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14. The cDNA inserts from p48 and p1bp were ligated into the mammalian expression vector pDC303 [B. Mosley *et al.*, *Cell* **59**, 335 (1989)], which resulted in plasmids FL1bp/CAV and 1bp/CAV, respectively. Plasmids (10 μg) were transfected into subconfluent monolayers of COS-7 cells and metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine as described [A. E. Namen *et al.*, *Nature* **333**, 571 (1988)]. Cells were lysed and IL-1β was immunoprecipitated with a rabbit antiserum. Proteins were analyzed by SDS-PAGE [U. K. Laemmli, *Nature* **227**, 680 (1970)]. The gels were treated with Enhance (DuPont Biotechnology Systems) and dried before autoradiography.
15. Radiolabeling and immunoprecipitation were carried out as above, except that [^{3}H]leucine (Amersham, 142 Ci/mmol, 0.1 Ci/ml) was used, and unlabeled human IL-1β (5 μg) was added before electrophoresis. After electrophoresis, the proteins were transferred to a polyvinyl difluoride membrane (ProBlott, Applied Biosystems) [P. Matsudaira, *J. Biol. Chem.* **262**, 10035 (1987)] and stained with Coomassie blue. The IL-1β band was excised from the membrane and loaded on an Applied Biosystems model 477A protein sequencer. The radioactivity released during each cycle of the sequencer was determined by scintillation spectroscopy.
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20. Chromosomal in situ hybridization was performed as described [L. A. Cannizzaro *et al.*, *Genomics* **6**, 197 (1990)]. Hybridizations were carried out at 37°C for 24 hours with an IL-1β convertase probe prepared from p1bp by nick-translation with [^{3}H]dNTP to a specific activity of 2 × 10^{7} to 4 × 10^{7} cpm/μg.
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23. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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26. We thank S. Dower and D. Cosman for critical review of the manuscript; T. Hollingsworth for DNA sequencing; D. Gearing for the cDNA library; and D. Chaplin and D. Pickup for communicating results prior to publication.

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Balancing Selection at Allozyme Loci in Oysters: Implications from Nuclear RFLPs

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Population genetic analyses that depend on the assumption of neutrality for allozyme markers are used widely. Restriction fragment length polymorphisms in nuclear DNA of the American oyster evidence a pronounced population subdivision concordant with mitochondrial DNA. This finding contrasts with a geographic uniformity in allozyme frequencies previously thought to reflect high gene flow mediated by the pelagic gametes and larvae. The discordance likely is due to selection on protein electrophoretic characters that balances allozyme frequencies in the face of severe constraints to gene flow. These results raise a cautionary note for studies that rely on assumptions of neutrality for allozyme markers.

Starch gel electrophoresis of soluble proteins has been the workhorse technique of population genetics for nearly 30 years. Although several studies have indicated that natural selection acts on particular allozyme loci (*1*–*6*), the working hypothesis of most population genetic applications has been that the majority of enzyme (as well as DNA) polymorphisms evolve as predicted by neutrality theory and can be interpreted accordingly for purposes of estimating population structure, gene flow, and genetic relatedness. Previous studies of the American oyster (*Crassostrea virginica*) in the southeastern United States revealed a remarkable contradiction between data from biparentally inherited allozyme loci, indicating little or no population subdivision (*7*), and maternally transmitted mitochondrial DNA (mtDNA), demonstrating a sharp genetic discontinuity between oyster populations from Atlantic Coast in contrast to Gulf of Mexico locales (*8*). Similar mtDNA surveys of a variety of coastal-restricted taxa, including horseshoe crabs, toadfish, black sea bass, diamondback terrapins, and seaside sparrows (*9*), demonstrate phylogenetic discontinuities between Atlantic and Gulf populations of these species, suggesting that similar historical processes are involved and that these biogeographic factors probably operated on American oysters as well.

