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## Mitochondrial DNA markers allow monitoring of oyster stock enhancement in the Chesapeake Bay

Received: 2 September 2003 / Accepted: 6 January 2004 / Published online: 19 February 2004  
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**Abstract** Overharvesting, habitat degradation, and disease have resulted in a century of decline for Atlantic Coast populations of the eastern oyster *Crassostrea virginica* (Gmelin). The introduction of oysters with superior disease resistance (e.g. oysters from different geographical areas, or genetically improved strains) may be useful in restoration efforts. In 1997 the Oyster Recovery Partnership and the University of Maryland Center for Environmental Science planted more than four million Louisiana oysters in the Choptank River, which flows into the Chesapeake Bay, Maryland, USA. These oysters, which may be distinguished from Atlantic oysters by diagnostic single-nucleotide polymorphisms (SNPs) in their mitochondrial DNA, were expected to display enhanced survival and reproduction as a result of their superior resistance to Dermo disease. A high-throughput, synthesis-by-sequencing technique (Pyrosequencing) was used to determine the mitochondrial haplotypes of spat collected in the Choptank River and nearby regions of the bay. Of 3,545 spat collected in 1999, 2000, and 2001, 3,349 (94.47%) possessed the North Atlantic haplotype, 176 (4.68%) had the South Atlantic haplotype, and 3 individuals (0.08%) had the Gulf Coast haplotype. Detection of newly recruited oysters possessing the Gulf Coast haplotype in the Choptank River confirmed the survival and reproduction of the outplanted Louisiana oysters. If appropriate genetic tags are available, effective monitoring of stock-

enhancement projects can be achieved with high-throughput molecular genotyping techniques.

**Electronic Supplementary Material** Supplementary material is available in the online version of this article at <http://dx.doi.org/10.1007/s00227-004-1312-z>

### Introduction

The eastern oyster *Crassostrea virginica* is an ecologically and economically important estuarine species. After more than a century of overharvesting and habitat degradation, Atlantic Coast oyster populations continue to suffer high mortality from epizootics caused by MSX (*Haplosporidium nelsoni*) and Dermo (*Perkinsus marinus*) (Ford and Tripp 1996). It has been argued that the combined impact of the MSX and Dermo diseases is the most important factor preventing oyster population recovery, and that rehabilitation cannot occur without enhancing disease resistance in native populations (Andrews and Hewatt 1957; Ford and Tripp 1996).

*C. virginica* populations vary in their degree of disease tolerance. Gulf Coast oysters are more tolerant of Dermo than Atlantic Coast oysters (Bushek 1994; Gaffney and Bushek 1996). Bushek (1994) observed higher Dermo infection levels in inoculated oysters from the Atlantic Coast (Maine, New Jersey, and Virginia) than in inoculated Gulf Coast (Texas) oysters held under common-garden conditions. The presence of heritable variation for Dermo resistance in *C. virginica* was further suggested by Oliver et al. (2000), who observed significant intercorrelations among full-sib families in Dermo resistance, field survival, and protease inhibitor levels. The enhancement of natural disease resistance of native populations by transplanting hatchery-reared, disease-tolerant oysters is a potential management solution for *C. virginica*. However, in view of the high cost and potential biological impact of restoration efforts, critical monitoring and evaluation are imperative.

