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Isolation by Distance in the Eastern Oyster, *Crassostrea virginica*, in Chesapeake Bay

COLIN G. ROSE, KENNEDY T. PAYNTER, AND MATTHEW P. HARE

From the Department of Biology, University of Maryland, College Park, MD 20742.

Address correspondence to M. Hare at the address above, or e-mail: matthare@umd.edu.

Abstract

Intensive efforts are underway to restore depleted stocks of *Crassostrea virginica* in Chesapeake Bay. However, the extent of gene flow among local populations, an important force mediating the success of these endeavors, is poorly understood. Spatial and temporal population structures were examined in *C. virginica* from Chesapeake Bay using eight microsatellite loci. Deficits in heterozygosity relative to Hardy-Weinberg expectations were seen at all loci and were best explained by null alleles. Permutation tests indicated that heterozygote deficiency reduced power in tests of differentiation. Nonetheless, genotypic exact tests demonstrated significant levels of geographic differentiation overall, and a subtle pattern of isolation by distance (IBD) was observed. Comparisons between age classes failed to show differences in genotype frequencies, allelic richness, gene diversity, or differentiation as measured by F_{ST}, contrary to predictions made by the sweepstakes hypothesis. The IBD pattern could reflect an evolutionary equilibrium established because local gene flow predominates, or be influenced in either direction by recent anthropogenic activities. An evolutionary interpretation appears justified as more parsimonious, implying that local efforts to restore oyster populations will have local demographic payoffs, perhaps at the scale of tributaries or regional subestuaries within Chesapeake Bay.

Marine species often have the capability of long-distance larval dispersal, and as a consequence show relatively low levels of population structure (Bohonak 1999). Larval duration in the water column prior to settlement explains a substantial fraction of the variation in average effective dispersal distances among species, despite the heterogeneity of methods used to estimate these parameters (Shanks et al. 2003; Siegel et al. 2003). Thus, genetic panmixia over small regional scales is a reasonable null hypothesis for marine species with long larval periods. It is well known that deviations from this trend occur because of rafting of adults (Johannesson 1988) or larval behavior (Baker and Mann 2003; Hill 1991; Shanks 1995), but it is difficult to predict how these mechanisms will interact with hydrography and selection to shape gene flow.

When a marine species is threatened or requires management, it is risky to assume that the potential for long-distance dispersal necessarily will be realized as large-scale gene flow (Cowen et al. 2000). The eastern oyster, *Crassostrea virginica* Gmelin, is broadly distributed in the western North Atlantic and was once abundant throughout Chesapeake Bay, a large estuary on the mid-Atlantic Coast of the United States (Figure 1). The oyster's reef-forming habit and large filter-feeding capacity historically made it a keystone member of the estuarine community (Jackson et al. 2001). Overfishing and disease have reduced the oysters to less than 1% of their historic numbers (Jordan and Coakley 2004; Newell 1988). Even so, the prolific fecundity of this species might allow for a rapid regeneration of historic numbers if not for the low density of remaining breeders in a severely degraded environment with intense disease pressure (Boesch et al. 2001a; Burreson and Ragone Calvo 1996; Jackson 2001). This suggests that when local restoration efforts are successful, the geographic scale of their effects will depend on the distribution of improved habitat and the extent of dispersal among those patches.

The eastern oyster has a life history conducive to high gene flow. Oyster larvae spend 2–3 weeks in the plankton (Kennedy 1996), conceivably traveling hundreds of kilometers before settlement. Thus, there is the potential that long-range gene flow provides demographic connections between areas with localized restoration activities. In Chesapeake Bay these activities include constructing reef habitat in protected sanctuaries, seeding reefs with broodstock, and developing and releasing disease-tolerant strains of the native oyster (Allen et al. 2003; Breitburg et al. 2000; Mann and Evans 2004). However, large-scale gene flow does not

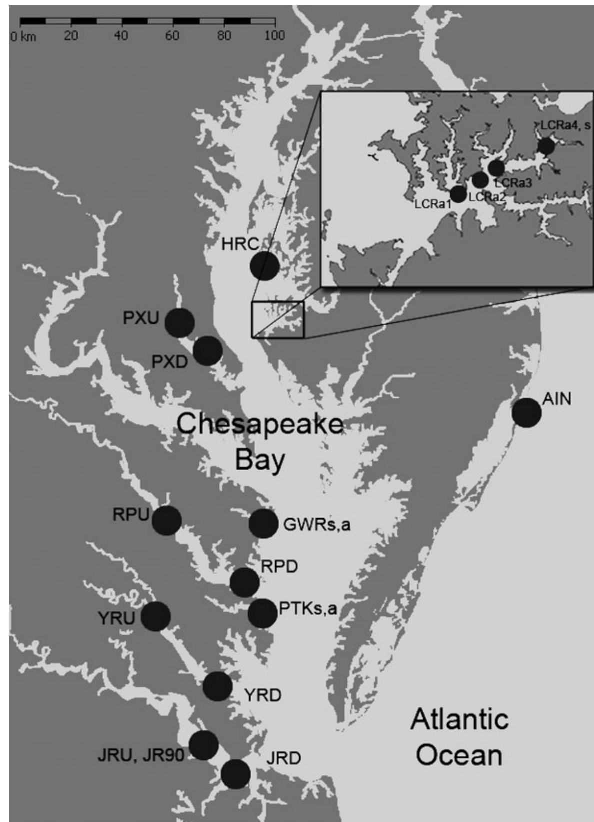

Figure 1. Map of Chesapeake Bay showing sampling sites and location abbreviations used in Table 1 and the text.

assure that restored reefs will be populated by migrants; optimal recruitment depends on matching the size and spacing of sanctuary reefs to the scale and pattern of dispersal (Botsford et al. 2003). Also, the potential genetic impacts from introducing disease-tolerant strains depend on the geographic scale of dispersal and subsequent interbreeding with wild stocks (Hare et al., in press).

Some Chesapeake Bay tributaries are "traplike" (Andrews 1979) with respect to oyster recruitment because of low flushing rates, restricted entrances, or retentive local circulation (Southworth and Mann 1998). Swimming behavior of oyster larvae in response to salinity, currents, or other cues (Dekshenieks et al. 1996; Finelli and Wethey 2003) can also promote retention (Southworth and Mann 1998; Tankersley et al. 1995). The best evidence for a traplike dynamic comes from the Great Wicomico River in Virginia where surface drifters and concentrations of oyster larvae both maintained their position or moved upstream (Southworth and Mann 1998). Without knowing the relative importance of physical factors and behavior to larval retention, the generality of local recruitment is uncertain. However, only certain tributaries will be retentive if it depends on hydrography, whereas behavioral retention mechanisms should promote local recruitment in most tributaries.

