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Authors

Hare, MP
Karl, SA
Avisé, JC

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Anonymous Nuclear DNA Markers in the American Oyster and Their Implications for the Heterozygote Deficiency Phenomenon in Marine Bivalves

Matthew P. Hare,* Stephen A. Karl,† and John C. Avise*

*Department of Genetics, University of Georgia; and †Department of Biology, University of South Florida

A puzzling population-genetic phenomenon widely reported in allozyme surveys of marine bivalves is the occurrence of heterozygote deficits relative to Hardy-Weinberg expectations. Possible explanations for this pattern are categorized with respect to whether the effects should be confined to protein-level assays or are genomically pervasive and expected to be registered in both protein- and DNA-level assays. Anonymous nuclear DNA markers from the American oyster were employed to reexamine the phenomenon. In assays based on the polymerase chain reaction (PCR), two DNA-level processes were encountered that can lead to artifactual genotypic scorings: (a) differential amplification of alleles at a target locus and (b) amplification from multiple paralogous loci. We describe symptoms of these complications and prescribe methods that should generally help to ameliorate them. When artifactual scorings at two anonymous DNA loci in the American oyster were corrected, Hardy-Weinberg deviations registered in preliminary population assays decreased to nonsignificant values. Implications of these findings for the heterozygote-deficit phenomenon in marine bivalves, and for the general development and use of PCR-based assays, are discussed.

Introduction

In allozyme surveys of natural bivalve populations, heterozygote frequencies often appear to be significantly below Hardy-Weinberg expectations (HWE) (reviews in Singh and Green 1984; Zouros and Foltz 1984). The magnitudes and patterns of these departures tend to vary across loci, as well as among populations and species. In an early review of this topic, significant heterozygote deficits at some allozyme loci had been documented in local populations of more than 25 species of bivalve molluscs (Zouros and Foltz 1984). Such observations are particularly enigmatic for these species because (a) most marine pelecypods are broadcast spawners and have pelagic larvae, such that large panmictic populations might be expected and (b) several of these species exhibit positive correlations between allozyme heterozygosity and fitness-related traits (e.g., growth rate, survival, and fecundity), such that heterozygotes might be selectively favored (Singh and Zouros 1978; Koehn and Shumway 1982; Vrijenhoek, Ford, and Haskin 1990; Pogson and Zouros 1994).

Several hypotheses regarding heterozygote deficits in marine bivalves have been advanced, but none alone appears capable of accounting fully for the phenomenon. For current purposes, these hypotheses may be grouped initially into two heuristic categories distinguished by whether the heterozygote-reducing effects are confined to protein-level assays of particular gene products or

whether they potentially extend genome-wide and, thus, would be reflected in appropriate assays at both the protein and DNA levels (table 1). The first category of explanations (I, table 1) involves biological "artifacts" that would compromise proper scorings of genotypes in protein-level assays (because the assay method might fail to distinguish some heterozygotes from homozygotes). The second category of explanations (II, table 1) includes population-level factors that should in principle be manifested genome-wide in appropriate assays at both the protein and DNA levels. One of these latter explanations (IId, table 1) fits less neatly into the category II. This involves natural selection, perhaps operating in underdominant fashion against heterozygotes at certain life-history stages or in a disruptive pattern producing microspatial heterogeneity in allele frequencies and thereby contributing to a Wahlund effect in local population samples. Such selective influences could be observed in both protein-level and DNA-level assays, but their effects would be confined to the loci under selection (and to other, tightly linked genes).

Several DNA-level assays used to describe variation in particular coding or noncoding stretches of the nuclear genome (nDNA) produce allelic and genotypic data analogous to (and in some respects more refined than) genetic information previously available from protein-level electrophoretic studies. These nDNA approaches include polymerase chain reaction (PCR) assays of single-locus minisatellites (VNTRs; Jeffreys et al. 1988) and microsatellites (SSRs; Weber and May 1989), randomly amplified polymorphic DNAs (RAPDs; Williams et al. 1991), exon-primed targeted digestions (Slade et al. 1993), and restriction site polymorphisms

Key words: Hardy-Weinberg, heterozygote deficiency, oysters, anonymous scnDNA markers, PCR.

Address for correspondence and reprints: Matthew P. Hare, Department of Genetics, University of Georgia, Athens, Georgia 30602. E-mail: harem@bscr.uga.edu.

Table 1
Examples of Potential Explanations for Heterozygote Deficiencies (Relative to Hardy-Weinberg Expectations) for Local Population Samples of Bivalve Molluscs

I. Effects confined to protein-level assays
a) null alleles due to post-transcriptional alterations
b) genetic imprinting, whereby alleles are differentially expressed depending on whether they are of maternal or paternal origin ^a
II. Effects expected to be registered in appropriate assays at both the protein and DNA levels
a) local inbreeding, including the possibility of occasional self-fertilization
b) other demographic or life-history patterns that produce spatial or temporal population substructure leading to a Wahlund effect
c) null alleles due to a high frequency of chromosomal deletions
d) selection against heterozygotes (effects likely to be locus dependent, rather than genomically pervasive)
III. Effects confined to PCR-nDNA assays
a) differential allelic amplification from target locus
b) amplification from multiple paralogous loci ^b

^a See Chakraborty (1989).

^b Expected to produce the appearance of heterozygote frequency excess rather than deficit (see text).

(RSPs) at anonymous low- or single-copy sequences (scnDNAs; Karl and Avise 1993). Such DNA markers are increasingly used in population genetics because moderately or highly polymorphic characters can be generated from multiple unlinked regions of the nuclear genome. Furthermore, many DNA-level polymorphisms are likely to be neutral or nearly so mechanistically (though not necessarily in an evolutionary-dynamic sense if linked to other markers under selection; see Avise 1991).

In some cases, nDNA and allozyme markers have shown strikingly different population genetic patterns in the same species. A pertinent example involves the American oyster (*Crassostrea virginica*), where a pronounced population subdivision concordant with earlier mitochondrial DNA results (Reeb and Avise 1990) was registered in RSPs at four anonymous scnDNA loci (Karl and Avise 1992), but a geographic uniformity was displayed in allele frequencies at multiple polymorphic allozyme loci (the protein data originally were interpreted to evidence high gene flow mediated by pelagic gametes and larvae [Buroker 1983]). This pronounced discordance between population genetic patterns at the DNA and protein levels was provisionally interpreted as suggestive of geographically uniform balancing selection at protein-coding loci (Karl and Avise 1992). Whether or not this explanation is correct, a sobering message is that different molecular markers can sometimes paint very different pictures of population structure and gene flow when interpreted under models of selective neutrality.

