and Mann (1998), but our 2002 data fall short of the return rate needed to measure the spatial pattern of enhancement throughout the GWR. Nonetheless, by any measure, the magnitude of population enhancement found in 2002 for the GWR was below expectations given the large, high density planting of DEBY oysters and the previous indirect evidence reported for successful enhancement in the GWR after identical placement of broodstock in 1997. Before interpreting the possible causes of apparently poor enhancement success and the implications of these results for oyster restoration procedures, we address the strengths and weaknesses of our genetic analyses.

*Robustness of assignment test results*

The accuracy of our assignments of individual recruits to wild versus DEBY source populations derives from the allele frequency differences between these groups at multiple highly polymorphic microsatellite loci. The lower allelic richness of DEBY oysters compared with wild confirmed that selection and/or hatchery amplification of DEBY broodstock had a substantial bottleneck effect on the genome and probably caused the allele frequency differentiation. The linkage disequilibrium found among microsatellite loci in DEBY reference samples also indicated inbreeding. If the LD was caused by physical linkage among loci then it would also be evident in the large wild reference sample, but it was not. For mtDNA, differences between DEBY and wild oysters also have resulted from the presence in DEBY broodstock of a highly distinct haplotype characteristic of *C. virginica* in the Gulf of Mexico (Reeb and Avise, 1990).

Several technical aspects of the assignment tests deserve comment. First, overall assignment accuracy was similar for the second batch of spat analyzed with only 4–6 microsatellite loci scored (95% versus 94%). This pattern has been reported previously (Roques et al. 1999; Bernatchez and Duchesne, 2000; Guinand et al. 2004) and likely results from the exclusion of loci that add as much noise as signal. Second, the accuracy analysis suggested that heterozygote deficiencies and/or LD in DEBY samples reduce assignment accuracy, but there is no evidence that these violations of assignment test assumptions biased the results. When we calculated assignment likelihoods using genetic distances to avoid the assumption of HWE there was a loss of accuracy relative to the Bayesian method, but the same individuals were assigned to the DEBY reference sample (results not shown). Finally, based on Bayesian assignments using permuted data it appeared that LD in DEBY samples was the main cause of reduced accuracy because randomizing genotypes within loci, which removes LD but does not eliminate deviations from HWE, increased accuracy as much as when alleles were randomized within loci, which removes both types of disequilibrium.

These technical considerations bolster the confidence in identification of a single oyster recruit as DEBY progeny. This individual oyster carried a mtDNA haplotype that was at a frequency of 0.45 in the GWR DEBY oysters and only 0.005 in wild oysters. Thus, its mother was most likely a DEBY oyster. The eight-locus nuclear genotype for this individual spat included four alleles that each occurred at less than 0.06 frequency in the wild reference sample and had frequencies five to seven times higher in the DEBY oysters. The combined presence of these five alleles (mtDNA and nDNA) makes Bayesian assignment of this individual to the DEBY source population highly preferred over assignment to the wild source. However, the multilocus genotype of this individual was statistically identified as F1 between wild and DEBY, so its father may have been a wild oyster.

Based on dive surveys on Shell Bar Reef, GWR, in September 2002, an estimated 68,800 wild (naturally set) oysters of 'market size' (≥76 mm) were present, mostly at the bottom of the reef (J Wesson, Virginia Marine Resources Commission, unpublished data). Assuming that market size oysters were all reproductive during summer 2002, and given that most (but probably not all) first-year DEBY oysters were male (Cox and Mann, 1992), ample opportunities existed for DEBY × wild reproduction. We identified an overabundance of F1-like spat in the September

and October samples, the same time frame in which the single individual was assigned to a DEBY source. These data are all consistent with enhanced recruitment primarily deriving from F1 'hybrid' offspring produced by late-season reproduction.

Higher resolution genetic data, including additional independent markers or sets of linked markers (Falush et al. 2003), will be needed to measure this form of enhancement with more confidence. Both types of improvement are underway. Guidelines based on power analyses with simulated data suggest that a total of ten to twenty independent loci may be required (Cornuet et al. 1999). Unfortunately, with respect to oyster microsatellite loci described thus far, their signal to noise ratios vary enough that assignment power will need to be empirically determined.

