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# Genetic Analysis of Selected Strains of Eastern Oyster (*Crassostrea virginica* Gmelin) Using AFLP and Microsatellite Markers

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**Abstract:** Amplified fragment length polymorphisms (AFLPs) and microsatellite markers were used to examine genetic variation and divergence in 4 selected strains (DBH, NEH, FMF, and CTS) and 1 wild population (DBW) of the eastern oyster *Crassostrea virginica* Gmelin. Eighty-six AFLP markers (from 3 primer pairs) and 5 microsatellite loci were used for the analysis of 30 oysters from each of the 5 populations. Microsatellite loci were considerably more variable than AFLPs. The observed heterozygosity ranged from 0.560 to 0.640 across populations for microsatellites, and from 0.186 to 0.207 for AFLPs. Both $F_{st}$ and $\phi_{PT}$ of microsatellite data and $\phi_{PT}$ statistics of AFLP data revealed significant divergence between all pairs of populations. There was no significant reduction in heterozygosity in all 4 selected strains; however, the number of alleles per locus was considerably lower in the selected strains than in the wild population. Two strains subjected to long-term selection for disease resistance shared frequency shifts at a few loci, which deserve further analysis to determine if they are linked to disease-resistance genes.

**Key words:** selection, disease resistance, genetic variation, AFLP, microsatellite, aquaculture, *Crassostrea* oyster.

## INTRODUCTION

The eastern oyster (*Crassostrea virginica* Gmelin) faces 2 major diseases along much of the Atlantic coast: MSX (caused by the parasite *Haplosporidium nelsoni*) and Dermo (caused by the parasite *Perkinsus marinus*). The 2 diseases, along with overfishing and habitat destruction, are among the leading causes for the collapse of the oyster fisheries in the mid-Atlantic region (MacKenzie, 1996). They also threaten aquaculture and restoration efforts. The development of disease-resistant strains of eastern oysters represents a major task for the scientific and aquaculture community.

Rutgers University has been breeding eastern oysters for disease resistance since the early 1960s (Haskin and Ford, 1979). The Rutgers breeding program was initially set up to select for resistance to MSX, and strong resistance was obtained after 6 generations of selection (Ford and Haskin, 1987). In 1990 an epizootic of Dermo diseases occurred in Delaware Bay, and the Rutgers MSX-resistant lines have been exposed to Dermo thereafter. In 1992 Rutgers' disease-resistant lines were regrouped into 2 geographic synthetic strains: NEH for lines originated from the Northeast and DBH for lines from Delaware Bay (S.K. Allen, Jr., personal communication). Both strains have

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demonstrated strong resistance to MSX and some resistance to Dermo (S.K. Allen, Jr., personal communication, Guo et al., 2003). At the Frank M. Flower Oyster Co., selective breeding, although largely unintentional, has produced oysters (referred to as the FMF strain hereafter) that show superior growth and markedly improved survival under the challenge of juvenile oyster disease (Farley et al., 1998). A new breeding program was initiated in Connecticut following outbreaks of MSX and Dermo in 1998 (I. Sunila, personal communication). The Connecticut selected strain (CTS) is relatively recent and has not been fully evaluated. Selection at Virginia Institute of Marine Science has also led to improvements in disease resistance in the eastern oyster (Andrews, 1968).

While the phenotypic performance of the selected strains is closely monitored, genetic differentiation in the selected strains is rarely studied and reported. A major concern for selected strains is inbreeding and the loss of genetic variability (Hedgecock and Sly, 1990; Gaffney et al., 1992; Hedgecock et al., 1992). Loss of genetic variation limits response to selection. Severe inbreeding may lead to poor larval survival and slow growth (Virjenhoek et al., 1990; Launey et al., 2001). Virjenhoek et al. (1990) conducted a genetic analysis of Rutgers MSX-resistant lines before the 1992 regrouping. They found that heterozygosity was basically unchanged, but rare alleles were lost in the selected strains. We assume that the regrouping of Rutgers disease-resistant lines in 1992 has resulted in genetic changes (presumably restoration of genetic variability) in the 2 synthetic strains, but no genetic analysis has been conducted.

The availability of several selected strains provides an opportunity to study genetic differentiation among them. Genetic analysis of selected strains may identify shared genetic changes as a response to selection. Alleles that show consistent increases in frequency may be linked to the traits under selection. In this study we conducted a genetic analysis of 4 selected strains and a wild population using amplified fragment length polymorphisms (AFLPs) and microsatellite markers. We report here significant divergence among the 5 populations and shared frequency shifts in selected strains at a few loci.

## Materials and Methods

### Samples and DNA Extraction

Four selected strains and one wild population were included for this study. The 4 selected strains were the following: DBH, Rutgers strain, originating from Delaware Bay and selected for MSX resistance since 1960 and Dermo resistance since 1990; NEH, Rutgers strain, originating from Long Island Sound and selected for MSX resistance since 1960 and Dermo resistance since 1990; FMF, Flower's Oyster Company strain, selected for fast growth and juvenile oyster disease resistance; and CTS, the third generation of Connecticut oysters selected for MSX resistance. The 2 Rutgers strains have a long and complicated history. Starting in 1960, over 30 lines were gradually established through selective breeding for resistance against MSX; some originated from the Delaware Bay, and some from Long Island Sound (Haskin and Ford, 1979). The current Rutgers strains, DBH and NEH, were established in 1992 by mixing surviving lines and wild oysters from each geographic population. Oysters sampled for this study were the third generation since the mixing in 1992. The FMF strain has been bred and used for commercial production for a long time, although its breeding history is unknown.

Oysters from all selected strains were hatchery-produced. DBH, NEH, and FMF were produced at Rutgers Cape Shore Facility using 20 females and 20 males for each strain. Eggs from 20 females were pooled and then divided into 20 aliquots, each fertilized by a different male. CTS seeds were obtained from a hatchery in Connecticut, and the number of parents used was unknown. The wild population, DBW, was a single year-class of wild oysters collected from Delaware Bay. All oysters were sampled at about 24 months, and there were no major disease-inflicted mortalities before sampling. Thirty individuals were randomly taken from each of the 5 populations. DNA was extracted from adductor muscle tissue using a QIAamp DNA mini kit (Qiagen Inc.) according to supplied protocols.

### Microsatellite Analysis

Five microsatellites, *Cvi8*, *Cvi9*, *Cvi11*, *Cvi12*, and *Cvi13*, which were developed by Brown et al. (2000), were used for this study. The microsatellites were fluorescence-labeled with either FAM or HEX (MWG Biotech Inc.) and scored on an ABI310 Genetic Analyzer. Polymerase chain reaction (PCR) was performed in a 15-μl volume containing 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 μM of each primer, approximately 50 ng of genomic DNA, and 0.5 U of *Taq* polymerase (Promega), using the following temperature profile: 94°C for 2 minutes, then 30 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 15 seconds.