There are several possible explanations for the apparent inconsistency between nuclear and cytoplasmic genetic structures in oysters. The discrepancy could be due to biological or demographic factors: (i) a higher rate of interpopulation gene flow mediated by sperm rather than by eggs; (ii) directional selection favoring different mtDNA haplotypes in the two regions; or (iii) a smaller effective population size for mtDNA that resulted in a faster rate of lineage sorting from the ancestral gene pool than was the case for most nuclear alleles (*10*). Alternatively, the apparent contradiction between the mitochondrial and nuclear genomes could be due to the following factors: (i) hidden variation within electromorph classes (cryptic allozymes), such that undetected allozyme differences truly distinguish Atlantic and Gulf populations; (ii) a slower rate of evolutionary change in allozyme frequencies; or (iii) balancing selection at multiple allozyme loci.

To distinguish between these two classes of competing hypotheses, we report here an analysis of restriction fragment length polymorphism (RFLP) in single-copy nuclear (scn) DNA. We constructed primers suitable for amplification of DNA by the polymerase chain reaction (PCR) for each of four anonymous nuclear loci (*11*), following a procedure described elsewhere (*12*). Nuclear DNA, isolated from each of 277 oysters collected at nine locations between Massachusetts and Louisiana (*13*), was amplified with the use of these primers (*14*). The amplified products were digested with restriction enzymes (*15*), and four restriction site polymorphisms interpretable as unlinked Mendelian variants at single genes were identified (*16*).

Restriction site polymorphisms from all four scn loci reveal significant shifts in allele frequency between the Gulf of Mexico and Atlantic collections of American oysters, with the differences between these two geographic regions generally much greater than those within either area (Table 1). The pattern contrasts strikingly with the geographic uniformity evidenced by the allozyme polymorphisms (Fig. 1). The pronounced genetic break in scnDNA appears along the eastern coast of Florida, as was true for the mtDNA phylogeographic break in oysters and other maritime species (*8*, *9*). The sample from Stuart, Florida, a geographically intermediate locale, generally exhibited transitional allele frequencies. A clustering of genetic distances based on the scnDNA allele frequencies further documents the dramatic population genetic separation between the Atlantic and the Gulf of Mexico oysters, the agreement with the mtDNA break, and the apparent contrast with the allozyme information (Fig. 2).

The similar pattern of geographic population subdivision registered in mtDNA and

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scnDNA (Figs. 1 and 2) indicates that biological factors differentially affecting mitochondrial and nuclear genomes in oysters probably cannot account for the original discrepancy between the mtDNA and allozyme data sets. Thus, the geographic uniformity in allozymes is not likely due to greater dispersal of male gametes or to the slow rate of evolutionary sorting of nuclear lineages from an ancestral gene pool, because effects of such demographic processes should also be reflected in the distributions of scnDNA alleles. Neither is the population genetic break in mtDNA attributable solely to directional selection on mtDNA, unless the kinds of selection pressures favoring different mtDNA haplotypes in the Atlantic versus the Gulf extend to a significant fraction of scnDNA genes as well. The case against contemporary directional selection itself being the sole force governing DNA genotypic distributions is strengthened further by the observation that the large number of mtDNA mutational differences accumulated between the Atlantic and the Gulf clades must have required a considerable period of historical population separation (*8*). Finally, it is difficult to imagine a selective pressure that could have molded in such consistent fashion the mtDNA phylogeographic partitions across species as different as the fishes, reptile, bird, arthropod, and bivalve mollusk that were assayed (*9*).

We are left with the conclusion that the original discrepancy in population genetic structure between the nuclear and cytoplasmic genomes of oysters most likely results from some nonrepresentative evolutionary genetic properties of the allozyme systems. Balancing selection on protein coding loci appears the most likely explanation. Geneticists working with mollusks have long been intrigued by strong associations observed between higher multilocus heterozygosity at allozyme loci and presumed components of