*Do 2002 results constitute effective enhancement?*

The enhanced recruitment documented in the GWR in 1997 as a result of high-density plantings of wild oysters at Shell Bar Reef, the same site as our 2002 planting, suggests that a similar magnitude effect might have been expected with the DEBY broodstock planting in 2002. Indeed, overall levels of recruitment in the GWR during 2002 were substantially higher than during the previous four years, but this was also true in multiple Virginia sub-estuaries where relatively small restoration plantings were made (Southworth et al. 2004). It is unlikely that the magnitude of enhancement observed in 1997 would be detectable as such in an overall good recruitment year. Thus, the high recruitment observed regionally during 2002 makes the genetic data from the GWR a critically needed direct measure of enhancement success. Unfortunately, by this genetic measure, the proportion of DEBY progeny among all spat tested that year suggests that the restoration planting provided no more than ten percent enhancement (assuming that all the spat identified as F1 progeny were accurately assigned) of 2002 recruitment in the Great Wicomico River.

Formal mark and recapture estimates are impossible because available census data are inadequate for estimating the ratio of wild and planted broodstock in the GWR. Even more uncertainty would accompany estimates of relative larval production that depend on unmeasured aspects of fecundity and density-dependent fertilization. Thus, it is impossible at this time to formally derive a null hypothesis for the expected proportion of DEBY recruits. However, in terms of the stated restoration goal of increasing oyster census size ten fold by 2010 (Chesapeake 2000 Agreement, http://www.epa.gov/r3chespk/), extrapolating from the local enhancement measured here suggests that large improvements are needed. Several non-mutually exclusive factors may have contributed to low enhancement of oyster recruitment: (1) DEBY broodstock too few or too young, (2) DEBY mortality, or (3) larval flushing.

The recruitment enhancement seen in the GWR during 1997 resulted from a planting of wild oysters (fishery buy-back) that were more numerous ($1.2\times10^6$), larger (90 mm average shell length), and therefore more fecund than the DEBYs planted in 2002 (see Introduction, Southworth and Mann, 1998). The small average size of DEBY broodstock (60 mm) in 2002 may mean that only a portion of them matured that year, maturation might have been delayed until late summer, and the majority of reproductive individuals were probably male (*C. virginica* is protandrous, Cox and Mann, 1992; Thompson et al. 1996). A biased sex ratio could have reduced overall fecundity or mating success; or, with wild females present, it could have generated a cohort consisting largely of F1 hybrids.

The second possibility is that post-planting mortality of DEBY oysters was high before most of them could reproduce. At the end of September there were no oysters visually identifiable as DEBYs (i.e., growing uniformly without attachment to a whole shell as a result of a 'cultchless' larval set on shell fragments in the hatchery) found during a dive survey of Shell Bar Reef conducted by the Virginia Marine Resources Commission (J Wesson, VMRC, unpublished data). Potentially high-impact mortality factors included poaching, predators such as cow-nosed rays (*Rhinoptera bonasus*), and parasitic disease. Poaching has not been reported as a problem in the GWR (J Wesson, VMRC, personal communication), and rays were not reported as a mortality factor in previous supplementation plantings of wild (Southworth and Mann, 1998) or cultchless oysters (Brumbaugh et al. 2000). However, rays are known to be common in Chesapeake Bay and were

implicated as a rapid source of mortality on plantings of cultchless oysters in the GWR in 2004 (J Wesson, VMRC, unpublished data). The parasites *H. nelsoni* and *P. marinus* were active in the GWR in 2002 and were probably causing some mortality in wild oysters (Ragone Calvo and Burreson, 2003). However, disease mortality leaves open 'box' shells and these were not observed in high numbers during the dive survey of Shell Bar Reef. Whatever the cause of mortality, the genetic identification of DEBY recruitment in late summer 2002 indicates that early mortality of planted oysters was not 100%.