## AFLP Analysis

AFLP analysis was conducted primarily according to AFLP Plant Mapping Protocol (Perkin Elmer, Applied Biosystems) with some modifications. Adapters, preselective primers, and selective primers were ordered from MWG Biotech Inc., and PCR reagents were purchased from Promega. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. Genomic DNA (approx. 0.5 μg) was digested with restriction enzymes *Eco*RI and *Mse*I, and ligated with relevant adapters overnight at room temperature. Preselective primers complementary to the adapter sequence only were used to amplify the restriction fragments created in the digestion-ligation step. Every 4 μl of diluted (20-fold) digestion-ligation product was amplified in a 20-μl reaction mixture containing 1.0 μl of each *Eco*RI and *Mse*I preselective primer, and 15 μl of PCR mix. Preselective PCR was run at a temperature profile of 1 cycle of 72°C for 2 minutes, 25 cycles of 94°C for 25 seconds, 56°C for 30 seconds, and 72°C for 2 minutes, and 1 cycle of 60°C for 30 minutes. Products from preselective PCR were diluted 20-fold with Tris/EDTA buffer and used as templates for selective amplification. Three pairs of selective primers, each containing 3 selective nucleotides at their 3′ end, were used for selective PCR, with the *Eco*RI selective primer being fluorescence-labeled (FAM or JOE). The 3 selective primer pairs were *Eco*RI-ACA/*Mse*I-CTA, *Eco*RI-ACA/*Mse*I-CTC, and *Eco*RI-ACT/*Mse*I-CAG.

## Electrophoresis and Data Collection

Electrophoresis and data collection were carried out on an ABI 310 Genetic Analyzer (Perkin Elmer). PCR products, 0.3 to 0.5 μl for microsatellites or 1.0 to 1.5 μl for AFLPs, were added to 0.2-ml sample tubes, each containing 12.0 μl of deionized formamide and 0.3 μl of GeneScan-500 size standard (Perkin Elmer). Samples were denatured at 95°C for 5 minutes and then immediately cooled on ice for 5 minutes before being loaded onto the ABI 310 Genetic Analyzer. Electrophoresis was conducted using POP4 polymer with the following parameters: injection for 10 seconds at 15 KV, running for 30 minutes at 13 KV and 60°C, for AFLPs; and injection for 2 seconds at 15 KV, running for 24 minutes at 15 KV and 60°C, for microsatellites. Data were collected using the GS STR POP4 A module in Data Collection Software (Version 1.0.2) and analyzed with GeneScan Analysis software (Version 3.1). Genotyper software was used to aid scoring genotypes, and electrophoretic histograms were manually examined for genotyping errors.

## Data Analysis

For microsatellite loci, the tests for departure from Hardy-Weinberg equilibrium (HWE) were performed by calculations of Wright's $F_{is}$ according to Weir and Cockerham (1984) using GENEPOP Version 3.3 (Raymond and Rousset, 1995). Markov chain-randomization procedure (Guo and Thompson, 1992) was used to calculate $P$ values. Dememorization number, number of batches, and number of iterations per batch were set at 1000. The genetic differentiation between populations ($\phi_{PT}$ and $F_{ST}$) and variance components of microsatellite diversity within and among populations for all pairs of populations were analyzed with analysis of molecular variance (AMOVA; Excoffier et al., 1992; Huff et al., 1993) in GenAlEx V5 (Peakall and Smouse, 2001).

AFLP loci were scored as peak (or band) presence (1 for *AA* or *Aa*) or absence (0 for *aa*) to create binary matrices. Only loci with clearly defined peaks were used for data analysis. The unbiased estimator of Lynch and Milligan (1994) was used to calculate $p$ (*A*) and $q$ (a) allele frequency at each locus. Expected heterozygosity was calculated with the assumption of AFLPs being independent nuclear loci at HWE. The number of fixed loci was counted in each population.

The genetic distance ($D$) for AFLP data was calculated according to the method described by Huff et al. (1993) and Peakall et al. (1995):

$$D = n[1 - (C/2n)],$$

where $n$ equals the total number of polymorphic peaks, and $C$ equals the number of bands shared by 2 individuals. Both peak presence and absence are considered informative for analysis. This is a Euclidean distance matrix and therefore appropriate for subsequent AMOVA. $\phi_{PT}$ genetic differentiation between populations was calculated, averaged over loci. All analysis was conducted using GenAlEx V5.

For both microsatellite and AFLP data, Nei's genetic distance matrices among populations were computed with the GENDIST program and used to construct unweighted pair-group method with arithmetic averages (UPGMA) trees with the NEIGHBOR program in the PHYLIP package (Version 3.56°C; Felsenstein, 1989). Bootstrap analyses with 200 replications were performed with the SEQBOOT

**Table 1.** Number of Alleles ($N_o$), Observed and Expected Heterozygosity ($H_o$ and $H_e$), and $F_{is}$ Statistics at Five Microsatellite Loci Five Populations of *C. virginica*

| Locus | DBW ($n$ = 30) | DBH ($n$ = 30) | NEH ($n$ = 30) | FMF ($n$ = 30) | CTS ($n$ = 30) |
|---|---|---|---|---|---|
| *Cvi-8* | | | | | |
| $N_a$ | 6 | 6 | 3 | 4 | 4 |
| $H_o$ | 0.300 | 0.133 | 0.067 | 0.276 | 0.367 |
| $H_e$ | 0.403 | 0.373 | 0.184 | 0.498 | 0.625 |
| $F_{is}$ | 0.271* | 0.649** | 0.647** | 0.548** | 0.427** |
| *Cvi-9* | | | | | |
| $N_a$ | 12 | 11 | 12 | 8 | 9 |
| $H_o$ | 0.867 | 0.966 | 0.833 | 0.724 | 0.767 |
| $H_e$ | 0.899 | 0.831 | 0.847 | 0.797 | 0.826 |
| $F_{is}$ | 0.053† | −0.145† | 0.033† | 0.109† | 0.088† |
| *Cvi-11* | | | | | |
| $N_a$ | 3 | 3 | 3 | 3 | 3 |
| $H_o$ | 0.643 | 0.633 | 0.433 | 0.357 | 0.567 |
| $H_e$ | 0.612 | 0.569 | 0.443 | 0.544 | 0.515 |
| $F_{is}$ | −0.033† | −0.095† | 0.038† | 0.359* | −0.084† |
| *Cvi-12* | | | | | |
| $N_a$ | 18 | 10 | 7 | 12 | 11 |
| $H_o$ | 0.633 | 0.767 | 0.690 | 0.733 | 0.400 |
| $H_e$ | 0.912 | 0.842 | 0.645 | 0.835 | 0.828 |
| $F_{is}$ | 0.321** | 0.106† | −0.052† | 0.138† | 0.529** |
| *Cvi-13* | | | | | |
| $N_a$ | 18 | 9 | 11 | 12 | 10 |
| $H_o$ | 0.711 | 0.700 | 0.833 | 0.900 | 0.700 |
| $H_e$ | 0.893 | 0.811 | 0.782 | 0.864 | 0.850 |
| $F_{is}$ | 0.219** | 0.154** | −0.049† | −0.025† | 0.193** |
| Average/sum | | | | | |
| $N_a$‡ | 57 | 39 | 36 | 39 | 37 |
| $H_o$ | 0.631 (0.202) | 0.640 (0.249) | 0.571 (0.255) | 0.598 (0.232) | 0.560 (0.190) |
| $H_e$ | 0.744 (0.214) | 0.658 (0.205) | 0.580 (0.232) | 0.708 (0.187) | 0.729 (0.173) |
| $F_{is}$ | 0.166† | 0.134† | 0.123† | 0.226† | 0.231† |

*$P < 0.05$; **$P < 0.01$. †Not significant. ‡Total number over 5 loci.

and CONSENSE programs, and consensus trees were drawn with DRAWGRAM program in the same package.