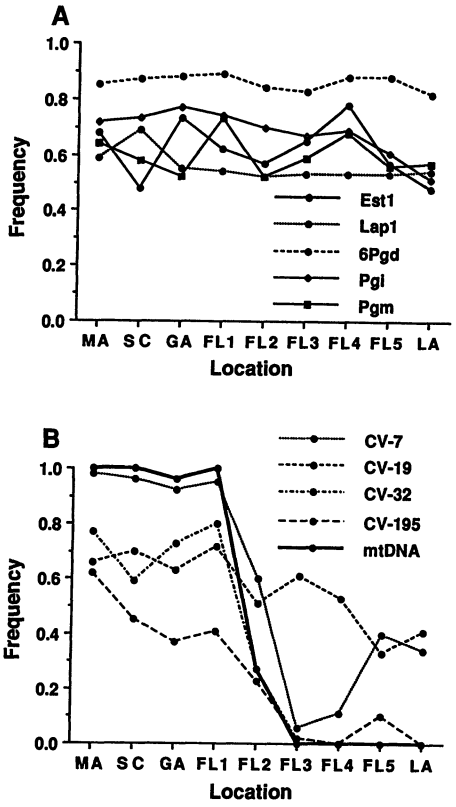

Fig. 1. Frequencies of the most common alleles at polymorphic allozyme loci (**A**) and DNA loci (**B**) along the coastline transect running from Massachusetts to Louisiana. Locations are abbreviated as in (*13*). Allozyme data from five loci were taken from (*7*) for collection sites near those used in the current study and are representative of the geographic uniformity also exhibited by six other highly polymorphic allozyme systems (*7*). Allozyme loci are as follows: Est1, esterase; Lap1, leucine aminopeptidase; 6Pgd, 6-phosphogluconate dehydrogenase; Pgi, phosphoglucose isomerase; and Pgm, phosphoglucomutase. DNA data are for frequencies of the two major mtDNA clades (heavy line) as reported in (*8*) and of the most common alleles at each of four scnDNA loci (CV-7, CV-19, CV-32, and CV-195) (present study).

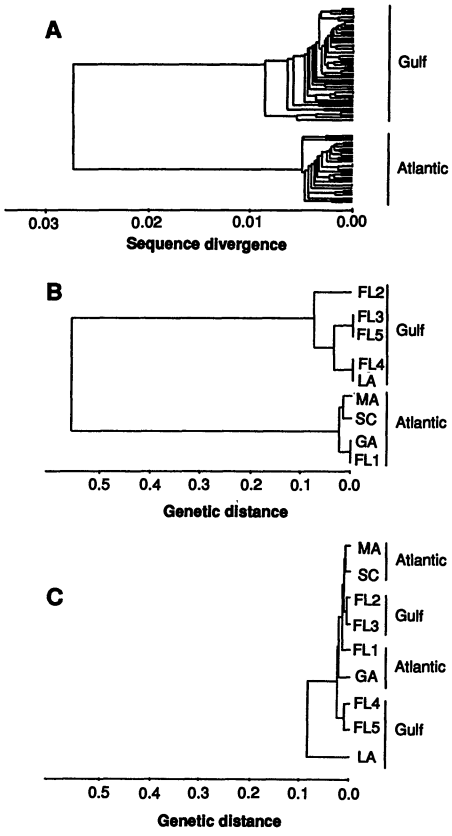

Fig. 2. Dendrograms based on mtDNA (*8*) (**A**), scnDNA (4 polymorphic loci, present study) (**B**), and allozymes (18 mostly polymorphic loci) (*7*) (**C**) in American oysters from the southeastern United States. Genetic distances were calculated as Nei's (*24*) unbiased estimator (for scnDNA and allozyme sample allele frequencies) or were taken from (*8*) for mtDNA haplotypes. Clustering of distance matrices was performed by the unweighted pair group method with arithmetic means. Location abbreviations are as in (*13*).