All prior discussions of heterozygote deficits in natural populations of marine bivalves have focused on allozyme polymorphisms and, thus, have potentially suffered from the biological artifacts that uniquely affect protein-level assays (I, table 1). Here, we present results of a survey of RSPs at four anonymous nDNA loci in population samples of the American oyster, a marine bivalve for which heterozygote deficiencies have previously been observed in protein assays (Zouros, Singh, and Miles 1980; Buroker 1983; Hedgecock and Okazaki 1984; Grady, Soniat, and Rogers 1989). Our initial intent was to expand on the previous study by Karl and Avise (1992) by more intensively sampling the area of a phylogeographic break near Cape Canaveral, Florida (Hare and Avise, unpublished data). However, in preliminary assays we found a pattern of deviation from HWE at the scored loci similar to that reported for allozymes in this species. Technical complications were also encountered that can affect genotypic scoring at the DNA level and, thus, an unanticipated digression reported upon here became the documentation of two DNA-level phenomena that can lead to artifactual genotypic scorings in assays that rely on PCR amplification. We describe some of the symptoms of these artifacts and provide general recommendations on how to recognize and ameliorate the difficulties. Finally, we discuss the relevance of the DNA findings to the heterozygote-deficit phenomenon in marine bivalves.

Materials and Methods

Population Samples

A total of 637 oysters from 26 locations throughout much of the range of *C. virginica* in the western North Atlantic was assayed (see table 3). In 1991, 361 oysters were sampled from 17 locations throughout eastern Florida, a region that in a previous study exhibited a pronounced transition in population allele frequencies at some loci between Atlantic and Gulf of Mexico regions (Karl and Avise 1992). The 1991 collections were made at roughly 40-km intervals along the intracoastal waterway from St. Augustine to Miami, Florida. In addition, 276 oysters from Karl and Avise (1992), originally collected in 1990, also were reanalyzed.

Laboratory Procedures

Techniques for the production of RSP data from scnDNA loci are detailed in Karl and Avise (1993). Briefly, primers capable of amplifying nDNA from *C. virginica* were designed from genomic clones represented at low copy number in the genome (as evidenced in dot blot experiments). Each primer pair amplified a product within which a single RSP was assayed (i.e., the informative restriction enzyme distinguishes two co-dominant alleles: cut and uncut). The primer pairs were

Table 2
Deviations of Heterozygote Frequencies from Hardy-Weinberg Expectations ($D = [H_o - H_e]/H_e$) for RSPs at Each of Four Loci Across 26 Sampling Locales of the American Oyster

POPULATION ^a	ORIGINAL PCR PRIMERS				NEW PRIMERS	
	CV-19.0	CV-195.0	CV-32.0	CV-7.0	CV-32.4	CV-7.7
MA* ^b ..	-0.061	0.380 ^c	-0.274	0.000	0.024	0.034
SC* ...	0.004	0.220	0.103	0.024	0.452	0.071
GA* ...	-0.294	0.156	-0.330	-0.786	0.226	0.167
SA	0.127	0.531	-0.377	0.000	0.040	0.083
FL	0.057	0.422	-0.159	0.114	0.270	0.213
SM ...	-0.068	0.519	-0.021	0.000	-0.040	0.049
SM2* ..	-0.152	0.294	-0.271	0.035	-0.181	0.196
OH	0.000	0.016	-0.067	0.061	0.286	0.028
DP	-0.136	-0.128	-0.476	-0.773	0.194	0.250
PI	-0.313	0.375	-0.138	0.318	0.057	0.094
SE	-0.283	0.305	-0.171	0.089	-0.063	0.250
VB	-0.064	-0.188	-0.022	-0.626	0.040	0.075
FP	-0.610	-0.181	-0.444	-0.667	-0.080	-0.254
ST	-0.501	0.025	-0.083	-0.764	0.147	-0.383
ST2* ...	-0.418	0.279	-0.016	-0.765	0.149	-0.154
JU	-0.159	0.323	-0.156	-0.625	0.216	0.061
PB	-0.156	-0.288	-0.770	-0.696	-0.231	-0.158
BB	-0.501	-0.189	-0.121	-1.000	0.219	0.286
DB	-0.658	-0.031	-0.182	-0.896	0.258	-0.220
PO	-0.249	-0.220	-0.008	-0.610	0.161	0.161
JL	-0.339	-0.144	-0.026	-0.750	0.054	0.057
OR	-0.067	-0.207	-0.327	-0.797	0.040	0.040
PC* ...	-0.006	0.000	0.000	-0.654	0.056	0.056
PA* ...	-0.235	-0.531	—	-0.715	0.010	-0.253
CA* ...	-0.149	—	—	-0.640	-0.222	-0.317
LA* ...	-0.268	—	—	-0.750	-0.132	-0.047

^a Localities defined in table 3.

^b Populations with asterisks were originally sampled by Karl and Avise (1992), and D values for the original primers were calculated from unpublished raw data in that study.

^c Bold and underlined values are significantly different from zero ($\alpha = 0.050$), and empty cells indicate allele fixation.

used to assay four RSPs, under assay conditions that differed somewhat (typically 0.5 μ l DNA, 12 pmol primer, 0.2 mM final dNTP concentration) from those in Karl and Avise (1992). The PCR reactions were also scaled down to a 25- μ l total volume.

Notations for these RSPs are nondecimal and italicized (*CV-7*, *CV-32*, *CV-19*, *CV-195*) when reference is made to the physical chromosomal locus (regardless of the primer pair used in the assay). A zero-decimal notation (e.g., *CV-7.0*) is used when referring to the four original primer pairs from Karl and Avise (1992), and higher decimal values (e.g., *CV-7.7*) are employed when referring to later-generation (internal) primers developed for two of these same loci (*CV-7* and *CV-32*) in the current study. These internal primers, intended for assays of the original RSPs in smaller amplification products, were designed using Oligo 4.0 (National Biosciences). Modified PCR assay conditions for the *CV-7.7*

and *CV-32.4* primer pairs included a $MgCl_2$ concentration of 3 mM, a total of 35 PCR cycles, and annealing temperatures of 60°C and 55°C for the two pairs, respectively.