## RESULTS

### Polymorphism and Heterozygosity

Eighty alleles were observed at the 5 microsatellite loci among the 150 oysters sampled from 5 populations, averaging 16 per locus. The number of alleles per locus varied greatly among loci, ranging from 3 at *Cvi-11* to 26 at *Cvi-12*. Locus *Cvi-8*, *Cvi-9* and *Cvi-13* had 10, 17, and 24 alleles, respectively. The observed and expected heterozygosity ($H_o$ and $H_e$) also varied among loci and to a lesser extent among populations (Table 1). The observed heterozygosity deviated significantly from the expected in 11 of the 25 cases (5 loci × 5 populations), as estimated by $F_{IS}$ HWE tests. All significant deviations were deficient for heterozygotes. The deviation was apparently more locus-specific than population-specific. For example, *Cvi-8* showed significant deviation in all 5 populations, while *Cvi-9* had none. The other 3 loci showed significant deviation in 1 to 3 populations. Across populations, 2 populations (DBW and CTS) deviated at 3 loci, 2 populations (DBH and FMF) at 2 loci, and 1 population (NEH) at 1 locus. Heterozygote

**Table 2.** Locus Information for 86 AFLP Markers Used in Population Analysis

| Locus | Primer set | Code[a] | Locus | Primer set | Code | Locus | Primer set | Code |
|---|---|---|---|---|---|---|---|---|
| 1 | E-ACA/M-CTA | C5f063 | 30 | E-ACA/M-CTC | C6f122 | 59 | E-ACT/M-CAG | F3f108 |
| 2 | E-ACA/M-CTA | C5f093 | 31 | E-ACA/M-CTC | C6f130 | 60 | E-ACT/M-CAG | F3f123 |
| 3 | E-ACA/M-CTA | C5f114 | 32 | E-ACA/M-CTC | C6f153 | 61 | E-ACT/M-CAG | F3f134 |
| 4 | E-ACA/M-CTA | C5f126 | 33 | E-ACA/M-CTC | C6f154 | 62 | E-ACT/M-CAG | F3f147 |
| 5 | E-ACA/M-CTA | C5f132 | 34 | E-ACA/M-CTC | C6f161 | 63 | E-ACT/M-CAG | F3f151 |
| 6 | E-ACA/M-CTA | C5f157 | 35 | E-ACA/M-CTC | C6f165 | 64 | E-ACT/M-CAG | F3f160 |
| 7 | E-ACA/M-CTA | C5f191 | 36 | E-ACA/M-CTC | C6f200 | 65 | E-ACT/M-CAG | F3f184 |
| 8 | E-ACA/M-CTA | C5f195 | 37 | E-ACA/M-CTC | C6f204 | 66 | E-ACT/M-CAG | F3f186 |
| 9 | E-ACA/M-CTA | C5f196 | 38 | E-ACA/M-CTC | C6f221 | 67 | E-ACT/M-CAG | F3f188 |
| 10 | E-ACA/M-CTA | C5f206 | 39 | E-ACA/M-CTC | C6f226 | 68 | E-ACT/M-CAG | F3f195 |
| 11 | E-ACA/M-CTA | C5f212 | 40 | E-ACA/M-CTC | C6f242 | 69 | E-ACT/M-CAG | F3f209 |
| 12 | E-ACA/M-CTA | C5f214 | 41 | E-ACA/M-CTC | C6f252 | 70 | E-ACT/M-CAG | F3f215 |
| 13 | E-ACA/M-CTA | C5f215 | 42 | E-ACA/M-CTC | C6f270 | 71 | E-ACT/M-CAG | F3f252 |
| 14 | E-ACA/M-CTA | C5f220 | 43 | E-ACA/M-CTC | C6f280 | 72 | E-ACT/M-CAG | F3f254 |
| 15 | E-ACA/M-CTA | C5f224 | 44 | E-ACA/M-CTC | C6f292 | 73 | E-ACT/M-CAG | F3f258 |
| 16 | E-ACA/M-CTA | C5f228 | 45 | E-ACA/M-CTC | C6f298 | 74 | E-ACT/M-CAG | F3f274 |
| 17 | E-ACA/M-CTA | C5f265 | 46 | E-ACA/M-CTC | C6f324 | 75 | E-ACT/M-CAG | F3f288 |
| 18 | E-ACA/M-CTA | C5f266 | 47 | E-ACA/M-CTC | C6f330 | 76 | E-ACT/M-CAG | F3f291 |
| 19 | E-ACA/M-CTA | C5f270 | 48 | E-ACA/M-CTC | C6f334 | 77 | E-ACT/M-CAG | F3f293 |
| 20 | E-ACA/M-CTA | C5f274 | 49 | E-ACA/M-CTC | C6f343 | 78 | E-ACT/M-CAG | F3f298 |
| 21 | E-ACA/M-CTA | C5f290 | 50 | E-ACA/M-CTC | C6f345 | 79 | E-ACT/M-CAG | F3f302 |
| 22 | E-ACA/M-CTA | C5f293 | 51 | E-ACA/M-CTC | C6f352 | 80 | E-ACT/M-CAG | F3f308 |
| 23 | E-ACA/M-CTA | C5f308 | 52 | E-ACA/M-CTC | C6f355 | 81 | E-ACT/M-CAG | F3f334 |
| 24 | E-ACA/M-CTA | C5f390 | 53 | E-ACA/M-CTC | C6f408 | 82 | E-ACT/M-CAG | F3f368 |
| 25 | E-ACA/M-CTA | C5f398 | 54 | E-ACT/M-CAG | F3f078 | 83 | E-ACT/M-CAG | F3f371 |
| 26 | E-ACA/M-CTC | C6f058 | 55 | E-ACT/M-CAG | F3f102 | 84 | E-ACT/M-CAG | F3f384 |
| 27 | E-ACA/M-CTC | C6f074 | 56 | E-ACT/M-CAG | F3f104 | 85 | E-ACT/M-CAG | F3f398 |
| 28 | E-ACA/M-CTC | C6f100 | 57 | E-ACT/M-CAG | F3f105 | 86 | E-ACT/M-CAG | F3f426 |
| 29 | E-ACA/M-CTC | C6f108 | 58 | E-ACT/M-CAG | F3f107 | | | |

[a]The locus code follows the commonly accepted protocol, with the first 2 characters referring to the primer pair and the last 3 digits indicating fragment size.

excess was observed in 7 of the 25 cases, but none was statistically significant (Table 1).