The presettlement movement of larvae only enables gene flow; the ensuing postsettlement processes of natural selection and variance in reproductive success determine which migrants leave offspring and the magnitude of effective gene flow (Hilbish 1996; Palumbi 1994). Hedgecock (1994) suggested that high fecundity and the stochasticity of larval viability can lead to extreme variance in reproductive success (a sweepstakes event) in marine organisms. Sweepstakes events could potentially create genetic heterogeneity among cohorts, or transiently among localities, when there is limited mixing of larvae among breeding populations. Two predictions of Hedgecock's sweepstakes hypothesis are (1) reduced variation within cohorts compared with the rest of the population (Hedgecock 1994) and (2) higher genetic heterogeneity over time in one location than seen spatially among breeding populations (Flowers et al. 2002; Li and Hedgecock 1998). Extreme variance in reproductive success has been hypothesized to explain genetic patterns observed in cod (Ruzzante et al. 1996) and oysters (Boudry et al. 2002; Hedgecock 1994; Li and Hedgecock 1998). In sea urchins sweepstakes events have been proposed in some populations (Addison and Hart 2004) and rejected in others (Flowers et al. 2002).

Selection against migrants can also limit gene flow despite high dispersal (Bertness and Gaines 1993; Johnson and Black 1984; Koehn et al. 1980; Schmidt and Rand 2001). In contrast to the demographic effects of larval retention and sweepstakes reproduction, which shape the distribution of polymorphism across the entire genome, genetic heterogeneity caused by selection is expected only at those loci linked to the genes under selection (Gilg and Hilbish 2003; Johannesson et al. 2004; Karl and Avise 1992; McGoldrick et al. 2000).

Each of the gene flow determinants described above has the potential to generate a complex patchwork of genetic connections that would complicate restoration planning. Using neutral genetic markers to avoid locus-specific patterns, high-resolution testing for population structure can be informative about the magnitude and spatial scale of gene flow. It takes very little gene flow over evolutionary time to homogenize populations, however, so population differences are only expected to accumulate in response to strong and consistent evolutionary barriers to gene flow (Palumbi 2003). Alternatively, anthropogenic effects could have homogenized Chesapeake oyster populations or created a patchwork of genetic differences. Human manipulation of these populations has included transplants within Chesapeake Bay (Meritt D, personal communication), introductions from the Gulf of Mexico (Carlton and Mann 1996; Milbury et al. 2004), and planting of juveniles produced in hatcheries from wild or selected-strain broodstock (Brumbaugh et al. 2000; Sorabella et al. 2002). Although many of these activities have been extensive in portions of Chesapeake Bay over the last few decades as measured by human efforts and resources expended, their impacts on oyster population genetics are unknown. It is conceivable that the genetic impacts have been minimal, for all the biological reasons listed above. If most oysters are not contributing offspring in any particular generation (sweepstakes) and intensive fishing pressure quickly culls transplanted oysters, then transplants that have important management benefits could have trivial effects on patterns of gene flow. Nonetheless, with anthropogenic and

Table 1. Oyster sample information. Spat and adult samples are designated with an "s" or "a" in the sample code. Sample sizes indicate the number of individuals analyzed

Sample code	Description	Sample size	Date collected	Latitude, longitude
AIN	Assateague Island	50	July 14, 2002	38°14′23″N, 75°08′44″W
GWRa	Great Wicomico River adults	90	November 1, 2002	37°49′N, 76°18′W
GWRs	Great Wicomico River juveniles	102	July 22, 2002	37°49′N, 76°18′W
HRC	Harris Creek	50	September 16, 2002	38°45′03″N, 76°17′45″W
JR90	James River (archived)	48	1990	37°03′N, 76°41′W
JRD	James River downriver	50	December 10, 2003	36°03′N, 76°41′W
JRU	James River upriver	38	December 10, 2003	37°04′10″N, 76°35′07″W
LCRa1	Little Choptank River adults—site 1	59	April 9, 2002	38°32′01″N, 76°14′38″W
LCRa2	Little Choptank River adults—site 2	50	April 9, 2002	38°32′37″N, 76°13′37″W
LCRa3	Little Choptank River adults—site 3	46	April 9, 2002	38°32′55″N, 76°13′04″W
LCRa4	Little Choptank River adults—site 4	57	April 9, 2002	38°34′05″N, 76°10′34″W
LCRs	Little Choptank River juveniles	163	June–August 2002	38°34′N, 76°10′W
PTKa	Piankatank adults	47	November 5, 2002	37°30′35″N, 76°20′32″W
PTKs	Piankatank juveniles	48	November 5, 2002	37°31′21″N, 76°21′12″W
PXD	Patuxent River downriver	50	October 18, 2002	38°23′31″N, 76°33′32″W
PXU	Patuxent River upriver	50	October 18, 2002	38°30′26″N, 76°40′11″W
RPD	Rappahannock River downriver	50	December 10, 2003	37°36′19″N, 76°24′45″W
RPU	Rappahannock River upriver	50	December 10, 2003	37°50′40″N, 76°45′40″W
YRD	York River downriver	50	December 10, 2003	37°15′15″N, 76°31′26″W
YRU	York River upriver	50	December 10, 2003	37°30′15″N, 76°47′51″W

evolutionary effects confounded, both sources of variation must be considered.

Isolation by distance (IBD), a pattern in which genetic differentiation increases with the geographic scale of comparison, is usually modeled as a stepping-stone pattern of gene flow in which migration only occurs among neighboring demes (Kimura and Weiss 1964). Recent simulations and theory indicate, however, that IBD can also emerge with a low level of long-distance migration if most recruitment is local (Palumbi 2003). Thus, if other assumptions are met, an IBD pattern provides a relatively robust indication that local gene flow predominates within the scale of study. IBD has been detected in several high-dispersal marine organisms, including fishes (Buonaccorsi et al. 2004; Castric and Bernatchez 2003; Gold et al. 2001; Planes and Fauvelot 2002; Pogson et al. 2001; Riginos and Nachman 2001), urchins (Palumbi et al. 1997), eels (Maes and Volckaert 2002; Wirth and Bernatchez 2001), and oysters (Launey et al. 2002).