A speculative hypothesis constitutes the third possibility, that a weather event flushed most of the DEBY larvae out of the GWR. Strong winds or heavy rains could influence the hydrodynamic characteristics that typically retain oyster larvae in the GWR (H Wang, Virginia Institute of Marine Science, personal communication). This flushing scenario is not far fetched in the context of restoration because reproduction of the DEBYs is likely to have been highly synchronous, putting all the DEBY larvae in the plankton simultaneously, and subjecting them as a group to the affects of storms during the 2–3 weeks before settlement. Synchronous spawning is characteristic of this species (Galtsoff, 1938), but may be even more likely for a young even-aged cohort of DEBY individuals because of their high relatedness or if they all require most of the summer for sufficient gametogenesis. The most extreme wind event measured during the entire 2002 summer lasted nine hours during a high tide cycle on August 28 with wind speeds averaging 44 km/h and bearing 81°, nearly straight up river. The high tide during the storm was the highest during August 2002 and approximately 0.24 m above the predicted height. The DEBY recruits successfully identified must have been in the plankton during August and/or September, coincident with this storm. Although this hypothesis cannot be falsified without a more detailed hydrographic model indicating the magnitude of tidal surge needed to flush the GWR, the coincidence illustrates how average hydrographic trends promoting larval retention may not apply to specific cohorts experiencing extreme weather events.

None of these three plausible explanations for low DEBY recruitment can be rejected, but our results indicate that one or more assumptions made during attempted restoration, namely that DEBY oysters are viable and fecund after planting and their larvae are retained in the GWR, were unmet in 2002. Deployment of selected-strain oysters will only provide reliable oyster enhancement when more is known about these critical factors, and when steps are taken to eliminate their potentially catastrophic effects (e.g., predation). Of course, it is impossible to control the weather and expensive to manage the sex ratio, but if the magnitude and probability of their effects are known, then their potential impacts can be incorporated into restoration plans.

### *Implications for oyster restoration*

Our results indicate that current oyster restoration procedures focusing on disease tolerant strains of *C. virginica* entail the use of genetically depauperate broodstock for supplementation. This has implications at two levels, the practicality of continued genetic monitoring (considered here) and the long-term consequences of supplementation (restoration assumptions 3 and 4 in the Introduction, also see below). Every time a selected line of oysters is used for restoration it requires hatchery amplification, and this has the potential for creating population bottlenecks if small numbers of broodstock are used or if there is high variance in reproductive success in the hatchery. In this study, two independently amplified groups of DEBY oysters, both derived from the same generation of a single selection line, revealed that hatchery amplification did, in fact, result in differentiation between DEBY seed planted in Maryland and Virginia, probably due to separate bottleneck effects. This differentiation could provide advantages for distinguishing among and monitoring local enhancement efforts. However, if also makes it necessary to analyze reference samples after each hatchery amplification, substantially increasing the effort and expense of applying assignment tests.

Unfortunately, there is a down side to the iterative bottlenecks that have increased our assignment accuracy and thereby facilitated direct monitoring of restoration efficacy in this study. The inbreeding imposed by these procedures typically has detrimental affects on average fitness (Bierne et al. 1998; Launey and Hedgecock, 2001). The consequences of inbreeding depression could be immediate, lowering average viability or

fecundity in the seed oysters used for restoration. Alternatively, over the long-term, population supplementation with inbred stocks can cause the genetic health of wild populations to deteriorate (Waples and Do, 1994; Wang and Ryman, 2001). These risks have not been quantified for oysters in Chesapeake Bay. They must be weighed against the potentially positive affects disease tolerant oyster strains might have on census numbers and on disease management.

Multi-million dollar restoration efforts currently presuppose that the GWR is dependably 'trap-like' and can serve as a local catchment basin for recruits from selectively bred disease tolerant stock. Measurable success at the recruitment stage, however, also requires that seed oysters survive until reproduction, have high fecundity, and that larval retention mechanisms operate consistently. One or more of these factors prevented the DEBY oysters from having a significant enhancement effect in 2002. Our results suggest that the current restoration strategy deserves more thorough evaluation in terms of the post-planting mortality, the sex ratio and fecundity of DEBY seed oysters, and the magnitude and consistency of larval retention. Research is continuing on all these fronts.

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