There was no significant difference in observed or expected heterozygosity between the wild and selected populations. The observed heterozygosity over 5 loci was 0.631 for the wild population (DBW) and 0.592 for the 4 selected strains. However, the selected strains had considerably or significantly fewer alleles than the wild population. The wild population, DBW, had 57 alleles, compared with 36 to 39 alleles in the selected strains (Table 1). The reduction in allele number is significant for NEH (by 36.8%) and CTS (by 35.1%). Both DBH and FMF had 31.6% reduction in allele number, which is not statistically significant at the 95% confident level ($P = 0.066$).

For the AFLPs, the 3 primer pairs generated a total of 133 clearly defined peaks, and among them 86% or 64.7% were polymorphic in at least 1 of the 5 populations. For brevity all AFLP loci were numerically labeled, but their primer pair and size information are presented in Table 2. The number of polymorphic markers per primer pair was 25, 28, and 33 for the 3 primer pairs used. The expected heterozygosity varied somewhat among primer pairs, with the primer pair E-ACA/M-CTA producing the highest levels of heterozygosity in all 5 populations (Table 3). However, there was a significant decrease in the number of polymorphic loci or increase in the number of fixed loci in 3 (DBH, NEH, and FMF) of the 4 selected strains, while the change was not statistically significant in CTS ($P = 0.481$).

**Table 3.** Expected Heterozygosity by Primer Pair and Number of Fixed Loci Over 86 AFLP Markers in Five Strains or Populations of *C. virginica*

| Heterozygosity | No. loci | DBW | DBH | NEH | FMF | CTS | Average |
|---|---|---|---|---|---|---|---|
| Expected heterozygosity | | | | | | | |
| E-ACA/M-CTA | 25 | 0.213 | 0.280 | 0.231 | 0.205 | 0.258 | 0.237 |
| E-ACA/M-CTC | 28 | 0.170 | 0.139 | 0.139 | 0.167 | 0.180 | 0.159 |
| E-ACT/M-CAG | 33 | 0.180 | 0.183 | 0.224 | 0.186 | 0.185 | 0.192 |
| Average | — | 0.188 | 0.201 | 0.198 | 0.186 | 0.207 | 0.196 |
| No. fixed loci | 86 | 9 | 18 | 15 | 19 | 11 | |

**Table 4.** Values for $\phi_{PT}$ (below diagonal) and $F_{ST}$ (above diagonal) from Microsatellite Data, and $\phi_{PT}$ Statistics from AFLP Data of All Pairwise Comparisons among Five Populations of *C. virginica*

| Population | DBW | DBH | NEH | FMF | CTS |
|---|---|---|---|---|---|
| Microsatellite ($\phi_{PT}/F_{ST}$) | | | | | |
| DBW | — | 0.028** | 0.096*** | 0.035** | 0.083*** |
| DBH | 0.0439** | — | 0.076*** | 0.069*** | 0.131*** |
| NEH | 0.1571*** | 0.1314*** | — | 0.105*** | 0.167*** |
| FMF | 0.0522*** | 0.1103*** | 0.1681*** | — | 0.085*** |
| CTS | 0.1254*** | 0.2000*** | 0.2546*** | 0.1260*** | — |
| AFLP: ($\phi_{PT}$) | | | | | |
| DBW | — | | | | |
| DBH | 0.0619** | — | | | |
| NEH | 0.1169** | 0.0818** | — | | |
| FMF | 0.0648** | 0.1054** | 0.1093** | — | |
| CTS | 0.1254** | 0.2000** | 0.2546** | 0.1260** | — |

**$P < 0.01$; ***$P < 0.001$.

The number of fixed loci was 9 in DBW, 18 in DBH, 15 in NEH, 19 in FMF, and 11 in CTS.

## Genetic Differentiation

There was significant differentiation among the 5 populations. All pairwise $F_{ST}$ and $\phi_{PT}$ statistics from microsatellite data were significant, suggesting that all 5 populations were significantly different from each other (Table 4). Similarly, pairwise $\phi_{PT}$ statistics from AFLP data revealed the same result—significant genetic differentiation between all pairs of populations. This finding is also supported by AMOVA analysis in which genetic difference among populations was significant (Table 5). When all populations were considered, genetic variance among populations was highly significant and accounted for 10% of the total variance for AFLP data and 13% for microsatellite data. Variances among the 4 selected strains and between DBH and its wild control (DBW) were also significant.

Consensus trees based on UPGMA analysis of genetic distance matrices from microsatellite and AFLP are presented in Figure 1 (A and B, respectively). Both trees separated the CTS strain from the other 4 populations and placed the DBW and DBH strains close to each other. The only difference between the 2 trees was that in the AFLP tree, NEH joined the DBW-DBH cluster first, followed by FMF, while in the microsatellite tree, FMF joined the DBW-DBH cluster first, followed by NEH.

## Allele Frequencies

Allele frequency of microsatellites varied considerably among the 5 populations. Frequencies of some alleles

**Table 5.** AMOVA of $\phi$ Statistics Using AFLP and Microsatellite Data from Five Strains or Populations of *C. virginica*

| | AFLP data | | | Microsatellite data | | |
|---|---|---|---|---|---|---|
| Strains | % | $\phi$ statistics | *P* | % | $\phi$ statistics | *P* |
| DBW, DBH, NEH, FMF, CTS | | | | | | |
| Among pops. | 10 | 0.100 | 0.001 | 13 | 0.132 | 0.001 |
| Within pops. | 90 | | | 87 | | |
| DBH, NEH, FMF, CTS | | | | | | |
| Among pops. | 11 | 0.114 | 0.001 | 17 | 0.167 | 0.001 |
| Within pops. | 89 | | | 83 | | |
| DBW vs. DBH | | | | | | |
| Among pops. | 6 | 0.062 | 0.001 | 4 | 0.044 | 0.002 |
| Within pops. | 94 | | | 96 | | |

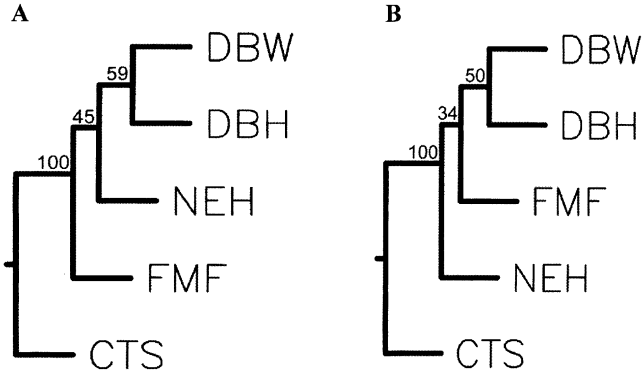


**Figure 1.** Consensus trees based on UPGMA analysis of genetic distance matrices from microsatellite (**A**) and AFLP (**B**) data. Numbers on the branches are bootstrap values (%) obtained from analysis with 200 replicates.