For purposes of cloning, amplification products from *CV-7* and *CV-195* were generated with various primers. In general, amplification products resulting from 35 PCR cycles were column-purified (Promega Wizard), ligated into pGEM-T vector (Promega), and used to transform competent JM109 *Escherichia coli* cells. Colonies were screened by amplifying the vector insert directly from the colony (Gussow and Clackson 1989) using (a) the primer pair that generated the insert (to check the restriction profile) and (b) one insert primer and one opposing vector primer (to determine orientation). Clones were sequenced (Sequenase; U.S. Biochemicals) from single-stranded template prepared from the vector (Promega protocol) or from PCR-amplified insert (fmol cycle sequencing [Promega] or automated sequencing [Applied Biosystems]). Except where specified, sequences were obtained in one direction only, with most ambiguities resolved by overlap between multiple sequencing reactions using different sequencing primers. Sequence alignments were done manually and attempted to minimize gaps and sequence differences.

Data Analysis

Genotypic proportions relative to HWE were summarized by $D = (H_o - H_e)/H_e$, where H_o and H_e are observed and expected frequencies of heterozygotes (the latter calculated using Levine's [1949] correction for small sample size). Chi-square statistics were computed as exact probabilities using the EXACT criterion of the HDYBG step in BIOSYS-I (Swofford and Selander 1981, version 1.7). Pairwise sequence similarity values were calculated using PAUP (Swofford 1993, version 3.1.1).

Results

Departures of heterozygote frequency from HWE in 26 populations are presented in table 2 for the four nDNA loci as assayed by the original Karl and Avise (1992) primers, and by the newly designed internal primers at *CV-7* and *CV-32*. Genotypic counts upon which these latter calculations were based are shown in table 3. Considering first the data produced by the original four primer pairs, the qualitative pattern of deviations from HWE was reminiscent of previously reported patterns based on an allozyme survey of oyster populations along the same coast (Buroker 1983): most gene-by-locality comparisons did not depart significantly from HWE (with our sample sizes); the statistically significant deviations predominantly involved heterozygote deficits, although some instances of heterozygote excess

Table 3
Numbers of Oysters Exhibiting Indicated RSP Genotypes at Two nDNA Loci in 26 Locales

Locus	POPULATION ^a																											
	MA*SC*	GA*	SA	FL	SM-	SM	2*	OH	DP	PI	SE	VB	FP	ST	ST-	2*	JU	PB	BB	DB	PO	JL	OR	PC*	PA*	CA*	LA*	
CV-7.7																												
AA ^b	28	19	22	16	18	19	18	17	1	3	7	5	9	6	4	3	2	1	2	1	1	1	1	0	3	1	2	
Aa	3	4	10	4	11	3	10	2	10	10	14	11	11	6	11	11	7	12	6	10	8	8	4	9	3	13		
aa	0	0	0	0	0	0	0	0	6	5	2	4	9	7	14	7	12	7	12	9	10	11	25	24	13	26		
CV-32.4																												
AA	23	8	20	11	10	11	20	7	0	1	3	1	5	0	1	0	2	0	0	1	0	1	0	3	1	11		
Aa	11	15	13	8	18	9	8	12	7	8	10	8	13	6	13	9	6	8	9	10	3	8	4	16	4	18		
aa	1	0	0	1	2	2	2	1	12	10	10	11	11	14	15	14	13	12	11	9	17	11	25	19	13	12		

^a Sample locations (asterisks, see footnote "b" in table 2): MA = Woods Hole, MA; SC = Charleston, SC; GA = Cumberland Island, GA; SA = St. Augustine, FL; FL = Flagler Beach, FL; SM-2 = New Smyrna Beach, FL; OH = Oak Hill, FL; DP = Delespine, FL; PI = Pineda, FL; SE = Sebastian, FL; VB = Vero Beach, FL; FP = Fort Pierce, FL; ST-2 = Stuart, FL; JU = Jupiter, FL; PB = West Palm Beach, FL; BB = Boynton Beach, FL; DB = Deerfield Beach, FL; PO = Pompano Beach, FL; JL = Ft. Lauderdale, FL; OR = North Miami, FL; PC = Port Charlotte, FL; PA = Panacea, FL; CA = Carabelle River, FL; LA = Grand Isle, LA.

^b A = uncut allele.

were also observed; and the departures from HWE occurred in a locality- and locus-specific pattern (table 2). With regard to the latter point, most noteworthy were the results for CV-7.0, where 15 of 26 population samples (58%) displayed significant heterozygote deficits. In the most extreme case (at Boynton Beach [BB]), no het-

erozygotes were recorded among the 20 individuals surveyed, despite the appearance of 8 and 12 homozygotes for the two alternative alleles.

Technical artifacts and misscorings of gels are potential sources of locus-specific deviations from HWE both in allozyme assays (Ayala et al. 1973; Buroker, Herschberger, and Chew 1975) and in the PCR-dependent assays reported here (table 1). In the PCR screening of RSPs, if a primer pair does not amplify all alleles at a locus, or if it amplifies alleles from multiple loci, then the amplification products are not a fair representation of the target (diploid, single-copy) Mendelian gene. Therefore, we next investigated the effects of these potential artifacts on the DNA-level assays, with special focus on the CV-7 locus where the heterozygote deficits were most pronounced.

Differential PCR Amplification

Polymorphism at a PCR priming site (particularly near its 3' end) is expected to cause different amplification efficiencies across alleles. The result of amplification from a diploid locus with priming site polymorphism will depend upon competition for replication between the two alleles in the reaction. PCR outcomes consistent with this "artifact" would include (a) an altered intensity of amplification from one allele or another, depending upon PCR conditions and (b) (when more than two primers are available for a locus) different apparent RSP genotypes at the locus, depending on the particular combination of primers employed. At the population level, the effect of priming site polymorphism would be to lower the estimated frequency of RSP heterozygotes below the true value, because some RSP heterozygotes would be misscored as homozygotes.

