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Biochemical Genetics of *Fundulus heteroclitus* (L.). I. Temporal and Spatial Variation in Gene Frequencies of *Ldh-B, Mdh-A, Gpi-B,* and *Pgm-A*[1]

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Natural populations of Fundulus heteroclitus *show extensive spatial variation in gene frequencies at four unlinked polymorphic loci. Large clinal changes in gene frequencies were found for* Ldh-B, Mdh-A, *and* Gpi-B, *whereas the spatial variation for the* Pgm-B *locus was small. Since the geographical area over which these clines are found is characterized by a steep thermal gradient, the clines in gene frequency are correlated with a directional change in mean water temperature. Maximum gene diversity of these four loci was correlated with annual fluctuations in water temperature. Temporal stability of the allelic frequencies was established for a 2–4 year period.*

KEY WORDS: *F. heteroclitus*; allozymes; cline; gene frequency; environmental temperature.

INTRODUCTION

The term "cline," originally described by Huxley (1939), refers to the directional change of a character of gene frequency within a species over geographical distance. Since the time of Fisher (1937) and Haldane (1948), population geneticists have attempted to explain the existence of these character gradients through various models. The sophistication of the models increased as each

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new generation of theorists expanded the range of biological phenomena considered (for review, see Felsenstein, 1976). With the advent of electrophoretic analysis, the demonstration of a clinal change in gene frequency for a single locus has appeared more frequently in the literature. Such clines are well documented in teleost populations, especially for the four enzyme systems we have investigated: lactate dehydrogenase (LDH), malate dehydrogenase (MDH), glucosephosphate isomerase (GPI), and phosphoglucomutase (PGM). In 1971, Johnson described a change in LDH allelic frequency along Puget Sound for the crested blenny, *Anoplarchus purpurescens*. Merritt (1972) reported a latitudinal cline for a LDH polymorphism in the fathead minnow, *Pimephales promelas*. During the same year, Powers (1972) described a cline in LDH for *Fundulus heteroclitus*. Clines in both LDH and PGM have been described for West Coast populations of the sockeye salmon, *Oncorhynchus nerka* (Utter *et al.*, 1974). A comparison between upstream and downstream trout populations showed changes in gene frequencies at the *Ldh* and *Mdh* loci (Utter *et al.*, 1974). Clinal and nonclinal variations in PGM and GPI were found in *Menida* species by Johnson (1974). Reports of allelic clines for other proteins in fish have also appeared, such as the esterase (*EstIII*) and hemoglobin (*HbI*) polymorphism in the eel pout, *Zoarces viviparus* (Frydenberg *et al.*, 1973; Christiansen and Frydenberg, 1974; Hjorth and Simonsen, 1975). Thus the occurrence of clinal variation in protein polymorphisms does not appear to be a rare phenomenon in fish populations.

In this article we present the spatial and short-term temporal variation in allelic frequencies for the four unlinked genetic loci of *Fundulus heteroclitus*: *Ldh-B*, *Mdh-A*, *Gpi-B*, and *Pgm-B*.[4]

MATERIALS AND METHODS

The range of the common killifish, *Fundulus heteroclitus*, extends from the Mantanzas River in Florida to regions as far north as Port au Port Bay in Newfoundland, Canada (Leim and Scott, 1966). Fish were collected at 22 localities along this geographical range, as shown in Fig. 1. Fish were caught in shallow inlets using commercially available minnow traps. The samples were frozen immediately using liquid nitrogen. Liver, eye, and white epaxial muscle were dissected in the laboratory and electrophoresed as previously described (Place and Powers, 1978). Only alleles with frequencies greater than 0.01 were tabulated. Alleles with frequencies of less than 0.01 were pooled with the data of the allozyme most similar in electrophoretic mobility.

The gene frequencies are tabulated from data obtained from pooled samples with no distinction made for age or sex.

[4] Nomenclature is as described in the accompanying article (Place and Powers, 1978).