(with frequency of 0.15 or higher in at least 1 of 5 populations) in the 5 populations are presented in Figure 2. Certain alleles were clearly more frequent in some populations than in others. At *Cvi-8*, for example, A204 was the most common allele (with a frequency of 0.53) in CTS, while A202 was the most common allele in the other 4 populations (frequencies >0.66). Allele A214 at *Cvi-8* was present in FMF at a frequency of 0.20, but was not detected in other populations. At *Cvi-12*, the 2 most common alleles were A106 and A124 in NEH (with frequencies of around 0.40), while CTS, DBW, and FMF had frequencies lower than 0.07 for these 2 alleles, with their most common alleles being A120 (0.32), A112 (0.15), and A112 (0.32), respectively. There were also significant differences in the frequency of some alleles between DBW and DBH. Allele A192 of *Cvi-13* had a frequency of 0.20 in DBH, but was not detected in DBW. Similar patterns existed for A106 (0.25 in DBH vs. 0.05 in DBW) and A128 (0.15 in DBH vs. 0.0 in DBW) at *Cvi-12*, and A127 (0.23 in DBH vs. 0.05 in DBW) at *Cvi-9*, where the frequencies were higher in DBH than in DBW.

The frequencies of the *A* allele at the 86 AFLP loci for the 5 populations are presented in Figure 3. There was good correlation in allele frequency among the 5 populations at most loci. The correlation in allele frequency between DBH and DBW was particularly good, and they had similar frequencies at most loci, but differed considerably in allele frequency at locus 5, 13, 18, 24, 52, 69, and 85 (Figure 3, A). The 2 Rutgers selected strains, DBH and NEH, had similar frequencies at most loci but differed significantly at locus 9, 19, 38, and 58 (Figure 3, B). Similarly, the selected strains derived from Long Island Sound, NEH, FMF, and CTS, had similar patterns of allele frequencies, but each population had a few loci with distinctive allele frequencies. CTS had higher frequencies at locus 3, 39, and 66. FMF had higher frequencies at locus 20 and 64. NEH showed higher frequencies at locus 19, 24, and 52 (Figure 3, C).

The 2 Rutgers strains, DBH and NEH, which were subjected to long-term selection for disease resistance, showed allele frequency shifts in the same direction at a number of microsatellite and AFLP loci (Table 6). Allele A106 at *Cvi-12*, for example, had a frequency of 0.05 in DBW, 0.05 in FMF, 0.07 in CTS, but 0.25 in DBH and 0.43 in NEH. The *A* allele at AFLP locus 24 had a frequency of 0.03 in DBW, 0.0 in FMF and CTS, but 0.52 in DBH and 0.33 in NEH. The same pattern of increased frequency was observed for 5 other loci. In contrast, DBH and NEH both had lower frequencies than the other 3 populations at AFLP locus 18 and 59 (Table 6).

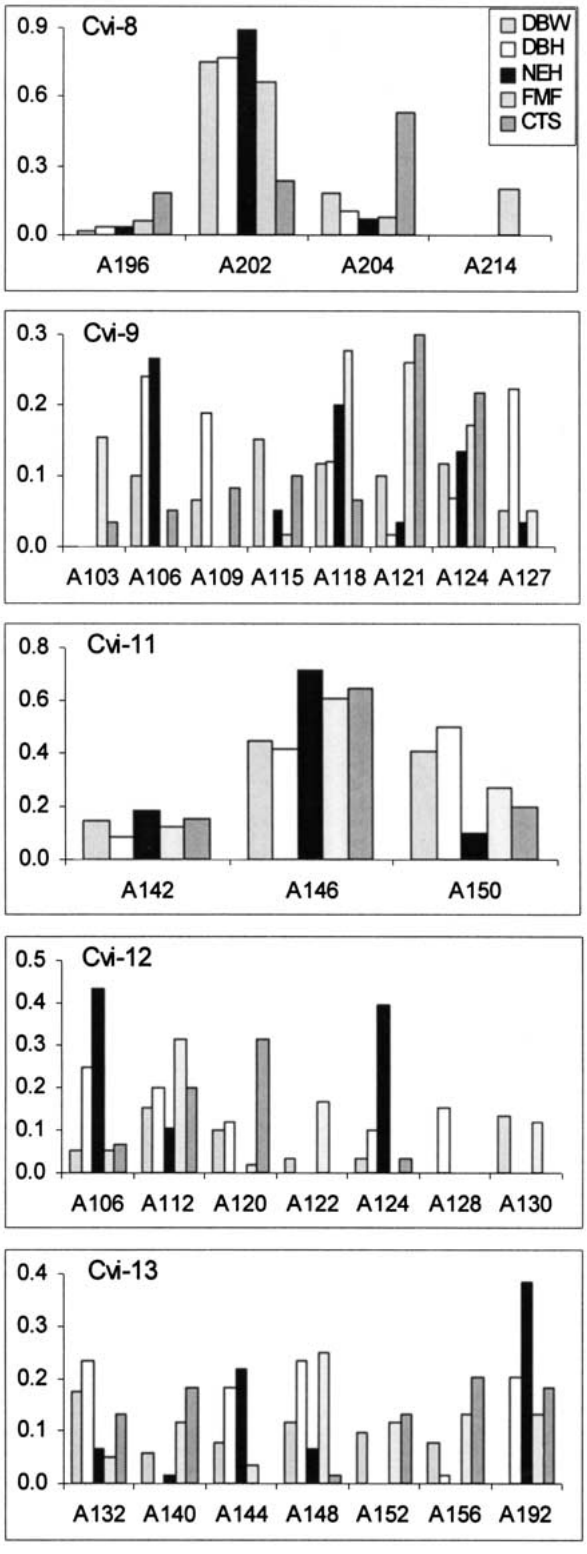


**Figure 2.** Allele frequencies (y axis) at 5 microsatellite loci in 5 strains or populations of *C. virginica*. Allele names are specified on the x axis.

## Discussion

### Population Divergence: Microsatellite Versus AFLP Markers

Microsatellites and AFLPs are different types of genetic markers, but both markers revealed significant genetic divergence among the 5 populations as measured by the pairwise $\phi_{PT}$ statistics (as well as $F_{ST}$ for microsatellites), and AMOVA analysis. This finding is not surprising since all 5 populations have been isolated from each other over at least 10 generations.