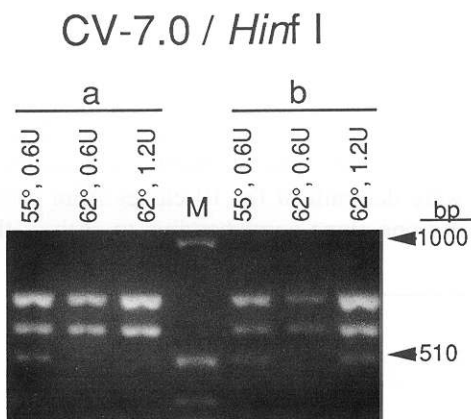
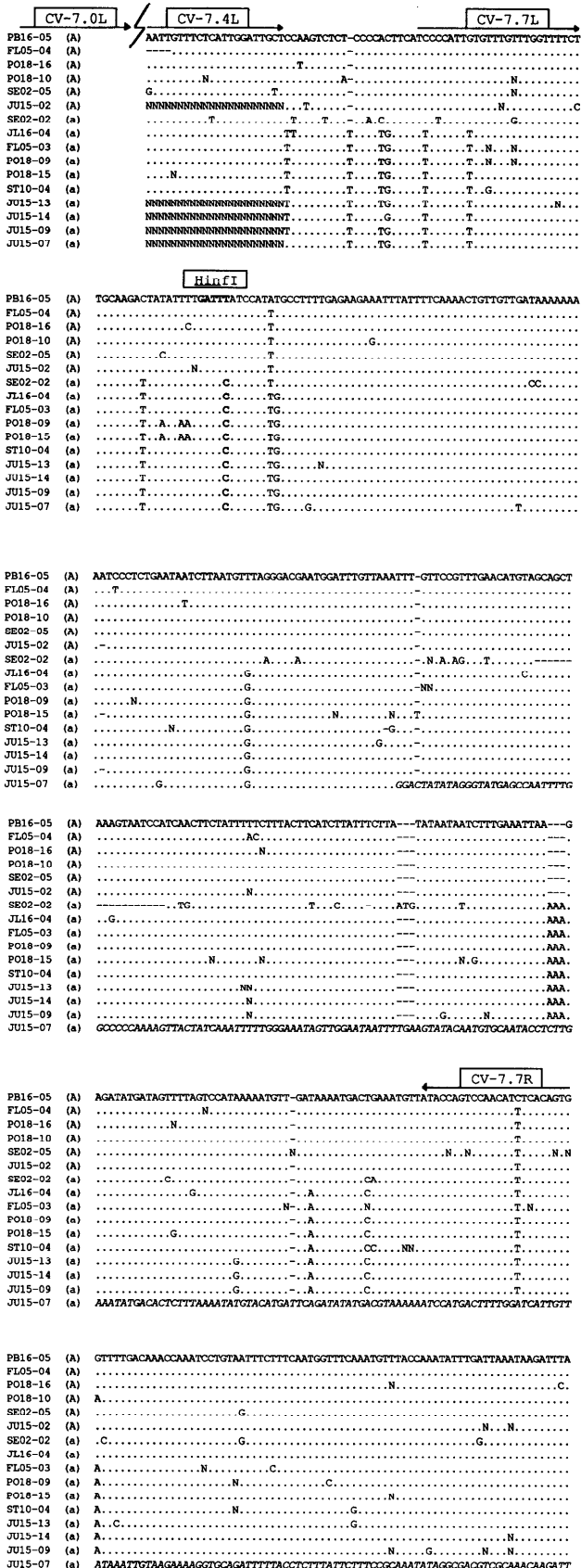


FIG. 1.—PCR condition-dependent *Hinf*I restriction profiles exemplifying differential amplification with the CV-7.0 primers in two heterozygous individuals ("a" and "b"). Visualization was with ethidium bromide, and M is a molecular weight standard. Generally, in CV-7.0 digestion profiles the top band is uniformly present and the lower two visible bands define the target RSP (in a true heterozygote, a small fragment of about 130 bp [off the gel] is cleaved from the middle fragment to produce the bottom fragment of length 510 bp [visible in lanes 1, 5, and 7]). For each of the two heterozygous individuals shown, nDNA was amplified using standard conditions (62°C annealing, 0.625 U *Taq*), lower annealing temperature (55°C), or higher *Taq* concentration (1.2 U). Genotypic scorings varied according to these conditions. For example, at 55°C, individual "a" gave the appearance of being a heterozygote, whereas at 62°C it gave the appearance of being homozygous for the uncut allele regardless of *Taq* concentration.



Redesigned primers that correct the problem of differential PCR amplification due to primer site polymorphism should (a) eliminate symptom “a” above, (b) produce a heterozygous RSP genotype if such was the case for any other primer pair at the locus (symptom “b”) and (c) increase the scored frequencies of RSP heterozygotes.

To address these issues empirically, initially a single individual whose genotype appeared to vary depending upon the annealing temperature was chosen for cloning (fig. 1). Small regions of a single clone were sequenced, and a new internal primer pair (CV-7.4) was designed (fig. 2). This primer pair amplified a much smaller DNA fragment (569 vs. 1,350 bp) still containing the target *HinfI* polymorphism. PCR condition-dependent genotypes were also observed with these new primers (data not shown). In addition, 28% of 300 individuals assayed were scored for different apparent genotypes depending on whether the CV-7.0 or CV-7.4 primers were used, with no consistent pattern of which pair produced a homozygous versus heterozygous genotype.

Reasoning that a comparison of alleles would reveal regions of sequence with relatively little polymorphism for the design of refined primers, seven putatively heterozygous individuals that showed symptoms of CV-7.4 priming site polymorphism were chosen for cloning. CV-7.4 alleles apparently refractory to amplification were targeted for cloning from each heterozygote by using some combination of the CV-7.0 and CV-7.4 primers capable of amplifying both alleles and cloning the amplification products thereby produced. The size and orientation of the insert, and its *HinfI* restriction fragment profile, were determined for 10 clones from each individual, and one clone corresponding to each of the two