Table 1. Frequencies (*f*), sample sizes (*n*) (differences in sample sizes were due to the inability to amplify DNA from some of the individuals with all of the primer pairs), and significance levels for heterogeneity χ^2 [$P(\chi^2)$] for the most common Atlantic allele in each population (*13*). A two-tailed Student's *t* test on the arcsine transformed frequencies was used to compute significance values (*P*) for the difference in mean allele frequency between Gulf and Atlantic samples. Significant differences also were found with a nonparametric Mann-Whitney test. The FL2 population was excluded from the analysis because it represents a genetically mixed mtDNA group and may be of hybrid origin.

Location	Locus							
	CV-7		CV-19		CV-32		CV-195	
	f	*n*	*f*	*n*	*f*	*n*	*f*	*n*
			Atlantic					
MA	0.98	(26)	0.66	(16)	0.77	(31)	0.62	(33)
SC	0.96	(22)	0.70	(23)	0.59	(22)	0.45	(21)
GA	0.92	(32)	0.63	(30)	0.73	(30)	0.37	(31)
FL1	0.95	(30)	0.72	(29)	0.80	(26)	0.41	(29)
FL2	0.60	(26)	0.51	(27)	0.27	(28)	0.23	(28)
Mean ± SD	0.952 ± 0.025		0.620 ± 0.043		0.723 ± 0.093		0.460 ± 0.112	
$P(\chi^2)$	0.547		0.741		0.087		0.025	
			Gulf					
FL3	0.06	(27)	0.61	(27)	0.02	(27)	0.02	(25)
FL4	0.40	(36)	0.33	(38)	0.00	(36)	0.09	(35)
FL5	0.11	(14)	0.53	(16)	0.00	(17)	0.00	(17)
LA	0.34	(35)	0.41	(26)	0.00	(27)	0.00	(40)
Mean ± SD	0.228 ± 0.168		0.470 ± 0.124		0.005 ± 0.010		0.030 ± 0.048	
$P(\chi^2)$	0.0001		0.006		0.395		0.004	
P (Gulf versus Atlantic)	0.0001		0.0237		0.0001		0.0004	

organismal fitness (for example, metabolic efficiency, growth rate) (*2*, *17–21*) and have concluded that allozyme polymorphisms themselves underlie physiological energetic differences by virtue of their influence on metabolite flux through central biochemical pathways (*5*, *22*). Such balancing selection on allozyme polymorphisms could counter the influence of genetic drift, even in the face of population subdivision due to historical demographic events or contemporary restrictions on gene flow that are reflected in geographically divergent frequencies of neutral genetic markers. Hidden molecular variation that would also distinguish Atlantic and Gulf oysters may exist at the allozyme loci, but this possibility can in a sense be subsumed under the hypothesis of balancing selection, provided that the selection operates with respect to the observed electromorph classes instead of the level of the hidden variation. A slower rate of evolution for allozymes as compared to scnRFLPs can also be eliminated, because both cases in oysters involve the population level sorting of ancestral polymorphisms that affect both equally.

Regardless of the specific underlying causes, the heterogeneity in geographic pattern among allozyme, scnDNA, and mtDNA data sets cannot be accommodated under a single evolutionary model involving either neutrality or balancing selection (*23*). In this example where an allozyme survey had suggested high levels of gene flow, dramatic population genetic separation nonetheless was present in both the nuclear and the cytoplasmic genomes. Results of this study emphasize the need for caution in inferring population genetic structure and gene flow from any single class of genetic markers.