Communicated by J.P. Grassle, New Brunswick

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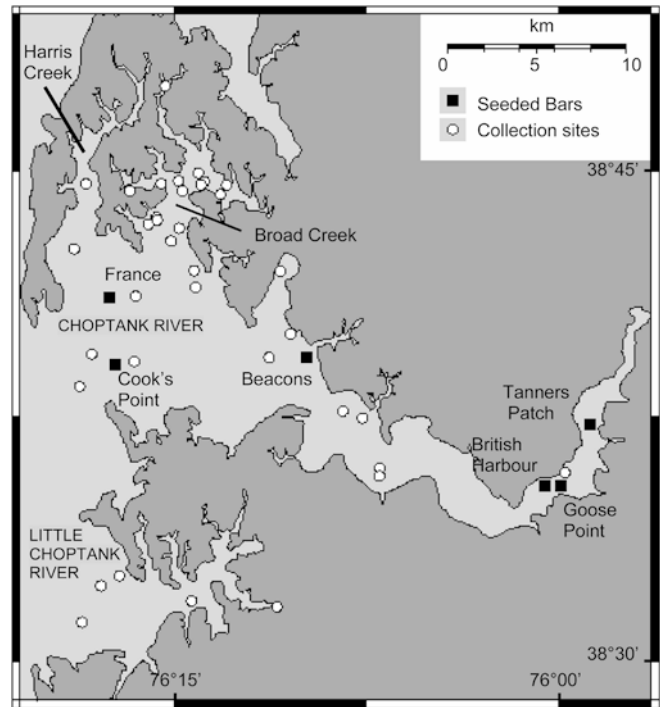
Eastern oysters exhibit natural genetic variation that may be exploited to discriminate between native oysters and enhancement stocks. Restriction fragment length polymorphism (RFLP) analysis of the entire *C. virginica* mtDNA molecule revealed the existence of two distinct haplotype assemblages corresponding to Gulf Coast and Atlantic populations, with an abrupt break on the Atlantic Coast of Florida near Cape Canaveral (Reeb and Avise 1990). Distinct regional variation has also been detected in the mitochondrial large subunit (16S) ribosomal gene of *C. virginica* (Ó Foighil et al. 1995; Wakefield and Gaffney 1996; Wakefield 1997). Major 16S mtDNA haplotypes are characterized by differences at two variable nucleotide positions (sites 288 and 296, Ó Foighil et al. 1995). The dominant haplotypes have the following sequences for the region 288–296 of Ó Foighil et al. (1995): North Atlantic 5'-TAAATTCTA-3', South Atlantic 5'-GAAATTCTA-3', and Gulf Coast 5'-GAAATTCTG-3'. The transition between the Gulf and Atlantic Coast 16S haplotypes occurs around Cape Canaveral (Avise 1992; Hare et al. 1996; Wakefield 1997). This large-scale population structure in *C. virginica* presumably reflects population history as well as contemporary physical and geographic boundaries to gene flow; similar patterns have been observed in other organisms such as the black sea bass (*Centropristis striata*) and the horseshoe crab (*Limulus polyphemus*) (Saunders et al. 1986; Avise 1992). In addition, a poorly defined transition zone for the 16S mtDNA gene of *C. virginica* is evident in the mid-Atlantic region, with distinct northern and southern Atlantic haplotypes differing by a single nucleotide (Ó Foighil et al. 1995; Wakefield 1997). The common 16S mtDNA haplotypes (North Atlantic, South Atlantic, and Gulf Coast) can effectively serve as genetic markers for assessing enhancement programs involving inter-regional transplants (Harrison 1989; Avise 1992; Gaffney 1996).

The goal of this project was to develop a high-throughput genetic protocol for detecting 16S haplotypes in *C. virginica* and to assess an oyster restoration effort conducted by the University of Maryland Center for Environmental Science and the Oyster Recovery Partnership. We used 16S mtDNA haplotypes to assess enhancement success by screening newly settled spat for the presence of Gulf Coast haplotypes among the native North and South Atlantic haplotypes of the Chesapeake Bay.

## Materials and methods

### Enhancement seeding

In October 1997 approximately  $4 \times 10^6$  hatchery-reared *Crassostrea virginica* (Gmelin) spat on shell were deployed on portions of six natural oyster bars throughout the Choptank River (Fig. 1), at densities of approximately  $500 \text{ m}^{-2}$ . Mean salinities in the Choptank River normally range from 5 to 8 at the most upstream sites (Tanners Patch, Goose Point, and British Harbour) to 10–15 at



**Fig. 1** Map of six bars in the Choptank River seeded with Louisiana oysters in 1997 and of spat collection sites from 1999 to 2001

France and Cook's Point bars. The Beacon Bar site normally experiences salinities between the upstream and downstream values. Oyster spat on shell that had tested negative for Dermo were deployed on specially prepared portions of the natural oyster bars. Spat were not tested for MSX, as this parasite has never been shown to occur at the Horn Point hatchery. Portions of each bar were delineated and covered with dredged fossil oyster shell. The seeded bars were each composed of two areas of dredged shell in a layer approximately 15 cm in depth in a flat configuration and one mounded site in which dredged shells were piled up to provide vertical relief of at least 1 m. As an exception, Tanners Patch was planted directly on a natural oyster bar with spat on shell, but without special bottom preparation.

### Sampling

To determine the survival and reproduction of the outplanted Louisiana oysters, juvenile oyster spat were collected throughout the Choptank River, its tributaries (Broad Creek and Harris Creek), and the Little Choptank River (Fig. 1; Electronic Appendix 1). Sampling efforts were focused on oyster bars known to be historically productive (Smith et al. 2003). Sampling commenced in 1999, 2 years after the 1997 planting.

*C. virginica* exhibits protandrous hermaphroditism, with a typical primary male phase after which a portion of the population transforms to reproduce as females (Coe 1934). It has been estimated that individuals in the Chesapeake Bay may take two to three seasons to transform to females and to significantly contribute to new recruitment (Coe 1934; Korrington 1952; Haley 1977, 1979; Hayes and Menzel 1981). As the 16S genetic markers are located in maternally inherited mtDNA, 2 years were allowed for the seeded individuals to reproduce as females in order to transmit the diagnostic Louisiana mtDNA haplotype to progeny. Sampling commenced in 1999, 2 years after the 1997 planting. Oysters were collected by dredging cultch with attached oysters from various natural oyster bars to target the fall recruitment period in 1999,

2000, and 2001. Dredged cultch was sorted manually and all spat <2 cm were collected. Due to variation in growth rate, it is not possible to discriminate with certainty between the new year class and any spat which may have resulted from the previous year's set with minimal growth; therefore, cohorts were collectively grouped and analyzed by collection date.