AFLPs are dominant markers and therefore less informative than microsatellites per locus. The large number of AFLP markers that can be easily generated may compensate for the low per-locus information. The 3 AFLP primer pairs used in this study produced 86 polymorphic markers, averaging 29 per primer set. The large number of markers may have provided wider coverage of the genome than the 5 microsatellites. The finding that AFLPs can be used for population analysis in the eastern oyster and lead to the same conclusions as microsatellites is encouraging. This finding may encourage further use of AFLPs for population analysis when microsatellites are unavailable in a laboratory or for a particular species. AFLPs have been used for population analysis in many species including finfish and other aquatic animals (Seki et al., 1999; Miller et al., 2000; David et al., 2001). One shortcoming of AFLP markers is that they are not as easily transferred among labs as microsatellites. The precise sizing of AFLP fragments, using automated DNA sequencers, can greatly increase their transferability. There is good evidence that AFLP fragments of the same mobility are homogenous and locus-specific (Qi and Lindhout, 1997; Waugh et al., 1997; Qi et al., 1998; Haanstra et al., 1999). The locus-specific nature of AFLPs is also supported by our observation that different populations had similar frequencies at most of the AFLP loci, although it is possible that 2 peaks may belong to the same locus (Li and Guo, 2004).

Microsatellites are clearly more variable than AFLPs. Microsatellite loci showed significantly higher levels of heterozygosity than AFLPs (0.560 to 0.640 vs. 0.186 to 0.207), owing to their hypervariability and multiallelic nature (Weber and Wong, 1993). The level of heterozygosity detected by AFLPs was similar to levels detected in the Kanab amber snail (Miller et al., 2000) and other organisms (Powell et al. 1996; Pejic et al., 1998; Maguire et al., 2002). The biallelic nature of AFLPs limits the

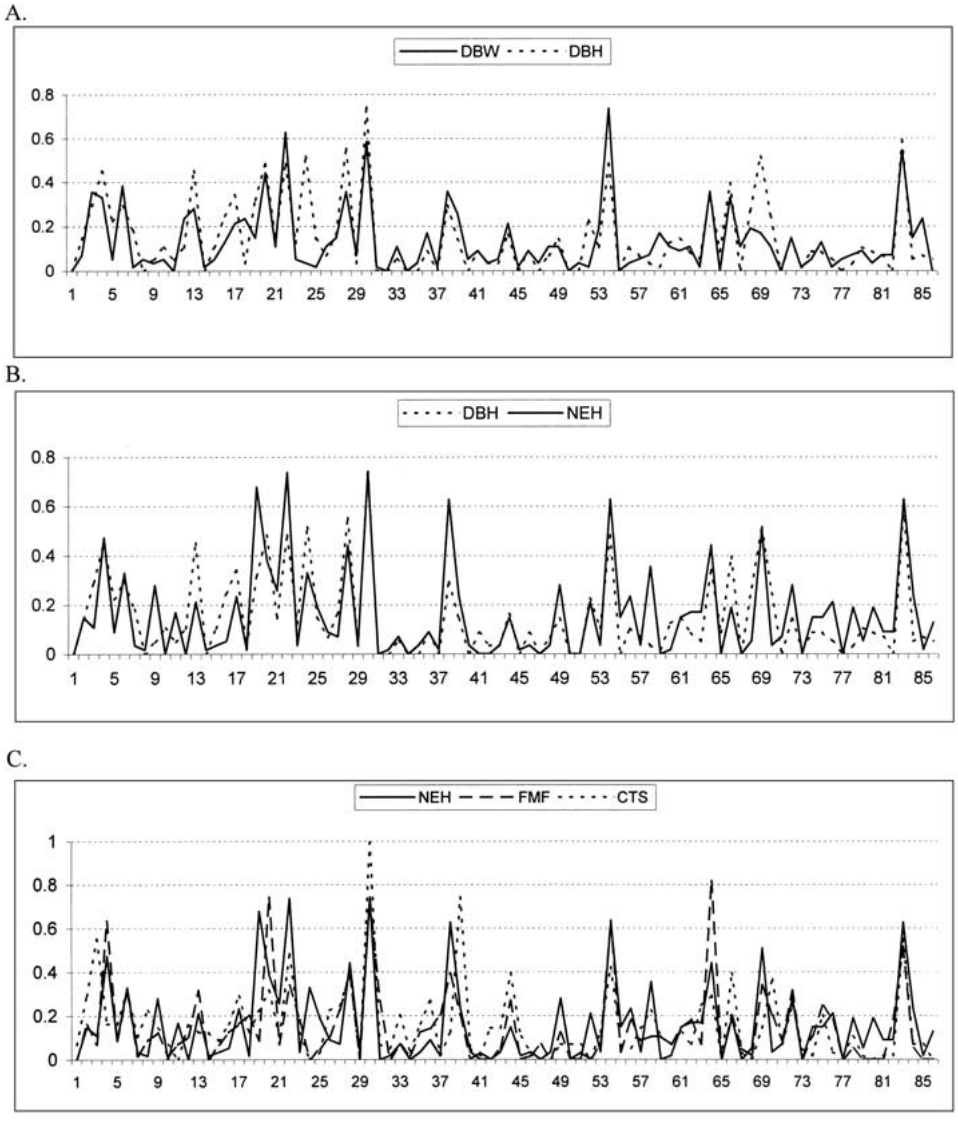


**Figure 3.** Comparison of frequencies (y axis) of the *A* allele at 86 AFLP loci (x axis) in 5 strains or populations of *C. virginica*.

**Table 6.** Allele Frequency at Loci Where Two Rutgers Strains Selected for Disease resistance (DBH and NEH) Show Concerted Changes

| Locus | DBW | DBH | NEH | FMF | CTS |
|---|---|---|---|---|---|
| *Cvi-09* A106 | 0.100 | 0.241 | 0.267 | 0 | 0.083 |
| *Cvi-12* A106 | 0.050 | 0.250 | 0.431 | 0.050 | 0.067 |
| AFLP locus *18* | 0.247 | 0.034 | 0.017 | 0.204 | 0.124 |
| AFLP locus *19* | 0.144 | 0.317 | 0.678 | 0.087 | 0.225 |
| AFLP locus *24* | 0.034 | 0.517 | 0.330 | 0 | 0 |
| AFLP locus *28* | 0.342 | 0.553 | 0.452 | 0.394 | 0.394 |
| AFLP locus *52* | 0.017 | 0.225 | 0.204 | 0 | 0 |
| AFLP locus 59 | 0.170 | 0.017 | 0 | 0.106 | 0.106 |
| AFLP locus *69* | 0.170 | 0.517 | 0.484 | 0.342 | 0.144 |

maximum level of heterozygosity that can be detected. A shortcoming of microsatellites is the relatively large number of null alleles and degree of segregation distortion found in oysters (Gaffney, 2002; Hubert et al., 2002; Reece et al., 2002). In this study 11 of the 25 microsatellite locus-populations showed significant heterozygote deficiency.