FIG. 2.—Aligned partial DNA sequences from locus CV-7 (only 449 nucleotides of the 5' end are shown). The far left column indicates the individual (e.g., JU15) and clone number (e.g., 13) from which the sequence was determined, followed (in parentheses) by the allelic designation for the target RSP. A dot denotes a nucleotide match with the top reference sequence, and any ambiguous nucleotides (N) or nucleotide substitutions are indicated. Only clones generated with the CV-7.0L primer provided sequence information for the CV-7.4L priming site. Each dash indicates a single nucleotide gap. PCR priming sites are labeled and indicated with arrows above the alignment, and the target *HinfI* site is boldface and labeled. Primer sequences used were CV-7.0L and R, as reported in Karl and Avise (1992). The internal primers in the present study were CV-7.4L = 5'-AATTGTTTCTCA-TTGGATTGCT-3'; CV-7.4R = 5'-ATCAT(C/T)GCCAGTCC-ATAAATG-3'; CV-7.7L = 5'-T(T/C)C(T/C)CATT(T/G)TG(T/G)TTGTTGGTTTTCT-3'; CV-7.7R = 5'-CACTGTGAAATGTTGGACTGGTAT-3'. The bottom sequence is aligned with the others at the 5' end only, after which an insertion sequence (shown in italics) prevented further alignment.

expected *HinfI* alleles was isolated and sequenced (fig. 2). In addition, sequences were generated from some clones that had unexpected restriction fragment profiles (i.e., undetectable in the diploid genotype; see below). Sixteen sequences were aligned, and “conserved” regions on either side of the target *HinfI* site were selected for the design of new primers (CV-7.7) that would amplify a 328-bp product (fig. 2). One of these new primers (CV-7.7L) was designed with 16-fold degeneracy at the 5' end to account for observed sequence polymorphism (see legend to fig. 2).

Unlike the results using the CV-7.0 and CV-7.4 primers, the CV-7.7 diploid *HinfI* restriction profiles were repeatable under a wide range of PCR conditions (using modified MgCl₂ and primer concentrations and annealing temperatures). Furthermore, the CV-7.7 primers successfully amplified DNA from 40 individuals that previously had failed to amplify with the CV-7.0 primers (and thus had not been included in the CV-7.0 results in Karl and Avise [1992]). However, 21 individuals that appeared heterozygous using the CV-7.0 or CV-7.4 primers were scored as homozygous with CV-7.7 (a violation of criterion “b” above). This may indicate that one or more alleles remain refractory to amplification with CV-7.7 primers. Alternatively, because the apparent genotypes of these individuals did not show PCR condition dependency with CV-7.7, perhaps the “heterozygous” restriction patterns from primer pairs CV-7.0 or CV-7.4 were due to amplification from a paralogous locus (see below).

Amplification from Multiple Paralogous Loci

PCR amplification of nontarget DNA from unrelated loci can occur by spurious and imperfect primer annealing under low stringency conditions, but because such PCR products are usually of unexpected size, this problem can be detected easily and can be circumvented by increasing reaction stringency. Of concern here is amplification from more than one member of a gene family. Amplification from multiple paralogous loci could inflate the apparent number of heterozygotes over the true value for the target RSP, because individuals homozygous at the target locus could be misscored as heterozygotes when an allele from a paralogous locus also amplified.

In the combined Karl and Avise (1992) and current study, 12 individuals (1.9%) assayed with CV-7.0 primers produced putatively heterozygous digestion profiles with additional unexpected fragments, indicating either a size polymorphism in one allele or amplification from more than two loci (fig. 3A). The seven individuals used for cloning CV-7.0/CV-7.4 products did not have aberrant diploid *HinfI* profiles such as in figure 3A. Yet when *HinfI* profiles were examined for a total of 30 clones

from each of four of these individuals, the profiles suggested that multiple unique products had been amplified and cloned (two from specimen SE02, three from JL03, and four each from PO18 and JU15; e.g., fig. 3B). In each of the latter three individuals, only a single representative of the unexpected clones was observed. Sequencing and alignment of the clones with unique restriction profiles from each of two specimens (PO18 and JU15) revealed high sequence similarities ($\geq 92\%$; fig. 2). Two JU15 clones with identical *HinfI* restriction profiles (9 and 14) were also sequenced and were found to differ by three nucleotide substitutions and a single nucleotide indel (99% similarity; fig. 2). These data are consistent with amplification from multiple paralogous loci but cannot exclude the possibility that clones differ due to a high rate of *Taq* polymerase error in the PCR reaction prior to cloning.

However, clone 7 from individual JU15 was larger than expected due to a large insertion flanked on the 5' end by a sequence 92%–99% similar to those of the other clones from JU15 (figs. 2 and 3B). A corresponding large product was not detectable in the JU15 PCR product used for cloning. The high sequence similarity in one region of the JU15–7 clone suggests homology with the two expected alleles. Furthermore, the insertion was not likely an artifact of *Taq* error. Therefore, we provisionally interpret clone JU15–7 as a paralogous product that amplified at a level detectable only by cloning.

The internal CV-7.7 primer pair amplified a 328-bp product in most individuals, under the conditions used for population screening. Additional unexpected PCR products were amplified in only 19 specimens (3.0%), and these were unaffected by more stringent primer annealing conditions of 64°C for 30 s per cycle. In addition to the 328-bp product, one fragment (180 or 250 bp) was amplified from each of 12 individuals, in each case producing diploid *HinfI* profiles consistent with an indel in one of two alleles. When indels such as these made scoring of the target restriction site uncertain or impossible, the individuals were excluded from population analyses. In seven individuals, a faint nontarget product (350 or 950 bp) was co-amplified under all conditions tested. In no case did this secondary product obscure scoring of the target RSP, and all three possible diploid genotypes were observed among these individuals. Individuals that appeared heterozygous at the target RSP served to confirm that the secondary product must have been amplified from another locus of unknown homology, rather than indicating an allele with an indel at the target locus.

Population-level Effects of Primer Redesign

The CV-7.7 primers increased the frequency of individuals scored as heterozygotes such that, unlike re-