SCUBA surveys were conducted by K. Paynter (University of Maryland) in the fall of 1998, 1999, and 2000 to assess the survival of the seeded oysters. Three surveys were completed each year, late summer to fall. Nine quadrats were surveyed at each seeded bar; three from each of the two flat areas and three from the mounded area. New spat, live oysters, gapers, and dead oysters within 1/9-m<sup>2</sup> quadrats were counted to estimate spat density and mortality; when possible, three quadrats were surveyed at each of the seeded bars (K. Paynter, University of Maryland, personal communication). Additionally, 60 adult and spat samples were collected from the survey sites. The 16S haplotypes of these oysters were determined in order to verify the survival of the Louisiana spat on the planting grounds.

#### Tissue preparation

Adductor muscle and gill tissue were excised for DNA extraction. Samples were preserved in 95% ethanol and stored at 4°C. A simple Proteinase K digestion was conducted to obtain DNA suitable for PCR (polymerase chain reaction) amplification. For each sample, 1–2 mm<sup>3</sup> oyster tissue was added to 45 µl PCR buffer (500 mM KCl+100 mM Tris-HCl, pH 8.0) and 5 µl Proteinase K (20 µg µl<sup>-1</sup>) in a 96-well PCR plate. The plate was incubated at 65°C for 1 h, followed by denaturation at 94°C for 15 min, and was subsequently subjected to centrifugation for 1 min at ~140 g. The resulting supernatant was used as template for amplification.

#### Pyrosequencing

Detection of the 16S mtDNA genetic marker was conducted using a technique developed by Pyrosequencing AB. Pyrosequencing is a synthesis-by-sequencing protocol that utilizes an enzyme cascade and real-time pyrophosphate (PPi) detection (Alderborn et al. 2000). All reactions were performed in 96-well plate format with a 50 µl volume. The PCR reaction, per sample, consisted of the following: 5 µl 10× PCR buffer without MgCl<sub>2</sub> (Sigma-Aldrich); 4 µl 25 mM MgCl<sub>2</sub> solution (Sigma-Aldrich); 1 µl 10 mM dNTP 1:1:1:1 (0.2 mM concentration each) (MBI Fermentas); 1 µl 10 µM Biotin-TEG-labeled, HPLC-purified forward primer (5'-Biotin-TEG-AGATTTTTGGGTGGG-3'); 1 µl 10 µM unlabelled reverse primer (5'-CGAACTTTACTAGAGGATTG-3'); 0.3 µl 5 U/µl JumpStart Taq polymerase (Sigma-Aldrich); 35.8 µl sterile deionized water; and 2 µl DNA template from the extraction protocol described above. The thermocycling profile used was: 94°C for 2 min, 50 cycles of 94°C for 45 s, 50°C for 45 s, 72°C for 1 min, and extension at 72°C for 5 min; 50 cycles were completed to exhaust the Biotin-TEG-labeled primer. The PCR amplification yielded a 140-bp fragment of the gene encoding 16S ribosomal RNA.

Immobilization of the biotinylated PCR product was completed using streptavidin-coated Sepharose beads (Pharmacia Amersham) to obtain single-stranded template. Products were denatured, washed, and annealed as specified by Pyrosequencing AB. The final product was added to a PSQ plate and hybridized with the reverse sequencing primer (5'-GAAGTCAGGTCAAATCTAGT-3') at 80°C for 2 min. Sequencing was performed using the PSQ 96.

The PSQ 96 is an automated analytical instrument that utilizes an enzyme cascade system composed of four enzymes and specific substrates that generate light upon dNTP incorporation and PPi release during DNA chain elongation (Ronaghi 2001). Based upon the intensity of light produced at each nucleotide position, the SNP Software AQ subsequently assigns haplotype identification. Two

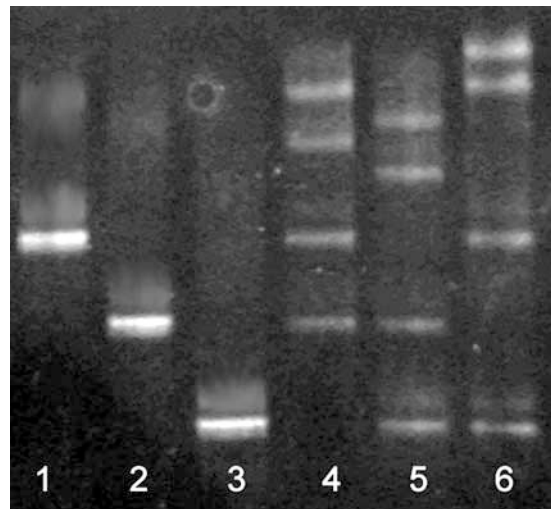
SNP sites were analyzed in this protocol; signal peaks at each of the SNP positions clearly indicate the haplotype of the oyster sample. Pyrosequencing was used to analyze the collected spat samples and an additional panel of control samples for verification of the technique (Milbury 2003).

#### Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) is capable of detecting small sequence variation in double-stranded DNA fragments, including single-nucleotide polymorphisms (Sheffield et al. 1990; Lessa 1992). The polyacrylamide gel contains denaturants (urea and formamide) distributed in a concentration gradient from the top to the bottom of the gel. Even single base changes in sequence may affect the melting profile of the molecule, altering its rate of migration through the gel. Previous research (Wakefield 1997) demonstrated that each of the three 16S mtDNA haplotypes of *C. virginica* produces distinct DGGE bands.

DGGE was performed to verify the 16S mtDNA haplotypes assigned by Pyrosequencing. Using PCR, oyster spat DNA was amplified to yield a 409-bp product, which included the region of DNA assessed by Pyrosequencing. The 25-µl PCR reaction consisted of the following: 2.5 µl 10× PCR buffer without MgCl<sub>2</sub> (Sigma-Aldrich); 2.0 µl 25 mM MgCl<sub>2</sub> solution (Sigma-Aldrich); 0.5 µl 10 mM dNTP 1:1:1:1 (0.2 mM concentration each) (MBI Fermentas); 0.5 µl 10 µM GC-clamped forward primer GC-16SAR (5'-CGCCGCCGCCGCTGTTTATCAAAAACAT-3') modified from Kessing et al. (1989) and 0.5 µl 10 µM reverse primer 16SOB (5'-ATTAGCCTGTTATCCCCGGCG-3') (Banks et al. 1993); 0.1 µl 5 U/µl Taq polymerase (Sigma-Aldrich); 17.9 µl sterile deionized water; and 2 µl template from the incubation/denaturation extraction protocol outlined above. The thermocycling profile used was: 94°C for 2 min, 35 cycles of 94°C for 45 s, 50°C for 45 s, 72°C for 1 min, and extension at 72°C for 5 min.

DGGE was used to analyze samples that were identified by Pyrosequencing as either Gulf Coast or rare haplotypes. To verify the genotypic assignments obtained by Pyrosequencing, an out-group heteroduplexing step (Campbell et al. 1995) was included in the DGGE protocol (Fig. 2). Heteroduplex formation was promoted by heating pooled PCR products (i.e. 6 µl PCR product combined with 6 µl of a North Atlantic reference standard amplicon) at 94°C for 5 min, immediately cooling them to -20°C, and



**Fig. 2** *Crassostrea virginica*. Denaturing gradient gel electrophoresis of 16S mtDNA showing heteroduplexed pairwise combinations [lane 1 North Atlantic (NA); lane 2 South Atlantic (SA); lane 3 Gulf Coast (GC); lane 4 NA+SA; lane 5 SA+GC; lane 6 NA+GC]

then slowly warming them to 4°C. Homo- and heteroduplex molecules were separated on 15–30% denaturing gradient polyacrylamide gels in 1× TAE running buffer [40 mM Tris acetate (pH 7.4), 20 mM glacial acetic acid, and 0.5 M Na<sub>2</sub> EDTA]. Electrophoresis was performed at a constant voltage (150 V) and temperature (60°C) for 4 h on a Bio-Rad DCode system. Gels were stained in 0.5 µg ethidium bromide ml<sup>-1</sup> for 10 min and visualized using a UV transilluminator and Kodak ID digital imaging system.

#### Restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) analyses of five additional mtDNA regions were conducted to assess mtDNA haplotype affinities of spat samples shown by Pyrosequencing to possess the Gulf Coast or rare 16S haplotypes. The regions assayed included: (1) cytochrome oxidase subunit I (COI; 718 bp amplicon); (2) cytochrome oxidase subunit III (COIII; 727 bp); (3) *cyt b*-COII, a 1,917 bp fragment spanning the 3' end of cytochrome *b* oxidase, cytochrome oxidase subunit II, and tRNA<sup>Ser</sup>; (4) ATP6-ND2, a 1,594 bp fragment spanning the 3' end of ATPase subunit 6, a 102 bp noncoding region, and the 5' end of NADH dehydrogenase subunit 2; (5) ND2-ND4, a 790 bp fragment spanning the 3' end of NADH dehydrogenase subunit 2, tRNA<sup>Arg</sup>, tRNA<sup>His</sup>, and the 5' end of NADH dehydrogenase subunit 4. Because Gulf Coast and Atlantic oysters show substantial mtDNA sequence divergence (Reeb and Avise 1990), we expected spat derived from Louisiana broodstock to show diagnostic Gulf Coast RFLP haplotypes for all mtDNA regions examined; any spat with Gulf-type (or rare) Pyrosequencing haplotypes that showed Atlantic RFLP patterns in the other mitochondrial regions would be considered mutant Atlantic haplotypes. PCR was conducted using the primer sets as described in Milbury (2003). The five amplicons were screened for diagnostic polymorphisms distinguishing Gulf from Atlantic oysters, using a variety of restriction enzymes: COI (*Mva*I, *Bsa*HI, *Hae*III), COIII (*Csp*6I, *Aff*III, *Bse*NI, *Bsa*HI), *cyt b*-COII (*Ase*I, *Bse*NI, *Bsi*HKAI, *Bsa*HI), ATP6-ND2 (*Apa*LI and *Ase*I), and ND2-ND4 (*Bst*NI and *Alu*I). Restriction digests were conducted as outlined in Milbury (2003).