Locus *Cvi-8* showed significant heterozygote deficiency in all 5 populations. The occurrence of null alleles may be one explanation for heterozygote deficiency (Brown et al., 2000), as heterozygotes involving a null allele are detected as homozygotes. In our study there was no amplification for 8, 2, 2, 1, and 4 specimens at locus *Cvi-8, 9, 11, 12*, and *13*, respectively, in our sample size of 150; most of them may be null-allele homozygotes at these loci. Null alleles may be caused by sequence variation at the primer site (Launey and Hedgecock, 2001). Because AFLPs are dominant markers, it is not possible to detect deviation from HWE in population analysis. AFLPs showed low levels of segregation distortion (8.2% over 282 loci) in a family of the eastern oyster (Yu and Guo, 2003).

Although the phylogenetic trees of the 5 populations are not highly informative because of the small genetic distances involved, the trees do confirm the genetic lineage of the selected strains. DBH was derived from Delaware Bay wild populations, and they were closest to each other on both the microsatellite and AFLP trees. NEH, FMF, and CTS are mostly decedents of Long Island Sound populations, and they were closer to each other than to DBH and DBW.

## Genetic Changes in Selected Strains

Neither microsatellites nor AFLPs detected significant loss of heterozygosity in the 4 selected strains compared with the wild population from Delaware Bay, which is somewhat surprising. When populations undergo isolation and selection, reductions in effective population size and genetic variability are often unavoidable. Heterozygosity is often used as a measurement for genetic changes. Significant losses of heterozygosity have been reported in hatchery populations of fish (Allendorf and Phelps, 1980; Ryman and Stahl, 1980), while other studies have shown no significant differences between wild and hatchery populations (Allendorf and Utter, 1979). No association was observed between selection for fast growth and loss of heterozygosity in the hard clam *Mercenaria mercenaria*. (Dillon and Manzi, 1987). Also, in the Pacific oyster *Crassostrea gigas*, heterozygosity of 2 hatchery-propagated stocks was found not statistically different from their progenitor population after 3 generations (Hedgecock and Sly, 1990).

All 4 selected strains in this study, however, showed significant loss of rare alleles as measured by microsatellites and by AFLPs. The selected strains had 31.6% to 36.8% fewer alleles at the 5 microsatellite loci sampled. The loss of AFLP alleles, as measured by the increase in the number of fixed loci, was 22% in CTS, 67% in NEH, 100% in DBH, and 111% in FMF. The loss of rare alleles is a common phenomenon in populations in captivity owing to small population size, genetic drift, and selection. In an analysis of selected lines from which DBH and NEH were derived, Virjenhoek et al. (1990) also found a fewer alleles in the selected stocks compared with their wild populations. Allele loss has been reported in other selected strains of aquatic species (Gosling, 1982; Dillon and Manzi, 1987; Launey et al., 2001). As this study and other studies have shown, loss of alleles is often more easily detected than decrease of heterozygosity in hatchery or selected stocks, probably because rare alleles are more sensitive than heterozygosity to genetic drift or selection (Nei et al., 1975; Hedgecock and Sly, 1990). The loss of alleles may be a better indicator of genetic changes in selected strains, although it is unknown how great the loss of alleles would have to be to cause severe inbreeding depression. None of the selected strains shows noticeable signs of inbreeding depression at this time. Because all 4 strains were subjected to selection, and 2 of the strains were derived from mixing of selected lines and wild populations, we did not attempt to estimate effective population size.

In addition to the loss of rare alleles, there were other changes in allele frequencies in the selected strains. Gaffney et al. (1992) have shown in the eastern oyster that selection or genetic drift in a single generation may cause significant differences in allele frequencies. Significant differences in allele frequencies were found between DBW and DBH, and among all populations. Further, the two Rutgers strains that were subjected to long-term selection for disease resistance showed frequency shifts in the same direction at some loci (see Table 6). Because of the large number of loci examined, genetic drift can produce similar patterns of allele frequency shifts at some loci by chances alone. We cannot exclude, however, the possibility that those changes are results of long-term selection, and the markers involved may be linked to disease-resistance genes. We do not regard the markers listed in Table 6 as being linked to disease-resistance genes, but rather consider them as "suspects" that deserve further investigation. We are in the process of checking the suspected loci for frequency changes over disease-inflicted mortality and their positions on the genetic linkage map (Yu and Guo, 2003), which may provide an opportunity to identify potential disease-resistance genes.

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## References

Allendorf, F.W., and Phelps, S.R. (1980). Loss of genetic variation in hatchery stock of cutthroat troutns. *Trans Am Fish Soc* 109:537–543.

Allendorf, F.W., and Utter, F.M. (1979) "Population genetics" In: *Fish Physiology Vol. VIII*, Hoar, W.S., randall, D.J., and Brett, J.R. (eds.), Academic press, New York, N.Y., pp 407–454.

Andrews, J.D. (1968). Oyster mortality studies in Virginia, VII: review of epizootiology and origin of *Minchinia nelsoni*. *Proc Natl Shellfish Assoc* 58:23–36.

Brown, B.L., Franklin, D.E., Gaffney, P.M., Hong, M., Dendanto, D., and Kornfield, I. (2000). Characterization of micro satellite loci in the eastern oyster, *Crassostrea virginica*. *Mol Ecol* 9:2117–2119.

David, L., Rajasekaran, P., Fang, J., Hillel, J., and Lavi, U. (2001). Polymorphism in ornamental and common carp strains (*Cyprinus carpio* L.) as revealed by AFLP analysis and a new set of microsatellite markers. *Mol Genet Genom* 266:353–362.

Dillon, R.T., and Manzi, J.J. (1987). Hard clam, *Mercenaria mercenaria*, broodstocks: genetic drift and loss of rare alleles without reduction in heterozygosity. *Aquaculture* 60:99–105.

Excoffier, L., Smouse, P.E., and Quattro, J.M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 131:479–491.

Farley, C.A., Lewis, E.J., Relyea, D., Zahtila, J., and Rivara, G. (1998). Resistance studies for juvenile oyster disease (JOD) 1997: some early insight into genetic aspects. *J Shellfish Res* 17:352–353 (Abstract).

Felsenstein, J. (1989). Phylip - Phylogeny Inference Package (Version 3.2). *Cladistics* 5:164–166.

Ford, S.E., and Haskin, H.H. (1987). Infection and mortality patterns in strains of oysters *Crassostrea virginica* selected for resistance to the parasite *Haplosporidium nelsoni* (MSX). *J Parasitol* 73:268–376.

Gaffney, P.M. (2002). Associations between microsatellites and repetitive elements in bivalve genomes. Presented at The International Conference on the Status of Plant, Animal and Microbe Genome Research X, January 12–16, 2002, San Diego, Calif.

Gaffney, P.M., Davis, C.V., and Hawes, R.O. (1992). Assessment of drift and selection in hatchery population of oysters (*Crassostrea virginica*). *Aquaculture* 105:1–20.