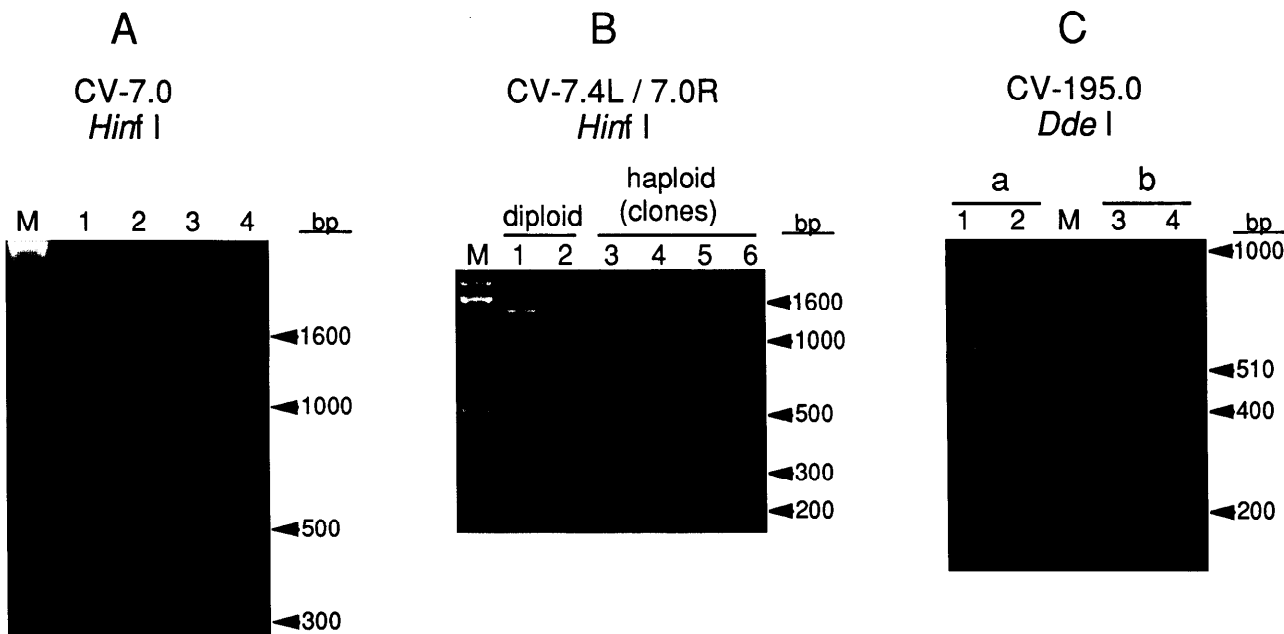


FIG. 3.—Digestion profiles evidencing amplification from multiple paralogous loci. Molecular weight size standards are designated by M. **A**, Amplification from genomic DNA of four apparently heterozygous individuals, one of which (lane 1) shows the digestion profile normally observed, and three of which (lanes 2–4) show anomalous profiles (with extra bands) observed in a few specimens. **B**, Amplifications from individual JU15 using primer pair CV-7.4L/CV7.0R. Lanes 1 and 2, amplification products from diploid genomic DNA, where lane 1 is before and lane 2 is after digestion by *Hinf*I. In lane 2, the top band is monomorphic and the lower two visible bands are defined by the target RSP (see legend to fig. 1). Lanes 3–6, amplification products from haploid clones (JU15-2, -9, -13, -7, respectively), where lanes 3 and 4 are digestion profiles expected based upon the diploid profile and lanes 5 and 6 show different anomalous profiles evidencing probable amplification from paralogous loci. **C**, Amplifications from two individuals (a and b) using primer pair CV-195.0. Lanes 1 and 3 are undigested, and lanes 2 and 4 are PCR products digested in parallel by *Dde*I at high concentration (5 U per 20 μ l reaction) in order to guard against the possibility of incomplete digestion. Lane 2 is the typical heterozygous restriction profile (the target RSP defines the bottom two visible bands) and lane 4 shows a restriction profile suggestive of amplification from multiple loci.

sults from the CV-7.0 primers, genotypic frequencies did not depart significantly from HWE in any population sample (tables 2 and 3). This contrast is seen most dramatically in the Boynton Beach (BB) collection, where a pronounced and statistically significant ($P < 0.01$) heterozygote deficit was displayed using the CV-7.0 primers, whereas a small, nonsignificant ($P = 0.33$) heterozygote excess was scored with CV-7.7. Thus, use of refined CV-7.7 primers, that appear to have ameliorated most symptoms of PCR “artifacts,” also generated RSP genotypic scorings in agreement with HWE.

Notwithstanding such differences in genotypic scoring, it should be also noted that the CV-7.7 primers generated an allelic frequency distribution across populations that remained nearly identical to that published for CV-7.0 (Karl and Avise 1992; Hare and Avise, unpublished data). Thus, a dramatic allele frequency shift (from approximately 0.2 in the Gulf of Mexico to nearly 1.0 in the Atlantic) continues to characterize the *Hinf*I polymorphism at CV-7. Furthermore, it is also important to note that statistically significant deviations from HWE were present in only 5 of the 31 allele frequency estimates of Karl and Avise (1992). Although some ar-

tifactual scorings likely occurred in that earlier study, the departures from HWE were not as common as those observed here using the CV-7.0 primers on some of the newly acquired samples (for reasons that remain unknown but presumably relate either to the differences in populations examined or to differences in details of laboratory procedure and scoring).

Characterization of Other nDNA Loci

CV-32. DNA sequence from the original genomic clone was used to design internal primers that amplified a much smaller product (264 versus 1,000 bp) containing the target *Nsi*I RSP. Primers CV-32.4L and CV-32.4R were two- and fourfold degenerate, respectively, at their 5' ends to account for ambiguities in the available sequence information. The CV-32.4 primers provided simple two-allele RSP data for all 637 individuals reassayed, including 50 specimens whose DNA had previously failed to amplify with the CV-32.0 primers. Among the 65 scoring differences involving CV-32.0 versus CV-32.4 primers, 97% resulted from amplification by CV-32.4 of a previously refractory *Nsi*I uncut allele (i.e., aa \rightarrow Aa). Furthermore, no individual scored

as heterozygous with CV-32.0 appeared homozygous using the CV-32.4 primer pair. Accordingly, with the redesigned primers, the single population that deviated from HWE with the original primers produced allele frequencies conforming to HWE, and heterozygote frequencies increased in most collections (tables 2 and 3). In agreement with results reported by Karl and Avise (1992), assays with CV-32.4 primers again indicated a dramatic change in allele frequency along the east Florida coast (Hare and Avise, unpublished data).

CV-195. The CV-195.0 primers amplified a 630-bp fragment from most individuals. However, in 21 specimens (3.3%), *DdeI* restriction profiles suggested the presence of size polymorphisms and/or amplification from more than one locus. In eight of these individuals, the *DdeI* fragments summed to more than twice the size of the single uncut product, suggesting multilocus amplification (fig. 3C).