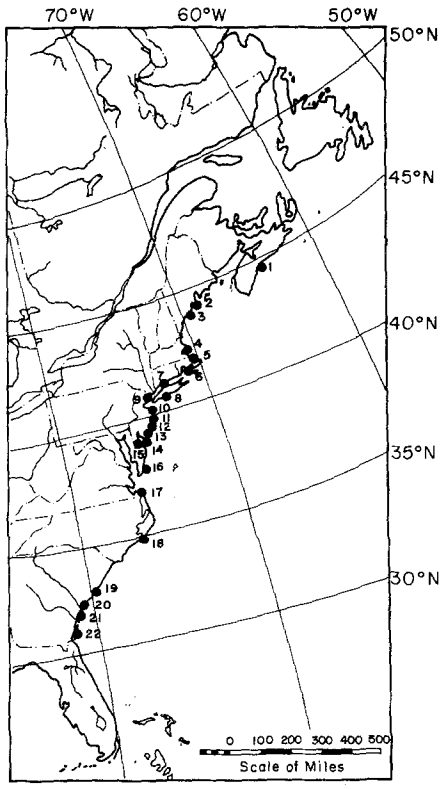

Fig. 1. Location of sampling stations for *Fundulus heteroclitus*.

Gene Diversity Index ($\bar{H}$)

The gene diversity index (expected average heterozygosity, $\bar{H}_{exp}$) was calculated according to Nei (1975). The heterozygosity at a locus is defined as $h = 1 - \sum x_i^2$, where x_i is the frequency of the ith allele. Hence the expected average heterozygosity ($\bar{H}_{exp}$) is defined as $\sum_{l=1}^{r} h_l/r$ where h_l is the heterozygosity of the lth locus and r is the number of loci. The variance of $\bar{H}_{exp}$ is

$$V(\bar{H}_{exp}) = \sum_{l=1}^{r} (h_l - H_{exp})^2$$

For populations that are in Hardy–Weinberg equilibrium this index is a measure of the proportion of heterozygous loci in an individual. The observed average heterozygosity ($\bar{H}_{obs}$) was estimated from the proportion of heterozygotes in the population. The sampling variance of this estimate at a locus is $\hat{h}(1-\hat{h})/N$ (Nei and Roychoudhury, 1974), where $\hat{h}$ is the proportion of heterozygotes in a sample of N individuals. The statistical shortcomings of this estimate are discussed elsewhere (Nei and Roychoudhury, 1974).

Table I. Spatial Pattern in Gene Frequencies

No.	Sample site	Latitude (°N)	*Ldh-B*[a]	*Ldh-B*[b]	*N*[a]	*Mdh-A*[a]	*Mdh-A*[b]	*N*[a]	*Gpi-B*[a]	*Gpi-B*[b]	*Gpi-B*[c]	*Gpi-B*[d]	*N*[a]	*Pgm-A*[a]	*Pgm-A*[b]	*Pgm-A*[c]	*Pgm-A*[d]	*N*[a]
1	Halifax, N.S.	44.63	0.047	0.953	96	1.000		98							0.990	0.010		98
2	Wiscasset, Me.	44.01	0.045	0.955	112	1.000		91		0.045	0.941	0.014	110		0.990	0.010		99
3	Boothbay, Me.	43.86		1.000	58													
4	Gloucester, Mass.	42.62	0.177	0.823	65													
5	Marshfield, Mass.	42.12	0.226	0.774	82	0.988	0.012	82		0.021	0.979		71		1.000			72
6	Woods Hole, Mass.	41.53	0.269	0.731	245[b]	0.978	0.022	245[b]		0.075	0.925		114		0.982	0.018		114
7	Stony Brook, N.Y.	40.93	0.250	0.750	60	0.952	0.048	280		0.105	0.895		280		0.970	0.030		280
8	Jones Beach, N.Y.	40.59	0.438	0.562	48													
9	Vince Lombardi, Rest Stop, N.Y.	40.70	0.472	0.528	36	0.500	0.500	54		0.157	0.843		54		0.947	0.053		38
10	Keansburg, N.J.	40.46	0.402	0.598	46					0.217	0.783		30					
	Sandy Point, N.J.	40.48	0.696	0.304	51	0.020	0.980	50		0.382	0.618		51		0.810	0.020	0.170	50
	Atlantic Highlands, N.J.	40.42	0.539	0.461	76													
11	Point Pleasant, N.J.	40.09	0.627	0.373	75	0.028	0.972	54		0.446	0.554		65		0.839	0.054	0.107	65
12	Leeds Point, N.J.	39.65	0.651	0.349	53	0.019	0.981	53	0.009	0.454	0.537		54	0.010	0.830	0.085	0.075	53
	Atlantic City, N.J.	39.35	0.656	0.344	48													
13	Stone Harbor, N.J.	39.06	0.699	0.301	73	0.007	0.993	74		0.500	0.500		74	0.021	0.750	0.157	0.072	70
14	Cape May, N.J.	38.94	0.670	0.330	47													
15	Woodland Beach, Del.	38.94	0.708	0.292	48		1.000	20		0.348	0.652		46		0.750	0.250		24
16	Wachapreague, Va.	37.61	0.782	0.218	174	0.020	0.980	174		0.573	0.427		48	0.033	0.717	0.250		60
17	Cape Henry, Va.	36.92	0.750	0.250	20	0.050	0.950	40		0.654	0.346		26		0.788	0.212		40
18	Beaufort, N.C.	34.73	0.893	0.107	131	0.058	0.942	52		0.722	0.278		72		0.833	0.167		63
19	Charleston, S.C.	32.77	0.908	0.092	87	0.100	0.900	80		0.727	0.273		66	0.007	0.879	0.106	0.007	66
20	Ladies Island, S.C.	32.30	0.984	0.016	31		1.000	31										
21	Savannah, Ga.	32.08	1.000		48													
22	Sapelo Island, Ga.	31.39	0.968	0.032	110	0.033	0.967	107	0.017	0.749	0.234		175	0.004	0.907	0.085	0.004	135