Gosling, E.M. (1982). Genetic variability in hatchery-produced Pacific oysters (*Crassostrea gigas* Thunberg). *Aquaculture* 26:273–287.

Guo, S.W., and Thompson, E.A. (1992). Performing the exact test of Hardy-Weinberg proportions for multiple alleles. *Biometrics* 48:361–372.

Guo, X., Ford, S.E., DeBrosse, G., Smolowitz, R., and Sunila, I. (2003) Breeding and evaluation of eastern oyster strains selected for MSX, Dermo and JOD resistance. J Shellfish Res 22: 353–354 (Abstract).

Haanstra, J.P.W., Wye, C., Verbakel, H., Meijer-Dekens, F., van den Berg, P., Odinot, P., van Heusden, A.W., Tanksley, S., Lindhout, P., and Peleman, J. (1999). An integrated high-density RFLP-AFLP map of tomato based on two *Lycopersicon esculentum* × *L. pennellii* $F_2$ populations. *Theor Appl Genet* 99:254–271.

Haskin, H.H., and Ford, S.E. (1979). Development of resistance to *Minchinia nelsoni* (MSX) mortality in laboratory-reared and native oyster stocks in Delaware Bay. *Mar Fish Rev* 41:54–63.

Hedgecock, D., and Sly, F. (1990). Genetic drift and effective population sizes of hatchery-propagated stocks of the Pacific oyster, *Crassostrea gigas*. *Aquaculture* 88:21–38.

Hedgecock, D., Chow, V., and Waples, R.S. (1992). Effective population numbers of shellfish broodstocks estimated from temporal variance in allelic frequencies. *Aquaculture* 108:215–232.

Hubert, S., Li, G., and Hedgecock. D. (2002). Development of a genetic linkage map, using microsatellite markers in the Pacific oyster *Crassostrea virginica*. Presented at The International Conference on the Status of Plant, Animal and Microbe Genome Research X, January 12–16, 2002, San Diego, Calif.

Huff, D.R., Peakall, R., and Smouse, P.E. (1993). RAPD variation within and among natural populations of outcrossing buffalograss [*Buchloe dactyloides* (Nutt.) Engelm]. *Theor Appl Genet* 86:927–934.

Launey, S., and Hedgecock, D. (2001). High genetic load in the Pacific oyster *Crassostrea gigas*. *Genetics* 159:255–265.

Launey, S., Barre, M., Gerard, A., and Naciri-Graven, Y. (2001). Population bottleneck and effective size in *Bonamia ostreae*–resistant populations of *Ostrea edulis* as inferred by microsatellite markers. *Genet Res* 78:259–270.

Li, L., and Guo, X. (2004). AFLP-based genetic linkage maps of the Pacific oyster *Crassostrea gigas* Thunberg. *Mar Biotechnol* 6:26–36.

Lynch, M., and Milligan, E.G. (1994). Analysis of population genetic structure with RAPD markers. *Mol Ecol* 3:91–99.

MacKenzie, C.L. Jr. (1996). History of oystering in the United States and Canada, featuring the eight greatest oyster estuaries. *Mar Fish Rev* 58:1–78.

Maguire, T.L., Peakall, R., and Saenger, P. (2002). Comparative analysis of genetic diversity in the mangrove species *Avicennia marina* (Forsk.) Vierh. (Avicenniaceae) detected by AFLPs and SSRs. *Theor Appl Genet* 104:388–398.

Miller, M.P., Stevens, L.E., Busch, J.D., Sorensen, J.A., and Keim, P. (2000). Amplified fragment length polymorphism and mitochondrial sequence data detect genetic differentiation and relationships in endangered southwestern U.S.A. amber snails (*Oxyloma* spp.). *Can J Zool* 78:1845–1854.

Nei, M., Maruyama, T., and Chakraborty, R. (1975). The bottleneck effect and genetic variability in populations. *Evolution* 29:1–10.

Peakall, R., and Smouse, P.E. (2001). GenAlEx V5: Genetic Analysis in Excel. Population Genetic Software for Teaching and Research. Canberra: Australian National University.

Peakall, R., Smouse, P.E., and Huff, D.R. (1995). Evolutionary implications of allozyme and RAPD variation in diploid populations of buffalograss (*Buchloe dactyloides* Nutt. *Engelm.) Mol Ecol* 4:135–147.

Pejic, L, Ajmone-Marsan, P., Morgante, M., Kozumplick, V., Castiglioni, P., Taramino, G., and Motto, M. (1998). Comparative analysis of genetic similarity among maize inbred lines detected by RFLPs, RAPDs, SSRs and AFLPs. *Theor Appl Genet* 97:1248–1255.

Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S., and Rafalski, A. (1996). The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. *Mol Breed* 2:225–238.

Qi, X., and Lindhout, P. (1997). Development of AFLP markers in barley. *Mol Gen Genet* 254:330–336.

Qi, X., Stam, P., and Lindhout, P. (1998). Use of locus-specific AFLP markers to construct a high-density molecular map in barley. *Theor Appl Genet* 96:376–384.

Raymond, M., and Rousset, F. (1995). GENEPOP (Version 1.2): population genetics software for exact tests and ecumenicism. *J Heredity* 86:248–249.

Reece, K.S., Morrison, C., Ribeiro, W.L., Gaffney, P.M., and Allen, S.K. Jr. (2002). Microsatellite markers for the eastern oyster *Crassostrea virginica*: linkage mapping and genetic monitoring of restoration projects. Presented at The International Conference on the Status of Plant, Animal and Microbe Genome Research X, January 12–16, 2002, San Diego, Calif.

Ryman, N., and Stahl, G. (1980). Genetic changes in hatchery stocks of brown trout (*Salmo trutta*). *Can J Fish Aquat Sci* 37:82–87.

Seki, S., Agresti, J.J., Gall, G.A.E., Taniguchi, N., and May, B. (1999). AFLP analysis of genetic diversity in three populations of ayu *Plecoglossus altivelis*. *Fish Sci* 65:888–892.

Vrijenhoek, R.C., Ford, S.E., and Haskin, H.H. (1990). Maintenance of heterozygosity during selective breeding of oysters for resistance to MSX disease. *J Hered* 81:418–423.

Waugh, R., Bonar, N., Baird, E., Thomas, B., Graner, A., Haypes, P., and Powell, W. (1997). Homology of AFLP products in three mapping population of barley. *Mol Gen Genet* 255:311–321.

Weber, J.L., and Wong, C. (1993). Mutation of human short tandem repeat. *Hum Mol Genet* 2:1123–1128.

Weir, B.S., and Cockerham, C.C. (1984). Estimating F-statistics for the analysis of population structure. *Evolution* 38:1358–1370.

Yu, Z., and Guo, X. (2003). A genetic linkage map of the eastern oyster *Crassostrea virginica* Gmelin. *Biol Bull* 204:327–338.