CV-19. The CV-19.0 primers amplified a 1,440-bp fragment polymorphic for a 175-bp indel and three *NsiI* sites (one of which is the target RSP in Karl and Avise [1992]). The possibility of PCR artifacts affecting genotype scoring at this locus has not been investigated.

Discussion

The original intent of this study was to reexamine, from the perspective of DNA markers, a well-known conundrum in marine bivalves involving the enigmatic phenomenon of heterozygote deficiencies as previously reported in allozyme assays. However, during this investigation, it became apparent that locus-specific "artifacts" on genotypic scoring can accompany PCR-based DNA-level assays. Thus, a revised interim goal (before the issue of heterozygote deficiency per se could be addressed) became to document these complications and determine how they might be circumvented in particular instances.

Artifactual genotypic scoring due to polymorphism at PCR priming sites in the American oyster was suggested initially by changes in apparent genotype as a function of varying PCR conditions. Construction and use of refined internal primers to assay the CV-7 and CV-32 RSPs eliminated most such PCR condition-dependent behavior and also increased the number of individuals scored as heterozygotes. Both results are consistent with an amelioration of artifacts due to priming site polymorphism. Amplification from multiple paralogous loci was suggested by diploid digestion profiles that displayed too many bands (relative to the known size of the uncut fragment). This interpretation was supported in one case by the sequences of cloned amplified DNAs from a single individual, which documented the presence of two expected target alleles plus a third sim-

ilar sequence with a large indel. Based on these findings, the following recommendations can be made for detecting, correcting, and avoiding PCR artifacts in the assay of RSPs at anonymous nDNA loci.

Genotypic Artifacts

Differential amplification of alleles that might be causing genotypic artifacts can be recognized during population screening most clearly when the intensity of a "cut" allele is below that expected given the intensities of other bands in a restriction profile (if the uncut allele is faint, the possibility of incomplete digestion must also be considered). Affected individuals may also show PCR condition-dependent genotypes in further experiments. However, not all kinds of differential amplification due to priming site polymorphism can be detected from these symptoms (e.g., Callen et al. 1993).

One obvious strategy to correct such artifacts is to design primers from sequences that display low polymorphism. A second strategy is to amplify smaller fragments, because there is some evidence that longer PCR products tend to amplify with reduced efficiency and yield (see Saiki et al. 1988). Conceivably, a primer mismatch that noticeably biases amplification toward one allele over another in a long product might have lower bias when the product is short (although we know of no empirical evidence to support this possibility, and our data do not directly test it). In any event, both strategies were used in our design of the CV-7.7 primer pair, and indeed they appear to have ameliorated the effects of priming site polymorphism. At the CV-32 locus, no information on polymorphism across alleles was used to design the internal CV-32.4 primers, yet they too increased the number of alleles amplified. The large reduction in PCR product size (from 1,350 to 328 bp for CV-7, and from 1,000 to 264 bp for CV-32) may, thus, have been a significant factor in the improved behavior of redesigned primers at these loci. However, because we cannot independently test with current data the effects of product length per se versus effects of the new priming site or primer degeneracy (all three of which changed with primer redesign), this conclusion must remain tentative.

Primers to anonymous loci are usually designed from genomic clones before the extent and nature of polymorphism are known, so screening of a few distantly related individuals (e.g., from geographically distant populations) is desirable to provide preliminary information on the presence of useful variation (Karl and Avise 1993). To increase the initial prospects of finding useful RSPs, primer design preferably maximizes PCR product size (within reasonable limits). However, after discovery of an RSP, results of this study suggest that

population screening might profit from the subsequent design of internal primers closer to the RSP.

Unfortunately, strategies to prevent artifacts due to priming site polymorphism may simultaneously exacerbate another source of PCR “artifact”—amplification from multiple paralogous loci. In other words, means to enhance primer amplification across multiple alleles at one locus may also increase the likelihood of cross-locus amplification when similar priming sequences are represented elsewhere in the genome. This complication of amplification from multiple paralogous loci is potentially more widespread than that of allele-specific amplification (which should be a difficulty only at highly polymorphic loci), although its extent will no doubt be influenced by the nature and abundance of duplicated loci in genomes, features that vary across taxa. Sporadic amplification from multiple paralogous loci is also an insidious complication of PCR-based assays because of the difficulty in detecting and differentiating paralogous products from those representing size-variant alleles at a single locus. Conditions favorable to the detection of paralogous amplification involve situations where the restriction profiles have an unexpected appearance based on preliminary population screening (e.g., a paralogous product may co-amplify with two different alleles in individuals heterozygous at the target locus, so that three or more “alleles” are observed in the restriction digest). Because of the impediments to detection, even an occasional occurrence of nontarget amplification may indicate more pervasive difficulties.

Using the CV-7.0 primers, the prevalence of paralogous amplification was unfortunately difficult to estimate even from clones, given the possible role of *Taq* errors in generating novel sequences. Anomalous diploid restriction profiles observed in 1.9% of the oysters assayed were suggestive of amplification from paralogous loci, although the possibility remains that these were attributable to size polymorphisms at the target locus (fig. 3A). It is noteworthy that none of the novel restriction patterns found among clones (putative paralogous amplification products) was observed in the diploid restriction profiles of individuals used for cloning. Thus, it appears that amplification products from paralogous loci may generally have been amplified at low efficiency such that they were irrelevant to the diploid genotypes as scored in most specimens.

In contrast, some individuals showed relatively unambiguous evidence of amplification from paralogous loci when assayed with the CV-195.0 primers (fig. 3C; note, however, that despite careful attempts at experimental controls, the possibility of incomplete digestion cannot altogether be eliminated as an explanation for aberrant digestion profiles). Apparently, variation exists among individuals in the proclivity to amplify across

paralogous loci and/or in the genomic representations of those loci. The former appears more likely a priori and might be expected when variation among individuals exists in the level of primer-site sequence heterogeneity across paralogous loci (theoretically giving each individual a unique distribution of probabilities for amplification from target and nontarget loci; Wagner et al. 1994). The internal CV-7.7 primers (even though one was degenerate) presumably maintained the competitive advantage that the target locus had over other paralogous loci in the PCR reactions with other primer pairs, as judged by the fact that only a small proportion (3.0%) of individuals had unexpected products amplified to detectable levels with CV-7.7 primers (similar to the 1.9% of individuals using CV-7.0). These unexpected results suggest that most of the overall improvement in CV-7.7 primers over CV-7.0 resulted from a correction of differential amplification due to priming site polymorphism and that this was accomplished without a radical alteration of the propensity to amplify products from paralogous loci. Our experience provides no obvious, practical strategy for primer design that is likely to narrow PCR amplification to a single locus.