## Results

For analysis of recruitment success, we determined the 16S genotype of samples collected within the Choptank River, its tributaries, and the Little Choptank River ( $N=3,545$  spat screened). Of these spat, 3,349 (94.47%) possessed the North Atlantic haplotype, 176 (4.68%) had the South Atlantic haplotype, and 3 individuals (0.08%) had the Gulf Coast haplotype (lower and upper 95% confidence intervals of 0.02% and 0.24%, respectively); an additional 17 individuals possessed rare 16S haplotypes (Table 1).

The rare haplotypes produced atypical pyrograms during Pyrosequencing. Three new variable sites were noted (Table 2). Incomplete reads were obtained for these rare individuals due to the designated iterative nucleotide administration cycle. (Alternative Pyrosequencing protocols would have enabled the detection of these unanticipated rare haplotypes, but at the expense of additional reagents and longer run times.) Despite the incomplete sequence, it remained possible to identify these individuals as possessing rare haplotypes.

DGGE analysis of the 16S region confirmed the Pyrosequencing results for both the Gulf Coast

and rare haplotype assignments. The putative Gulf Coast individuals exhibited the same DGGE banding patterns as oysters collected from the Gulf Coast, while the individuals scored as rare 16S haplotypes by Pyrosequencing also exhibited unique DGGE profiles.

RFLP analyses of other mitochondrial gene regions were consistent with the 16S identifications by Pyrosequencing. Restriction profiles for the five gene regions described previously were analyzed for each individual exhibiting a Gulf Coast 16S mtDNA haplotype (Table 3). Individuals with rare 16S haplotypes showed North Atlantic profiles across the other five gene regions. Spat exhibiting a Gulf Coast 16S haplotype showed characteristic Gulf Coast RFLP profiles, with the exception of one individual. This individual possessed a North Atlantic RFLP profile, indicating that the Gulf Coast 16S haplotype was a rare mutation in a North Atlantic mitochondrial genome. The remaining three spat with 16S Gulf Coast haplotypes exhibited some variation in RFLP profiles, but all were clearly members of the Gulf Coast haplotype group (Table 3). These three individuals composed 0.08% of the 3,545 samples analyzed.

The three individuals with Gulf Coast RFLP and 16S haplotypes were collected from British Harbour (October 1999), Mulberry Point (September 2000), and Neavitt Shell (April 2001). Exact R×C tests (StatXact, Cytel) detected no significant heterogeneity in the distribution of the three major 16S haplotypes within or among the sampling regions outlined in Table 1.

## Discussion

Through the use of high-throughput molecular techniques, we determined the 16S haplotype of several thousand oysters within the Choptank River and its surrounding tributaries. The majority (95%) of the *Crassostrea virginica* population possessed the North Atlantic haplotype. Roughly 5% possessed the South Atlantic haplotype.

In total, we found three individuals with the Gulf Coast haplotype, presumably progeny of the oysters planted in the Choptank River in 1997. Based upon survival data, and the proportions of oyster haplotypes observed in newly recruited spat, we can estimate the contribution of the 1997 Louisiana planting to local recruitment. Approximately 4.1 million oysters were planted on six seeded bars at a density of 500 ind. m<sup>-2</sup> in 1997. However, not all planted oysters survived to reproduce in 1999, 2000, and 2001. Dive surveys in 1998–2000 revealed a substantial reduction in out-planted oyster density (Table 4), which was most likely due to mortality, though we cannot exclude the possibility of clandestine harvesting. After 1 year, oyster abundances at the planted sites had decreased by between 14% (British Harbour) and 89% (Cook's Point). By 1999, oyster abundances were further reduced to

**Table 1** *Crassostrea virginica*. 16S haplotype frequencies by collection site (NA North Atlantic; SA South Atlantic; GC Gulf Coast)