Previous work has examined genetic variation in *C. virginica* from Chesapeake Bay. In a study by Buroker (1983), samples from 10 Chesapeake Bay oyster bars revealed significant genetic differentiation across 32 allozyme loci, with mean $F_{ST} = 0.016$. Principal component analysis clustered the oysters into four groups whose distribution did not correlate with any obvious environmental variables, and IBD was rejected. Other Chesapeake Bay studies found no significant genetic heterogeneity but also had low power (Brown and Paynter 1991; Rose 1984).

Here we test for population structure in Chesapeake Bay *C. virginica* using eight microsatellite loci. To test for IBD, specimens were collected from across Chesapeake Bay at various spatial scales. To test for sweepstakes events, temporal comparisons were made between juveniles and adults. We also test for anthropogenic effects where possible.

Materials and Methods

Sampling Design

Because temporal and spatial processes of differentiation could act at any geographic scale, spatial samples were collected from sites separated by aquatic distances ranging from one to hundreds of kilometers. A total of 1,228 specimens were collected from 16 locations in or near Chesapeake Bay (Table 1, Figure 1). All adults were collected by dredge or diver from natural subtidal reefs that have not been manipulated (e.g., transplants, juvenile oyster plantings) for several years prior to our collections. Juvenile oysters (spat) and adults were collected during the same year in the Piankatank, Great Wicomico, and Little Choptank rivers. Spat in the Great Wicomico and Little Choptank rivers were sampled by serially deploying clean oyster shell "collectors" for 2- or 4-week periods, respectively, from June through September. Collectors were examined by eye for spat. Spat in the Piankatank River were collected by dredge. All oysters were stored on ice until gill and mantle tissue, or whole spat, were preserved in 95% ethanol. Shell height of spat ranged from 2 to 25 mm, consistent with young of the year. Archived samples collected from the James River in 1990 were obtained from P. Gaffney, University of Delaware.

Two *C. virginica* strains artificially selected for disease tolerance have been planted in Chesapeake Bay for restoration purposes since 1999 (Brumbaugh et al. 2000). The two strains, known as CROSBreed and DEBY, were bred for resistance to the protozoan parasites *Perkinsus marinus* and *Haplosporidium nelsoni* (Ragone Calvo et al. 1997). A reference sample of DEBY strain oysters was obtained in 2002 from the progeny of generation 4 broodstock produced at the Center for Environmental Science, University of Maryland. A reference sample of CROSBreed strain, generation 5, was obtained from K. Reece, Virginia Institute of Marine Science (VIMS).

DNA Extraction, Amplification, and Genotyping

Approximately 20 mg of gill or mantle tissue was used for DNA extraction from adults using the DNeasy 96 Tissue kit (Qiagen Inc., Valencia, CA) following the protocol for animal tissues. CROSBreed, GWRa, and GWRs samples were extracted with a FastPrep FP120 instrument (BIO 101, Vista, CA) using a FastDNA kit (BIO 101) (see Reece et al. 2004). Genomic DNA was diluted to 50 ng/μl based on spectrophotometry.

Oysters were genotyped for eight microsatellite loci previously developed by Brown et al. (2000) and Reece et al. (2004). Five loci have perfect repeat motifs (one di-, one tri-, and three tetranucleotides), and three loci have imperfect repeat motifs (1 di-, 1 tri-, 1 tetranucleotide). The primers (reported with optimized annealing temperature and $MgCl_2$ concentration) are *Cvi*9 (52°C, 1.7 mM), *Cvi*12 (52°C, 1.7 mM), *Cvi*1i24b (52°C, 2.5 mM), *Cvi*2g14 (52°C, 2.5 mM), *Cvi*2i23 (51.5°C, 1.5 mM), *Cvi*2i4 (47°C, 2.8 mM), *Cvi*2j24 (touchdown, 1.7 mM), and *Cvi*1g3 (touchdown, 1.7 mM). Reaction conditions for polymerase chain reaction (PCR) in a total volume of 7.5 μl included final concentrations of 1× Invitrogen buffer (no $MgCl_2$), 100 μM deoxynucleoside triphosphate, and 200 nM each for forward and reverse primers, one of which was fluorescently labeled. Thermocycling involved one cycle of 95°C denaturing for 1 min; 30 three-step cycles including 95°C for 30 s, annealing temperature for 30 s, and 72°C for 20 s; and then a final extension at 72°C for 10 min. Touchdown thermocycling began with 10 three-step cycles in which annealing started at 60°C for 1 min and dropped by 1°C each cycle, followed by 30 cycles of 95°C for 15 s, 50°C for 1 min, and 72°C for 45 s. After amplification, 8.82 μl HiDi formamide and 0.18 μl Genescan-500 ROX size standard (Applied Biosystems, Foster City, CA) were combined with 1 μl PCR product for fragment analysis.

PCR products were electrophoresed with an ABI-Prism 3100 genetic analyzer (Applied Biosystems) and allele sizes were estimated using GENESCAN version 3.7 and GENOTYPER version 2.5 (Applied Biosystems). Electropherogram peaks were examined before assigning genotypes. If a single peak was detected and it was greater than 500 relative fluorescent units (RFUs), the specimen was labeled a homozygote for that allele. For a specimen to be labeled a heterozygote, both peaks had to be at least one repeat unit apart and greater than 100 RFUs; if the two peaks were of different heights, the shorter peak was scored only if its height was >10% of the taller one. If no peaks were present, a second PCR was performed; if electropherogram peaks were still absent then the locus was considered nonamplifying.

Data Analysis

To minimize missing data, we removed 30 specimens (2.4%) from the data set, distributed across 10 samples, because they had more than two nonamplifying loci. To quantify deviations of genotype frequencies from Hardy-Weinberg expectations among and within samples, we calculated the unbiased F_{ST} estimator θ and the F_{IS} estimator f (Weir and Cockerham 1984) using FSTAT version 2.9.3.2 (Goudet 2001). To test for overall genetic subdivision, θ was calculated for all samples except JR90 (a sample collected in 1990). Ninety-five percent confidence intervals (CI) were calculated using 15,000 bootstrap replicates across loci. Pairwise comparisons were performed between all samples except for JR90, which was only compared with JRD. Number of alleles, allelic richness, and gene diversity were also calculated with FSTAT. Unbiased estimates of P values for G-based exact tests of genotypic frequency differentiation (Goudet et al. 1996) were calculated using GENEPOP version 3.4 (Raymond and Rousset 1995b). To determine the independence of the microsatellite loci, we tested for genotypic linkage disequilibrium among each pair of loci using GENEPOP (10,000 dememorization steps, 1000 batches, 10,000 iterations per batch in the Markov chain).