The fact that the CV-7.7 primers produced products consistent with amplification from a single locus in most individuals underscores an important distinction between genomic copy number and the Mendelian “behavior” of a PCR marker. To function as a Mendelian marker in any PCR-based assay, primers must recognize and specifically prime unique sequences in the genome. The genomic copy number of the surrounding and intervening sequence is not at issue. Indeed, it is this principle that permits PCR-based assay of highly repetitive DNA sequences (such as mini- and microsatellites) in a single-locus fashion. Our data suggest that the CV-7 locus is physically duplicated, but sequence differences among the paralogous loci, and differential priming by CV-7.7 primers across those loci, seem to allow for Mendelian assays of a single target locus. This lack of correspondence between physical and assayed copy number means that techniques such as dot blotting and Southern blotting (Southern 1975), which are aimed at establishing genomic copy number, will not necessarily provide definitive information on the number of binding sites that the primers will recognize. Given this, it is difficult to imagine how, when dealing with PCR assays of natural populations, a researcher could be totally certain that a locus under consideration is strictly single copy. It should be further emphasized that this attribute is not limited to anonymous nuclear loci but can occur whenever PCR is employed.

To avoid the inherent circularity of confirming the Mendelian behavior of molecular markers solely via agreement of population allele frequencies with HWE

(i.e., in the absence of direct verification of genotypic scoring by pedigree analysis), an alternative line of support can derive from consideration of restriction profiles themselves. Clean diploid genotypes in which the restriction fragments sum to twice the size of the uncut PCR product are the best assurance of amplification from a single locus. Exceptions should be tested for PCR condition dependency, which is expected with spurious primer annealing and with competition for annealing between priming sites within or between loci.

Although this report has attempted to take a critical view of the application of anonymous nuclear DNA assays in natural populations, we should also emphasize that highly useful information to population genetic studies can nonetheless be obtained with this approach. The complications experienced with certain of the oyster *scnDNA* primers have not generally been detected in other *scnDNA* studies, including marine turtles (Karl, Bowen, and Avise 1992), deep-sea hydrothermal-vent tube worms, or hydrothermal-vent clams (Karl, unpublished data). Nor do the occasional amplification difficulties reported here significantly affect any of the broader conclusions about phylogeographic population genetic structure in oysters themselves (Hare and Avise, unpublished data). Rather, the complexities reported herein should sound a general cautionary note for contemplated applications of PCR-based assays to the genetics of natural populations.

Implications to Heterozygosity Studies

A precedent exists for reexamining, from a DNA-level perspective, enigmatic phenomena in marine bivalves previously noticed in allozyme surveys. Pogson and Zouros (1994) used markers from eight anonymous *scnDNA* loci (obtained from cDNA probes in Southern blots) to reassess a previously observed correlation between allozyme heterozygosity and growth rate in a cohort of scallop *Placopecten magellanicus*. They found no correlation between growth rate and the degree of heterozygosity at these anonymous DNA markers, a result interpreted as provisional evidence against the associative overdominance hypothesis for the allozyme patterns.

In our case, the initial rationale for the present study was that if heterozygote shortages relative to HWE were confined to protein-level assays, they might reflect differential allelic expression or post-transcriptional effects resulting in the idiosyncratic appearance of "null" alleles at some allozyme loci, whereas if they consistently appeared in DNA-level assays also, more general population processes (such as inbreeding and/or the Wahlund effect) might be implicated.

One important finding from the current study is that sources of "artifactual" genotypic scoring can apply to

DNA-level as well as to protein-level assays. Thus, to the list of potential factors contributing to HWE departures in table 1 must be added a third category—effects confined to DNA-level assays—which includes differential PCR priming across alleles and amplification from multiple paralogous loci. These factors will have opposite influences on RSP departures from HWE, with the former tending to produce the appearance of heterozygote deficit and the latter the appearance of heterozygote excess. Technical artifacts that can obscure the assay of heterozygotes have also been reported for microsatellite markers (Pemberton et al. 1995) and VNTRs assayed on Southern blots (Chakraborty and Jin 1992).

There is no biological reason to expect that these opposing biases would necessarily cancel out such as to lead to an agreement of genotypic proportions with HWE in large population samples. Thus, agreement with HWE by even a moderate fraction of nDNA loci in populations of oysters (for example) would argue against the proposition that the heterozygote deficit phenomenon results from genomically pervasive factors such as the Wahlund effect. Rather, factors idiosyncratic to particular loci would be implicated (similar conclusions have been reached in earlier allozyme studies [Zouros and Foltz 1984; Gaffney 1990]). It must also be emphasized, however, that the statistical power to detect departures from HWE is notoriously low for the small or moderate sample sizes commonly used (such as those analyzed in this study; Ward and Sing 1970). Therefore, agreement with HWE in such tests cannot be taken as definitive evidence against mild but genomically pervasive heterozygote-reducing influences.

In the current study, the redesign and use of internal primers at the *CV-7* and *CV-32* loci produced restriction profiles with the appearance of Mendelian markers. Furthermore, using the internal primers, the genotype frequencies in local population samples of oysters proved invariably to be in accord with HWE. Nonetheless, special caution is needed to avoid the circular reasoning of using agreement with HWE as a criterion for assessing the validity of a genotypic assay, when HWE itself underlies the biological phenomenon under investigation.

On the one hand, the current agreement of DNA-level genotypic proportions with HWE suggests that the heterozygote-deficit phenomenon previously reported in allozyme surveys of marine bivalves is not genomically pervasive (at least in the American oyster) and, hence, that population-level considerations such as the Wahlund effect are less likely to be responsible. On the other hand, ironically, the pronounced meso-spatial heterogeneity in allele frequencies revealed in these same DNA-level assays (along the east Florida coastline) implies a much wider window of opportunity for Wahlund effects

than had been evidenced in earlier allozyme surveys, where allelic frequencies have shown remarkably little variation geographically. Whether such opportunity for Wahlund influence has been commonly realized in marine bivalves remains to be determined. The pronounced spatial heterogeneity in PCR-revealed allele frequencies in east Florida oysters will be the subject of a sequel presentation.

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