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11. Sequences of the primers 5′ to 3′ are as follows: CV-7: AAGCTTTAGCCTTCAACTCAGACAA, AAGCTTTAAGGTAGAAGCAAATTA; CV-19: TAAGTTGTTTCTGATCCTTTTG, CTTTATTTTTTACCATTGCC; CV-32: GGAAGCTTTATTATCTAACAGTCT, GGAAGCTTACAAAACAAGCTCGGCTA; CV-195: GGATC AGAAGGAAAGCAACAGCAC, AACGTTTGATGGAACAAGGGAAACT.
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13. Locations and sample sizes for American oysters in the present study are as follows: MA, Woods Hole, MA (n = 35); SC, Charleston, SC (23); GA, Cumberland Island, GA (33); FL1, New Smyrna Beach, FL (30); FL2, Stuart, FL (29); FL3, Port Charlotte, FL (29); FL4, Panacea, FL (39); FL5, Carabelle River, FL (18); and LA, Grand Isle, LA (41). Living oysters were collected and placed on wet ice for transportation to the laboratory. Total cell DNA was extracted from mantle-gonad tissue by homogenizing in 50 mM tris-HCl (pH 8.0), 100 mM EDTA, and 100 mM NaCl followed by one cycle of phenol, phenol-chloroform, and chloroform extraction. RNA was removed by ribonuclease A digestion for 3 hours followed by a repeat of the organic extractions described above. We precipitated DNA by adjusting the aqueous fraction to a final concentration of 300 mM sodium acetate and 70% ethanol; it was then vacuum-dried and resuspended in 1 × TE (10 mM tris-HCl, pH 8.0, 1 mM EDTA).
14. In general, 1 μl of genomic DNA was amplified in a 100-μl reaction volume with 25 pmol of each primer and 2.5 units of *Taq* polymerase, according to manufacturer's instructions (Promega), with the addition of $MgCl_2$ and bovine serum albumin to a final concentration of 2.5 mM and 0.1 μg/μl, respectively. PCR cycling parameters consisted of an initial denaturation at 95°C for 2 min, 40 cycles of denaturation at 94°C for 1 min with annealing at 55° to 62°C for 1 min and extension at 72°C for 2 min, and a final extension at 72°C for 7 min.
15. Approximately 10 μl of the amplified DNA was digested directly with 5 units of each restriction enzyme in a 20-μl reaction according to the manufacturer's directions. Electrophoresis was performed in 2.5% agarose gels stained with ethidium bromide (180 ng/ml). Restriction patterns were visualized with shortwave ultraviolet light. Polymorphisms were indicated by the gain or loss of fragments in the restriction profiles.
16. To eliminate the possibility that our nuclear loci mistakenly may have represented mtDNA polymorphisms, we probed a Southern (DNA) blot of the amplified scnDNA products with purified oyster mtDNA. No bands appeared in the autoradiogram except in control lanes. The hypothesis of mtDNA contamination is further discounted by the nature of the scnDNA polymorphisms themselves, which involved diploid genotypes with general conformance to Hardy-Weinberg expected genotypic frequencies. This result further supports the idea that the loci are inherited in a Mendelian fashion. In addition, all pairwise comparisons of the four loci show insignificant deviations from gametic equilibrium [B. S. Weir and C. C. Cockerham, in *Mathematical Evolutionary Theory*, M. E. Feldman, Ed. (Princeton Univ. Press, Princeton, NJ, 1989), pp. 86–110].
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Identification of *ras* Oncogene Mutations in the Stool of Patients with Curable Colorectal Tumors

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Colorectal (CR) tumors are usually curable if detected before metastasis. Because genetic alterations are associated with the development of these tumors, mutant genes may be found in the stool of individuals with CR neoplasms. The stools of nine patients whose tumors contained mutations of K-*ras* were analyzed. In eight of the nine cases, the *ras* mutations were detectable in DNA purified from the stool. These patients included those with benign and malignant neoplasms from proximal and distal colonic epithelium. Thus, colorectal tumors can be detected by a noninvasive method based on the molecular pathogenesis of the disease.

Colorectal cancer is the third most common malignancy in the world, with 570,000 new cases expected in 1992. In the United States alone, over 60,000 people will die from colorectal cancer this year (*1*). Whereas individuals with advanced disease have a poor prognosis, colorectal tumors diagnosed at an earlier stage can usually be cured by surgical or colonoscopic excision (*2*). Methods to detect surgically resectable tumors could therefore reduce deaths from this disease (*3*). The only noninvasive test for such a purpose involves testing stool for blood, but the appearance of hemoglobin in stool is not specific for neoplasia (*4*, *5*).

Tumor-derived mutations in oncogenes and suppressor genes potentially provide more specific markers (*6*). Mutations in

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