In order to test for an association between genetic and geographical distances, the natural logarithm of the shortest pairwise aquatic distances (shortest route over water) were correlated with pairwise values of $\theta/(1-\theta)$ between all samples except JR90 (Rousset 1997). Distances between adults and spat in the Great Wicomico River, Little Choptank River, and Piankatank River samples were changed from 0 to 1 km for the log transformation. We used the Mantel test (Mantel 1967) for correlation between the two distance matrices based on 10,000 permutations as implemented in the ISOLDE program in GENEPOP (Raymond and Rousset 1995b). GENEPOP was also used to compute the regression line describing the relationship between $\theta/(1-\theta)$ and the natural logarithm of distance.

We tested for evidence that stuttering or large allele dropout was affecting microsatellite genotypes. Stuttering refers to a tendency by *Taq* polymerase to amplify fragments of multiple sizes in addition to the correct one, especially from dinucleotide repeats (Shinde et al. 2003). Large allele dropout is the preferential amplification of shorter alleles from heterozygotes (Wattier et al. 1998). Both these artifacts affect the distribution of heterozygosity among allele size classes in predictable ways. To test for a deficiency of heterozygotes carrying alleles differing in size by one repeat unit (stuttering) and for an excess of specimens that are homozygous for small alleles (large allele dropout), we randomized genotypes for each locus within samples using MICRO-CHECKER version 2.2.1 (Van Oosterhout et al. 2004).

As a result of several generations of selection, the DEBY and CROSBreed oyster strains have genetic signatures that are distinct from wild oysters (Hare et al., in press). Because regional plantings of selectively bred oysters could affect an IBD pattern, multilocus genotypes of 49 CROSBreed and 82 DEBY oysters were used as reference samples for assignment tests with the oysters collected in this study. Using the Bayesian method of Rannala and Mountain (1997) in GENECLASS2 version 2.0.b (Piry et al. 2004), each presumed wild oyster was removed from the total collection and treated as unknown for testing against CROSBreed, DEBY, and the remaining N-1 wild samples. This assignment method assumes Hardy-Weinberg equilibrium but is fairly robust to deviations (Cornuet et al. 1999). Applying a low-stringency assignment criterion to be conservative,

Table 2. Per-locus and global allelic richness, gene diversity, Weir and Cockerham estimates of F_{IS} (f) and F_{ST} (θ), and exact tests of genotypic differentiation

	Cvi9	Cvi12	Cvi1i24b	Cvi2g14	Cvi2i23	Cvi2i4	Cvi2j24	Cvi1g3	All loci
Number of alleles[a]	24	32	26	37	40	28	21	10	218
Allelic richness[a]	14.2	14.1	14.6	21.8	21.1	17.0	11.1	6.47	15.0
Gene diversity[a]	0.905	0.853	0.888	0.947	0.895	0.923	0.866	0.618	0.862
F_{IS}[a]	0.182*	0.176*	0.404*	0.087*	0.017*	0.164*	0.163*	0.233*	0.175*
F_{ST}[a]	0.001	0.002	0	0	−0.002	0.001	0.002	0.002	0.001
Exact test[a] (P value)	0.460	0.146	0.064	0.798	0.991	0.052	0.056	**<0.0001**	**0.0001**
F_{ST} (GWRa and GWRs)	−0.001	0.003	0.006	0	−0.001	0	−0.003	−0.004	0
Exact test (P value)	0.167	0.155	0.220	0.440	0.649	0.051	0.891	0.868	0.253
F_{ST} (LCRa1–4 and LCRs)	−0.002	−0.001	0.001	0.001	−0.001	0.002	0.008	−0.003	0.001
Exact test (P value)	0.623	0.767	0.058	0.262	0.547	0.340	**0.013**	0.987	0.146
F_{ST} (PTKa and PTKs)	−0.005	−0.007	−0.001	−0.005	−0.006	0.014	−0.005	−0.001	−0.002
Exact test (P value)	0.655	0.754	0.473	0.832	0.977	**0.018**	0.632	0.660	0.662
F_{ST} (JRD and JR90)	−0.005	0	0.02	−0.003	−0.003	−0.003	0.006	0.008	0.002
Exact test (P value)	0.703	0.253	0.118	0.548	0.417	0.400	0.294	**0.020**	0.119

[a] All populations except JR90.

* $P < .001$; Bold type indicates $P < .05$ (Fisher's method).

specimens that had a lower negative log likelihood assignment score for CROSBreed or DEBY versus the wild reference sample were removed from the data set as possible selected-strain oysters. Similar assignment methods implemented in IMMANC version 5.0 (Rannala and Mountain 1997) were used to calculate, for each "wild" individual, the probability of being an F_1 offspring of a selected strain by wild cross, and individuals with $P > .95$ were removed. The IBD analysis was repeated after each culling.

We examined the effect of heterozygote deficiency on tests of genotypic differentiation by randomizing alleles within samples using GENETIX version 4.05.2 (Belkhir et al. 2001). The randomized data had levels of heterozygosity that were similar to Hardy-Weinberg expectations, but without changing the allele frequencies or homozygous null frequencies. The number of significant pairwise θ and exact tests were compared between the original and permuted data.

A power analysis for F_{ST} was done by randomly subsampling a data set consisting of two identically sized samples (each sample had $N = 100$). The first sample combined Patuxent River samples (PXD and PXU) and the second combined York River samples (YRD and YRU). Using the POPTOOLS version 2.6.2 (Hood 2004) add-in for Microsoft Excel, multilocus genotypes from each sample were randomly subsampled without replacement to create 200 replicate data sets for each of 15 subsample sizes. The subsampled data were analyzed in FSTAT, and mean F_{ST}, mean upper 95% CI, and mean lower 95% CI were calculated for the replicates at each subsample size.

We used temporally spaced samples from the James River, JRD and JR90, to estimate the effective population size of oysters. N_e was calculated using the moments-based method of Waples (1989) in NEESTIMATOR version 1.3 (Peel et al. 2004) and using the pseudolikelihood method of Wang (2001) in MLNE version 1.1 (Wang 2005). Assuming a 2-year generation time (Hedgecock 1994), we calculated N_e across six generations. Both methods assume that the samples are from a single isolated population; for the pseudolikelihood method we set the maximum N_e at 10,000 (due to computational constraints).

Average squared dispersal distance between parent and offspring, σ^2, was calculated using the method of Rousset (1997). Under a two-dimensional stepping-stone model, the inverse of the IBD regression slope is equal to $4D\pi\sigma^2$, where D is the density of the effective number of individuals (Rousset 2003). The area of Chesapeake tributaries was estimated using SCION IMAGE version 4.0.3.2.