Sampling location	1999				2000				2001				Total (all years)				Total
	NA	SA	GC	Rare	NA	SA	GC	Rare	NA	SA	GC	Rare	NA	SA	GC	Rare	
Harris Creek area																	
Harris Creek	54	1			61	3		1	25	3			140	7		1	
Total													140	7		1	148
Broad Creek Area																	
Great Bar	9	2			164	3			48	2		1	221	7		1	
Spat 36									23	2			23	2			
2001 Dredge Shell									286	9		5	286	9		5	
Neavitt Shell					184	6			330	10	<b>1</b>		515	16	<b>1</b>		
Brown Bar	40	2											40	2			
Ware Pt	5												5				
Deep Neck	30	1		1	366	22		1	34	2			430	25		2	
Leaderham Creek					9	1			7	1			8	2			
France COE2	17												17				
France-Dive	7	1		2									7	1		2	
Mulberry Pt	14	2			190	9	<b>1</b>		588	43		2	792	54	<b>1</b>	2	
Limey's Lump									183	11			182	22			
Cedar Pt									34	3			34	3			
Inside Cedar Pt									181	8		2	181	8		2	
Hambleton Island									37	2			37	2			
Spat 35									5				5				
Drum Point-Spat 29									28				28				
Total													2,811	153	2	14	2,980
Choptank River area																	
Cook's Pt-Dorchester	2												2				
Cook's Pt-Talbot	28	1		1	11				5				44	1		1	
Cook's Pt-Dive	1												1				
Cook's Pt COL2					12				36	1			48	1			
France Shell COL3					16								16				
Royston	4	2							66	3		1	70	5		1	
Lighthouse Shell COL5					3								3				
Batchelor's Pt	2				7								9				
Ferry Bar	2												2				
Chloras Shell COL4					24	2			1				25	2			
Beacon's Pt	1				13	2							14	2			
Sandy Hill	1	1			1				18				20	1			
British Harbour				<b>1</b>												1	
Goose Pt					23	1							23	1			
Total													277	13	1	2	293
Little Choptank River area																	
Ragged Pt DNR	31												31				
Ragged Pt	6												6				
AQA Lot#1 DNR	48	2											48	2			
Casson's	15	2											15	1			
Grapevine DNR	21												21				
Total													121	3			124
Total (all areas)	338	17	1	4	1,084	49	1	2	1,935	100	1	11	3,349	176	3	17	3,545

**Table 2** *Crassostrea virginica*. Sequence data obtained by Pyrosequencing. Nucleotide identity is indicated by a dash (X missing data)

	Nucleotides									
	T	A	A	A	T	T	C	T	A	A
North Atlantic	T	A	A	A	T	T	C	T	A	A
South Atlantic	G	-	-	-	-	-	-	-	-	A
Gulf Coast	G	-	-	-	-	-	-	-	-	G
Rare 1	C	-	-	-	-	-	-	-	-	A
Rare 2	T	-	-	-	-	-	-	-	-	G
Rare 3	X	-	G	-	-	-	-	-	-	A
Rare 4	X	G	-	-	-	-	-	-	-	A
Rare 5	T	G	-	-	-	-	-	-	-	A
Rare 6	T	-	-	-	-	-	-	C	-	A

12–26% of the original numbers. In 2000, abundance estimates ranged from 1% to 9% of the number originally planted. These estimated densities for 1999 and

2000 exclude new spat recruiting to the planted sites, as the proportional contribution of native and Louisiana recruitment remains unknown. It appeared that the new

**Table 3** *Crassostrea virginica*. RFLP profiles of common haplotypes (North Atlantic, South Atlantic, and Gulf Coast) compared to a representative Louisiana (LA) broodstock sample and an individual collected directly from the seeded site. The last three profiles are from individual spat possessing the 16S Gulf Coast haplotype

Sample site	COI			COIII				Cyt <i>b</i> -COII				ATP6-ND2		ND2-ND4	
	<i>Mva</i> I	<i>Bsa</i> HI	<i>Hae</i> III	<i>Csp</i> 6I	<i>Aff</i> III	<i>Bse</i> NI	<i>Bsa</i> HI	<i>Ase</i> I	<i>Bse</i> NI	<i>Bst</i> HKAI	<i>Bsa</i> HI	<i>Apa</i> LI	<i>Ase</i> I	<i>Bst</i> NI	<i>Alu</i> I
North Atlantic	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
South Atlantic	A	A	A	A	B	A	A	A	A	A	A	A	B	A	A
Gulf Coast	B	B	B	B	B	C	B	B	B	B	B	B	A	B	B
Gulf Coast	B	B	B	C	A	C	A	B	B	B	B	B	B	B	B
LA broodstock	B	B	B	B	B	C	A	B	B	B	B	A	B	B	B
Adult-seeded bar	B	B	B	B	B	C	B	B	B	B	B	B	B	B	B
British Harbour	B	B	B	B	B	C	B	B	B	B	B	B	B	B	B
Broad Creek #1	B	B	B	B	B	C	B	B	B	B	B	B	B	B	B
Broad Creek #2	B	B	B	B	B	C	A	B	B	B	B	A	B	B	B

**Table 4** *Crassostrea virginica*. Estimated numbers of Louisiana oysters planted in October 1997 at six sites. The 1997 counts are based on numbers of seed deployed. Subsequent year estimates are based on abundances estimated from dive surveys, excluding the individuals believed to have been recruits to the planting sites. Dive surveys were not completed at Tanner's Patch; numbers in parentheses are estimates based upon mortality observed at the other sites

	1997	1998	1999	2000
Goose Point	1,136,000	808,000	207,000	31,000
British Harbour	1,142,000	982,000	276,000	31,000
Beacon's Pt	508,000	250,000	130,000	25,000
France	508,000	209,000	78,000	47,000
Cook's Pt	508,000	56,000	59,000	5,000
Tanner's Patch	304,000	(157,000)	(58,000)	(12,000)
Total	4,106,000	2,462,000	808,000	151,000

recruits accounted for approximately 14%, 16%, and 22% of the live oysters on the planted beds in 1998, 1999, and 2000, respectively (K. Paynter, University of Maryland, personal communication). Dive survey data were unavailable for 2001.