Results

Genetic Variation and Hardy-Weinberg Equilibrium

A total of 1,198 individuals were analyzed. All eight microsatellite loci were highly variable in terms of gene diversity (0.618–0.947) and number of alleles (10–40; Table 2). All samples had roughly the same allelic richness and the same proportion of rare alleles to total number of alleles (mean = 30%; Table 3). The number of singleton alleles, those observed only once in the entire data set, ranged from 0 to 5 per sample, whereas only two alleles were private, occurring more than once but found only in one sample (Table 3).

No significant genotypic linkage disequilibrium was detected between loci ($P > .05$ in each case). All samples, including all three spat samples, had significant Hardy-Weinberg deviations in the direction of heterozygote deficiency. Over all samples, the F_{IS} estimator f was found to be statistically different from zero for each locus (all $P < .001$) and over all loci ($P < .001$; Table 2). There was no evidence of stuttering or large allele dropout at any locus. Although insertions and deletions are abundant in the flanking sequences of six of the loci (Reece et al. 2004), no correlation was found between the level of polymorphism reported for the flanking regions (2.0–5.8%) and F_{IS} (Pearson $r = -.34$, 5 df, $P = .507$) as would be expected if null alleles were caused by polymorphic nucleotides in the PCR priming sites.

Table 3. Number of alleles and mean allelic richness across all loci in each population. Singleton alleles were those that appeared only once in our analysis. Private alleles occurred more than once but were found only within one sample. Rare alleles were at less than 2% overall frequency

		Number of alleles				
Population	*N*	Singletons	Private	Rare	Total	Mean allelic richness
AIN	50	0	0	36	128	14.8
GWRa	90	3	1	55	148	15.1
GWRs	102	2	0	54	147	15.2
HRC	50	2	0	28	120	14.0
JR90	48	2	0	47	136	15.0
JRD	50	0	1	39	129	14.8
JRU	38	2	0	32	119	15.0
LCRa1	59	2	0	43	134	14.9
LCRa2	50	1	0	37	127	14.4
LCRa3	46	0	0	30	120	14.8
LCRa4	57	1	0	38	130	14.6
LCRs	163	5	0	62	155	14.2
PTKa	47	0	0	31	120	14.9
PTKs	48	2	0	35	127	14.7
PXD	50	1	0	35	126	14.6
PXU	50	0	0	35	125	15.0
RPD	50	3	0	38	129	15.5
RPU	50	0	0	43	134	15.3
YRD	50	3	0	43	133	15.0
YRU	50	2	0	41	130	16.0
All populations	1198	31	2	126	220	15.1

Genetic Differentiation

Over all samples (excluding JR90), exact tests of genotypic differentiation detected significant ($P < .05$) population structure at one of the eight microsatellite loci and highly significant ($P = .0001$) population structure across all loci (Table 2). Genotypic exact tests demonstrated statistically significant differences ($P < .05$) at 35 of the 171 pairwise comparisons (21%). After sequential Bonferroni correction for multiple comparisons (Holm 1979), three of the pairwise comparisons remained statistically significant ($\alpha = 0.05$). The global estimate of θ was low ($\theta = 0.001$) and not significantly different from zero (Table 2). We calculated θ for all pairs of samples and found that 19 of the 171 comparisons (11%) were significantly different from zero ($P < .05$), but none remained statistically significant after sequential Bonferroni correction ($\alpha = 0.05$).

Adult and spat oysters from the same locality showed no significant difference in gene diversity (sign test comparing adults and spat in three locations, eight loci each, df = 23, $P = .308$), allelic richness ($P = .541$), or genotypic frequency as measured by exact tests ($P > .05$; Table 2). Adult oysters collected in the James River more than a decade apart, JRD and JR90, also did not have significantly different genotypic frequencies (Table 2).

Isolation by Distance

A significant association ($P = .009$) was found between pairwise estimates of genetic structure ($\theta/(1-\theta)$) and the natural logarithm of aquatic distance for all samples (excluding JR90) (Table 4). A regression of $\theta/(1-\theta)$ and aquatic distance with all loci combined is shown in Figure 2. Analysis of individual loci revealed statistically significant correlations for two of the eight loci, and six of the eight loci had positive regression slopes (Table 4). The IBD pattern remained significant ($P < .05$) after setting negative values of $\theta/(1-\theta)$ to zero (data not shown), after removing any one sampling site from the data set (data not shown), or after removing any one locus (Table 4). Furthermore, the pattern of IBD remained significant after combining all downriver and upriver adult samples within tributaries or combining coincident spat and adult samples in GWR and PTK (data not shown). When all four adult samples from LCR were combined with LCR spat, IBD remained nearly significant ($P = .057$). Finally, multilocus assignment tests identified 23 of the study oysters as CROSBreed or DEBY strain individuals or an overlapping set of 156 as F_1 progeny between wild and selected-strain crosses. The IBD slope remained positive and significant ($P = .036$) after removing the 23 oysters from the data set, but removing the larger subset of nonwild oysters reduced the slope slightly (0.00066) and made the Mantel test nonsignificant ($P = .068$).

Sample Size and Power

Using 100 samples each from the York River and Patuxent River, we detected significant population structure ($\theta = 0.0034$, $P < .05$). Randomly drawing 200 replicate samples at each of several subsample sizes, the mean value of θ was unaffected by subsample size (as expected for an unbiased estimator of F_{ST}), but the 95% CI increased as subsample size decreased (data not shown). If we consider θ to be significant when the mean lower CI does not overlap zero, a sample size of 90 or greater was necessary to statistically detect the low observed levels of differentiation.

Table 4. IBD parameters for each locus, all loci combined, and all loci minus one. IBD slope and r^2 were calculated from regression of $\theta/(1-\theta)$ against log distance. Mantel's test was performed to determine the significance of the relationship (P) between genetic differentiation and aquatic distance

	Per locus			One locus excluded		
Locus	IBD slope	r^2	P	IBD slope	r^2	P
*Cvi*9	0.0010	.030	**.032**	0.0006	.076	**.025**
*Cvi*12	0.0017	.056	.059	0.0005	.057	**.037**
*Cvi*1i24b	0.0002	.001	.415	0.0007	.114	**.002**
*Cvi*2g14	0.0004	.016	.160	0.0007	.082	**.016**
*Cvi*2i23	−0.0002	0	.683	0.0008	.096	**.006**
*Cvi*2i4	−0.0001	.002	.706	0.0008	.119	**.001**
*Cvi*2j24	0.0009	.024	.231	0.0006	.065	**.019**
*Cvi*1g3	0.0020	.020	**.046**	0.0005	.075	**.035**
All loci	0.0007	.093	**.009**			

Bold type indicates uncorrected $P < .05$.

The data were permuted to investigate the effect of Hardy-Weinberg deviations on power to detect differences by exact tests and θ. A greater number of statistically significant pairwise comparisons were observed in the permuted data than in the original data for both measures of differentiation (Table 5). These results suggest that Hardy-Weinberg deficits reduced power to detect population differentiation. Mantel tests detected an IBD pattern in the permuted data set ($P = .005$) with slightly greater statistical power than with the original data set ($P = .009$). The regression of $\theta/(1-\theta)$ against the natural logarithm of distance led to a nearly identical slope and r^2 compared with the unpermuted data (Table 5).

Effective Population Size

The moments-based estimate of oyster N_e in James River was 535 (95% CI: 234–6061), whereas the pseudolikelihood estimate was 1,516 (95% CI: 422–10,000). Only the lower confidence interval is informative in the pseudolikelihood estimate because the upper limit was arbitrarily set to 10,000.

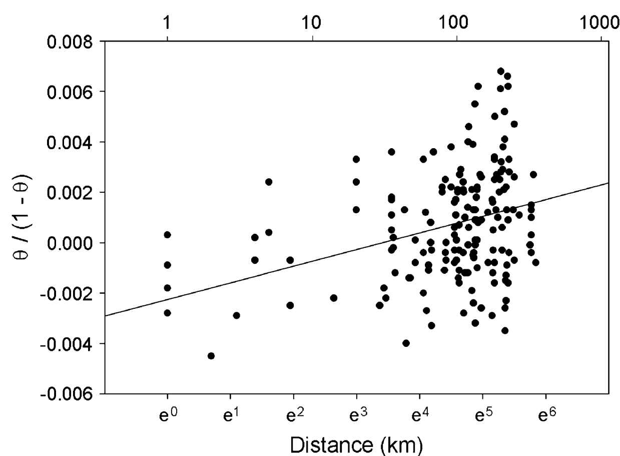

Figure 2. IBD in Chesapeake Bay oysters. Multilocus estimates of pairwise differentiation are plotted against logarithm of aquatic distances. The regression is $y = 0.0007x - 0.0023$ and the distance between subpopulations ranges from 1 to 345 km.

Dispersal Distance

To estimate the σ^2 from the IBD slope, we first determined the N_e/N ratio for James River from the likelihood estimate of $N_e = 1{,}516$ and the harmonic mean of James River population estimates over 1998–2002, $N = 1.8 \times 10^9$ (VIMS 2003, CBOPE Web site). This estimate of N_e/N, 8.42×10^{-7}, is consistent with the estimate from Hedgecock et al. (1992). Total N_e for Chesapeake Bay was estimated to be 2,611 by assuming the N_e/N ratio is uniform across Chesapeake Bay and multiplying the James River ratio by the total number of oysters in the Chesapeake, 3.1×10^9 (harmonic mean of estimates from 1998 to 2002; VIMS 2003, CBOPE Web site). The total N_e divided by the area of the tidal waters in Chesapeake Bay, approximately 11000 km^2 (Boesch et al. 2001b), gives an average density of 0.24 oysters/km^2. Based on this density estimate and the inverse of the IBD slope (1,429), average squared dispersal distance is approximately $\sigma^2 = 479$ km^2.

Discussion

In this study we have examined the magnitude and pattern of genetic differentiation among several eastern oyster populations in Chesapeake Bay. We found evidence for spatial but not temporal genetic heterogeneity. Most significantly, genetic differentiation increased with geographic distance within the bay. If this pattern is the result of an evolutionary equilibrium, it provides support for the assumption of local recruitment that underlies current strategies for oyster restoration. However, genetic differences contributing to this association were small, so before elaborating on the biological meaning and significance of IBD we discuss the robustness of these findings.

Power

Choice of molecular marker and sampling design both affect the ability to detect differentiation between populations.

Table 5. Effect of permutation on tests of differentiation and IBD. Exact tests and θ were calculated for 171 pairwise comparisons of 19 populations. Sequential Bonferroni correction was calculated with $\alpha = 0.05$

		Pairwise test of differentiation					
		θ		Exact test			
	F_{IS}	No. significant ($P < .05$)	No. significant (Bonferroni corrected)	No. significant ($P < .05$)	No. significant (Bonferroni corrected)	IBD slope	IBD r^2
Original data	0.175	19	0	35	3	0.0007	.093
Permuted data	0.003	78	6	117	85	0.0007	.100

Microsatellites are markers that permit a high level of statistical power because of their high heterozygosity (Estoup et al. 2002; Hedrick 1999), but homoplasy can downwardly bias F_{ST} estimates for loci with high mutation rates (Balloux and Goudet 2002; Balloux et al. 2000; O'Reilly et al. 2004). The only individual locus to show significant genotypic differentiation, *Cvi*1g3, also had the lowest number of alleles and gene diversity (Table 2), implicating homoplasy caused by high mutation rates as a constraint on differentiation at the other seven loci. However, F_{ST} was not any higher for *Cvi*1g3, so in this case the different statistical results may depend on the relative power of rare and moderate frequency alleles (Waples 1998).

For highly differentiated populations, population structure can be statistically detected even with small sample sizes. However, this is generally not the case for marine populations with high gene flow (Ruzzante 1998). When differentiation is low, exact tests have greater power than F_{ST} to reject homogeneity (Goudet et al. 1996; Raymond and Rousset 1995a), and this was true here with testing at the genotypic rather than allelic level. While exact tests provide a powerful statistical test, they do not provide information about the degree of gene flow. In order to analyze geographic patterns of gene flow, we relied on estimates of F_{ST}.

When the number of migrants per generation is high ($N_em > 10$), which our data suggest is the case for Chesapeake Bay oysters, F_{ST} is estimated with low precision (Neigel 1996). Thus, with typical sample sizes, values of θ may be statistically indistinguishable from zero (Kalinowski 2002). Nonetheless, the power requirements differ for statistically testing F_{ST} between any pair of populations versus testing for an association between F_{ST} and another variable across many pairwise comparisons. Specifically, in this latter case the statistical significance of any particular pairwise comparison is less important than the absence of a systematic bias related to sample sizes, as with θ (Cockerham and Weir 1993; Weir and Cockerham 1984). In addition, Peterson and Denno (1998) found that the likelihood of detecting IBD increased with the number of populations sampled in a study, and power also depends on adequate sampling at multiple spatial scales (Palumbi 2003). Therefore, rather than sampling the >90 specimens per location that our power analysis showed were necessary to statistically detect differences between pairs of populations, we sampled approximately 50 specimens from a larger number of populations at multiple spatial scales to test overall patterns of gene flow in Chesapeake Bay. Of course, low precision could obscure an IBD pattern, but it should not falsely generate IBD because sampling error is independent of the proximity of the collection sites.