Crude estimates of recruitment success can be calculated given several assumptions regarding the native population size and sex ratio. Assuming the population had come to a balanced (1:1) sex ratio by 1999, about half of the surviving Louisiana oysters ( $4 \times 10^5$ ) would have reproduced as females and passed along the mtDNA Gulf Coast haplotype to their progeny. The expected frequency of individuals possessing the Gulf Coast haplotype, assuming a reproductive output comparable to native oysters, would simply be half the number of surviving Louisiana oysters divided by the total number of spawning females in the system (equal to half of the total of the estimated wild stock in the Choptank River plus half of the surviving Louisiana oysters). According to the Maryland Department of Natural Resources (J. Vanisko, personal communication), there are an estimated  $89 \times 10^6$  adult oysters in the Choptank River (at a density of 0.5 oysters  $m^{-2}$ ); however, this is a rough estimate, so we have calculated reproductive success using a range of  $30$ – $100 \times 10^6$  adult oysters. If there were about  $4 \times 10^5$  reproductive Gulf

Coast females in 1999, we would have expected approximately 0.9% (or 0.8–2.4%) of the 1999 year class in the Choptank River to carry the Gulf Coast 16S haplotype. By 2000 the Gulf Coast population had decreased to an estimated 151,000, with about 75,500 reproducing females, assuming a balanced sex ratio. Therefore, 0.08% (or 0.2–0.5%) of new recruits should have possessed the Gulf Coast haplotype in 2000. We therefore would have expected to detect three to nine Gulf Coast oysters in our 1999 sample set and two to six in our 2000 sample set. Dive survey data were unavailable for 2001; therefore, estimates of expected recruitment for that year could not be calculated.

We observed recruitment of progeny from the planting at a level of 1 oyster  $year^{-1}$  in 1999, 2000, and 2001, fewer than the expectation of three to nine in 1999 and two to six in 2000. This suggests that the contribution of those Louisiana oysters that survived to reproduce as females was less than that of the native oysters. In terms of absolute abundance, the impact of the enhancement effort appears to have been modest, largely as the result of high (>95%) mortality of the outplanted Louisiana oysters. At the same time, the survivors did contribute spat to the resident population, in roughly the anticipated numbers. Planting seed with higher potential survival, such as selected lines derived from locally adapted populations, might make more substantial contributions to the resident oyster population. It is also important to note that even numerically modest contributions from selected (e.g. disease tolerant) broodstock may enhance local population fitness by the addition of favorable genes.

Of the three oysters detected in the Choptank River that originated from the stocking program, two were found in Broad Creek, a site exhibiting higher larval retention than other areas of the Choptank River (Krantz and Meritt 1977; Meritt 1977; Kennedy 1980; Boicourt 1982). Future stocking with oyster strains exhibiting superior disease resistance may be more effective in producing a more concentrated population of disease-tolerant oysters if the founder population is established within areas of high larval retention and survival. Although the surviving oysters did contribute

spat to the native stocks of *C. virginica*, the overall impact of stock enhancement was low as a result of the substantial reduction in abundance of the outplanted seed oysters during their first 3 years.

Unauthorized harvesting of some of the planted bars may have contributed to some extent to this reduction; however, extensive on-site mortality due to environmental stresses and diseases such as MSX also seems likely. In another study, three additional Louisiana oyster strains ( $\sim 4,800$  oysters strain<sup>-1</sup>) were outplanted into the Yeocomico and Rappahannock Rivers on the west side of the Chesapeake Bay in 1999. Compared to native oysters, the Louisiana strains suffered greater mortality following exposure to extreme cold during low tides (S. Stickler, unpublished data). In addition, although Gulf Coast oysters are more tolerant of Dermo than Atlantic Coast oysters, they demonstrated a higher susceptibility to MSX (S. Stickler, unpublished data). MSX prevalence is generally slight in the upper tributaries of the Chesapeake Bay, where mean annual salinities of 8–12 are physiologically limiting for MSX (Ford 1985; Abbe and Sanders 1988; Ford and Tripp 1996; S. Jordan, Maryland Department of Natural Resources, personal communication). However, recent drought conditions have raised salinities in several of these tributaries, and, as a result, MSX has periodically appeared in the Choptank River (D.W. Meritt, unpublished data). Thus, although Louisiana oysters may be valuable as a source of genes for improved resistance to Dermo, they may be poor candidates for direct use in stock-enhancement programs in the mid-Atlantic.