Heterozygote Deficiency

The microsatellite loci used here showed large heterozygote deficits relative to Hardy-Weinberg expectations (positive F_{IS}). Heterozygote deficiency can be explained by Wahlund effects, inbreeding, natural selection, or null alleles (and other technical artifacts), but the large F_{IS} values make some of these hypotheses untenable. The Wahlund effect, a reduction in heterozygosity resulting from sampling across subdivided populations, cannot be a major contributor to the heterozygote deficiency because dramatic population structure is lacking. Even the selected strains of oysters that have been released into Chesapeake Bay are only moderately differentiated from wild stocks and therefore inconsistent with Wahlund-induced F_{IS} higher than 0.05. Finally, inbreeding is an unlikely source for such large F_{IS} values because sweepstakes events were negligible and because *C. virginica* is dioecious.

Natural selection cannot be dismissed as easily, but it seems unlikely to be the sole cause for heterozygote deficiencies because it would have to be acting across all eight unlinked loci. Zouros et al. (1980) proposed that background selection against deleterious alleles might cause heterozygote deficiency at linked genetic markers in oysters. Strong selection against deleterious alleles (genetic load) has been used to explain segregation distortion in studies of the Pacific oyster, *Crassostrea gigas* (Bierne et al. 2000; Boudry et al. 2002; Launey and Hedgecock 2001; McGoldrick and Hedgecock 1997; McGoldrick et al. 2000), and the eastern oyster (Yu and Guo 2003). Background selection reduces within-deme heterozygosity, potentially amplifying between-deme population structure (Charlesworth et al. 1997; Pamilo et al. 1999). However, there is no reason to expect that population structure induced by background selection would be positively associated with geographic distance between populations.

Null or nonamplifying alleles are a plausible explanation for heterozygote deficiencies in the data. Polymorphisms at priming sites could have created differences among alleles in their amplification efficiency in PCR, resulting in a global

deficiency of microsatellite heterozygosity. Hedgecock et al. (2004) detected null alleles in 49 of 96 microsatellite loci in the Pacific oyster and calculated that the minimum level of sequence polymorphism in the priming region was 1.2%. Data from Reece et al. (2004) showed that the DNA sequences flanking six of the microsatellite loci used in this study (*Cvi*1i24b, *Cvi*2g14, *Cvi*2i23, *Cvi*2i4, *Cvi*2j24, and *Cvi*1g3) had a mean sequence polymorphism of 3.6% (2.0–5.8%). Given this high level of polymorphism surrounding PCR priming sites for these loci, it is likely that null alleles contribute to the heterozygote deficiency described in this study. Although we failed to detect a correlation between sequence variation near the priming sites and F_{IS} in the six loci, the estimates of sequence variation from Reece et al. (2004) were relatively imprecise, based on an average of 3.7 alleles sequenced per locus.

Primer redesign can correct heterozygote deficiency if null alleles are to blame, as was done for an anonymous nuclear locus by Hare and Avise (1996). In highly polymorphic species, however, a large number of sequences must be considered to assure that polymorphisms do not affect priming sites. Thus, the redesign of PCR primers for the *Cvi*2g14 and *Cvi*1g3 loci by Reece et al. (2004) corrected some null alleles discovered by pedigree analysis but may not have prevented additional PCR null alleles in wild populations.

Corrections for null alleles are frequently applied to data sets, but common methods (e.g., Brookfield 1996; Chakraborty et al. 1992) assume a single null allele. These correction methods are inappropriate for the oyster data because multiple PCR null alleles may occur at different frequencies, and Hardy-Weinberg deviations may derive from both technical and biological factors (Foltz 1986; McGoldrick et al. 2000). Instead, we performed a permutation test to address whether heterozygote deficiency introduced bias or reduced statistical power in our estimates of F_{ST} and exact tests of genotypic differentiation. Results indicated that heterozygote deficits led to reduced power in tests of differentiation, but the IBD pattern was not sensitive to these effects.

Sweepstakes

Comparisons of adult and juvenile oysters in three tributaries of Chesapeake Bay demonstrated that sweepstakes events, if they happen, are not frequent or strong (see Table 3). The strongest evidence against sweepstakes reproduction was that juvenile and adult oysters had no statistical difference in allelic richness, the most sensitive indicator of recent bottlenecks (Spencer et al. 2000). Methods used here would have missed rare or localized sweepstakes events. Nonetheless, given these results and the high polymorphism observed at the microsatellites and their flanking sequences (Reece et al. 2004), we conclude that sweepstakes reproduction does not lower effective population size of Chesapeake Bay oysters as dramatically as previously hypothesized (Hedgecock 1994). This conclusion does not negate the expectation that high fecundity elevates variance in reproductive success and lowers N_e/N (Hedrick 2005) but merely rejects extreme sweepstakes events.

Effective Size

Hedgecock et al. (1992) measured temporal genetic variance between eastern oyster adults sampled one generation apart in the James River, Virginia, and used moments-based methods (Pollak 1983; Waples 1989) to estimate $N_e = 30.0$ (95% CI: 13.5–60.8). Both our estimates of James River N_e are substantially larger than Hedgecock's. Using the same estimation method as Hedgecock et al. (1992), we can reject $N_e < 234$ in James River. However, when many alleles are at low frequency, as with our data, moments-based estimates can be biased (Turner et al. 2001; Waples 1989), whereas likelihood-based estimates perform well (Wang 2001). Thus, the likelihood estimate of $N_e = 1{,}517$ is probably more accurate. This effective size is still consistent with a very small N_e/N ratio and high variance in reproductive success, but it is not compatible with dramatic sweepstakes events.

Isolation by Distance

The populations of *C. virginica* in Chesapeake Bay have a statistically significant population structure consistent with IBD, but genetic differentiation explained a small fraction of variation in aquatic distance and the regression slope was shallow. Hedrick (1999) raised the question of whether subtle microsatellite divergence has evolutionary meaning. Faint substructure could result from recent nonequilibrium processes or from random noise due to sampling error (Waples 1998). However, Palumbi (2003) suggested that low levels of genetic differentiation can be verified by demonstrating a relationship between genetic relatedness and distance because sampling error is unlikely to produce a significant IBD pattern. In this study, locus- and site-specific artifacts seem unlikely to have created IBD because the pattern remains significant after individual populations or loci are removed.