For many coastal ecosystems, supplementation of natural populations with hatchery-produced organisms is becoming a standard, albeit sometimes controversial, practice. Because most enhancement programs are costly, effective methods for evaluating their success or failure are sorely needed (Carvalho and Hauser 1994; Ward and Grewe 1994). Early attempts to evaluate the success of stocking projects with genetic markers employed protein (allozyme) electrophoresis (Garcia de Leaniz et al. 1989; Moran et al. 1991; Crozier and Moffett 1995; King et al. 1995; Kristiansen et al. 1997). For example, allozyme surveys demonstrated that a 20-year program of brown trout restoration in Spain was unsuccessful in enhancing the wild population (Moran et al. 1991). However, two factors limit the application of allozyme markers to genetic monitoring of marine and estuarine stock-enhancement programs: (1) the limited number of polymorphisms available for most species makes it difficult to obtain informative allozyme “tags” for hatchery stocks and (2) scaling-up to high-throughput methods is difficult. Modern DNA-based methods do not suffer from these drawbacks, and are likely to see increasing use as the techniques become more familiar to fisheries biologists.

Mitochondrial DNA polymorphisms typically assayed by restriction enzyme analysis of amplified target regions superseded allozymes as molecular markers for population profiling (e.g. Grewe et al. 1994; Wilson and

Donaldson 1998). However, PCR–RFLP of mtDNA is also difficult to scale-up for high-throughput processing; more importantly, this approach can only detect polymorphisms occurring in recognition sites of available restriction enzymes and may miss significant sequence variation. For example, in the case of the oyster, genetic variation in the 16S mitochondrial gene that differentiates North Atlantic, South Atlantic, and Gulf Coast populations is not detectable by RFLP analysis. Small and Chapman (1997) surveyed the 16S mtDNA gene region of *C. virginica* with a large set of restriction endonucleases, but were unable to detect variants previously discovered by direct sequencing (Ó Foighil et al. 1995). This variation is readily detected using DGGE, but the method is time-consuming and not suited to running more than several dozen samples per day.

We found the SNP analyses used in this research were also useful in examining other regions of the Chesapeake Bay for previous stocking efforts. Small-scale hatchery supplementation with Gulf Coast *C. virginica* strains has been carried out by the state of Maryland in the Tangier Sound (S. Jordan, Maryland Department of Natural Resources, personal communication). Sampling revealed a higher frequency of the South Atlantic haplotype in this region and the detection of two individuals with Gulf Coast haplotypes. This haplotype composition may reflect years of regional transfers and enhancement (Carlton and Mann 1996); large movements of oysters from the Carolinas began in the 1940s (Andrews and McHugh 1957; Haven et al. 1978). Because the upper region of the Tangier Sound is largely leased bottom, extensive oyster transplantation activities have occurred in this area and may explain the increased frequency of the South Atlantic haplotype as well as the rare occurrence of the Gulf Coast haplotype. Given the detection of Gulf Coast haplotypes in the Tangier Sound, we cannot ignore this region as a possible source for the Gulf Coast haplotypes in the Choptank River. The reverse may be true as well, presenting the possibility that these two Gulf Coast haplotypes in Tangier Sound may have in fact resulted from the 1997 Louisiana planting. However, much research has suggested that larvae of *C. virginica* exhibit vertical migration patterns such that their transport moves them upstream on flood tides (Carriker 1951; Andrews 1983; Kennedy 1996), thus retaining larvae in the Choptank River system.

Although a wide variety of genetic markers are available for use in genetic monitoring of stocked populations, we believe that SNPs are particularly promising, for several reasons. SNPs are abundant in both nuclear and mitochondrial genomes, providing a virtually unlimited source of genetic variants that may serve to identify hatchery-propagated populations. Unlike many of the more traditional DNA markers, SNPs are readily adapted to automated high-throughput processing, allowing thousands of individuals to be scored for multiple markers in a reasonable amount of time.

Various platforms such as Pyrosequencing are available for high-throughput SNP scoring (Kwok 2001).

The Pyrosequencing SNP analysis protocol provided an efficient means for assessing the Choptank River oyster restoration project. The method was found to be accurate and efficient in differentiating the Gulf Coast mtDNA haplotype from the native haplotypes, without requiring gel-based procedures, labeled primers, or labeled dNTPs. We are currently developing approaches for high-throughput genetic monitoring protocols that can detect enhancement stocks derived from local broodstock. These methods can, in principle, be readily applied to other species, making it feasible to monitor survival, reproduction, and recruitment in any stock-restoration program.

Marine stock-enhancement programs have been conducted around the globe for more than a century, despite a striking lack of empirical data on their effectiveness. Modern genetic monitoring methods now make it feasible to determine the survival and reproductive contribution of enhancement plantings. We used a high-throughput genetic technique (Pyrosequencing) to screen Chesapeake Bay oyster populations for the presence of newly recruited spat derived from outplanted hatchery seed, and demonstrated that the enhancement stock made a modest but detectable contribution to the resident population, despite substantial mortality. For organisms that are not easily physically tagged, high-throughput genetic analysis can provide an effective and practical approach for evaluating the efficacy of stock-enhancement efforts in marine and estuarine organisms.

**Acknowledgements** We would like to recognize S. Tobash and C. Epifanio for their invaluable support and assistance. We would also like to thank K. Paynter and T. Koles for the dive survey data and input. This research was funded by the NMFS Saltonstall-Kennedy Program, Delaware Sea Grant, the Paul R. Austin Student Sea Grant Fellowship, and the Oyster Recovery Partnership.

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