Oyster transplants among Chesapeake tributaries could have genetically homogenized populations, reducing the strength of an evolutionary equilibrium IBD pattern. Alternatively, it is conceivable that the IBD pattern was created by a particular combination of anthropogenic impacts. There are several reasons why anthropogenic effects are likely to be minimal in our data. First, we took great care to collect oyster samples from locations relatively unaffected by restoration activities and oyster transplanting. Second, plantings and transplants are often designed to have large impacts on local census numbers for fisheries or restoration, but this does not necessarily mean that the planted oysters successfully reproduce at a scale that would leave a genetic trace. This is especially true when there are targeted harvests of transplanted oysters. Finally, in cases where the population genetic consequences of oyster manipulations are predictable, we can test for these effects to assess the magnitude of their impacts.

The hatchery mass spawns that produce oysters for planting use a limited number of parents and have a potential for skewed parental contributions that lower allelic diversity (Launey et al. 2001). Thus, plantings of hatchery-produced oysters, done on a large scale, are predicted to lower allelic richness near the planting site. This could increase genetic relatedness locally while accentuating differences regionally

(through independent, hatchery-induced bottlenecks), conceivably generating a pattern of IBD. However, allelic richness was uniformly high across sites and equally high inside and outside the Chesapeake Bay.

Large-scale plantings of genetically distinct disease-tolerant *C. virginica* (DEBY and CROSBreed selection lines, Ragone Calvo et al. 1997) could also create an IBD pattern if different practices in Maryland and Virginia contributed to regional differentiation, while individual plantings homogenized local populations. Our results are equivocal on this matter because some loss of power is expected when over 10% of the total sample is removed to conservatively eliminate the effects of selected-strain introgression. Thus, it is possible that a combination of management activities has created an IBD pattern, but an evolutionary explanation for IBD seems more parsimonious. Finding an IBD pattern in Chesapeake Bay with other kinds of genetic markers, such as mitochondrial DNA, or among populations along the U.S. Atlantic Coast, would help confirm the appropriateness of applying an evolutionary interpretation here.

Evolutionary IBD develops as equilibrium is reached between gene flow and genetic drift (Wright 1943). The modern distribution of oysters in Chesapeake Bay arose in the last 12,000–18,000 years after the most recent glacial advance (Grumet 2000). Assuming a generation time of 4 years, there may have been as few as 3,000 generations for Chesapeake oysters to reach equilibrium. While this is implausible for species with low levels of migration, it is possible when the proportion of migrants (m) is high because the time to equilibrium is inversely related to migration (Crow and Aoki 1984). More specifically, if mutation rate is much smaller than m and $1/N_e$ is much smaller than 1 (both reasonable assumptions for oysters), then the time required for F_{ST} to go half way to equilibrium is approximated by $(\ln 2)/(2m + 1/2N_e)$. To illustrate the strong dependence on migration rate, suppose that oysters in Chesapeake Bay have $N_e = 100{,}000$. Then $m = 0.0001$ ($N_e m = 10$) requires 3,381 generations to get half way to equilibrium, while $m = 0.001$ ($N_e m = 100$) requires only 346 generations. Our low estimates of F_{ST} for Chesapeake Bay oysters reflect high rates of migration ($N_e m \equiv 250$) that could have generated migration-drift equilibrium since the Pleistocene. Furthermore, during the approach to equilibrium, IBD is manifest initially at relatively small spatial scales (Slatkin 1993). Thus, it is feasible for oysters to be at migration-drift equilibrium and show IBD within Chesapeake Bay. Also, under an equilibrium interpretation, the finding of IBD within Chesapeake Bay should not be sensitive to the degree of local recruitment occurring within the tributaries we sampled. That is, the pattern of IBD will be dictated by the least retentive tributaries whether they are sampled or not.

Spatial Scale of Dispersal

If an IBD pattern indicates that local gene flow predominates within Chesapeake Bay, how local is local? The average squared dispersal, $\sigma^2 = 472$ km^2, is roughly equivalent to 4% of the entire Chesapeake Bay or the area within a large tributary (e.g., area of James/Elizabeth rivers = 747 km^2). This estimate defines a geographic scale encompassing the bulk of dispersal from a central point source, implying that recruitment of oysters in Chesapeake Bay is local within tributaries or regional subestuaries. This single-generation value is a long-term evolutionary average that may encompass some interannual variation in dispersal distances. A two-dimensional IBD measure of σ^2 depends on population density but is independent of the shape of the distribution of dispersal distances (Rousset 1997). At higher oyster densities characteristic of Chesapeake Bay before 1900, the same slope would indicate a smaller average squared dispersal. A given σ^2 can result from lots of short-range dispersal or a little longer-range dispersal (Rousset 1997), so a measure of average dispersal distance is impossible to calculate from σ^2 without simulations based on particular distributions of dispersal distances. Ongoing studies are expected to help define average dispersal distance by contributing direct estimates from a point source (Hare et al., in press) and by estimating dispersal distributions from individual-based models of larval behavior and hydrographic mixing (North E, University of Maryland Center for Environmental Science, personal communication).

Conclusions

There are many potential explanations for differences between potential and realized dispersal (Cowen et al. 2000; Ehrlich and Raven 1969; Hilbish 1996; Pogson et al. 2001; Slatkin 1987). Hydrodynamic features within Chesapeake Bay tributaries are often cited as a primary mechanism determining local recruitment (Andrews 1979; Mann 1988). However, retentive characteristics such as low flushing rate or tidal gyres are only strongly expressed in a few tributaries and are therefore not likely to be the primary factor generating IBD at the scale of Chesapeake Bay. This reasoning implies that larval behavior may be as important as hydrography, making local recruitment the rule, not a tributary-specific phenomenon.

What is the relevance of this evolutionary equilibrium pattern of gene flow to restoration practices? Very few successful migrants are needed on average to homogenize populations over an evolutionary timescale (Wright 1931), so even slight genetic differentiation (such as at larger scales in Chesapeake Bay) indicates that gene flow is trivial over the ecological timescale relevant to restoration (Palumbi 2003; Waples 1998). IBD in Chesapeake Bay oysters therefore suggests that impacts from population enhancement efforts will be concentrated near where resources are invested.

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