[a] *N*, Sample size.
[b] Whitt (1970).

Surface Water Temperatures

Surface water temperatures were obtained from the Coast and Geodetic Survey Publication 31-1 (U.S. Department of Commerce, 1960), which summarized seawater temperatures and salinities based on observations made in Atlantic harbor and coastal waters. The ΔT parameter (see Fig. 8) represents the mean maximum water temperature minus the mean minimum water temperature.

RESULTS

Spatial Pattern of Variation

The allelic frequencies of the four polymorphic loci examined electrophoretically are given in Table I. Their spatial patterns of variation are graphically shown in Figs. 2, 3, 4, and 5. The *Ldh-B*, *Mdh-A*, and *Gpi-B* loci all demonstrate large clinal variation in allelic frequencies. The *Pgm-A* locus, however, shows a relatively small change in gene frequencies for its three alleles. Tests of goodness of fit (χ^2) of observations to Hardy–Weinberg expectations show good agreement for all comparisons. Seventy out of 71 samples did not deviate significantly from expected ($\chi^2_{[1,\,0.05]}=3.84$).

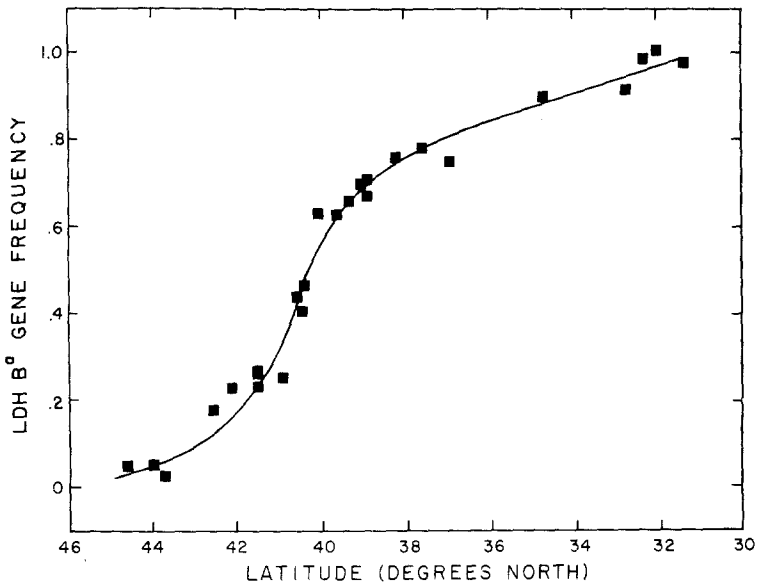

Fig. 2. Geographical variation in the *Ldh-B*^{a} gene frequency of *F. heteroclitus*.

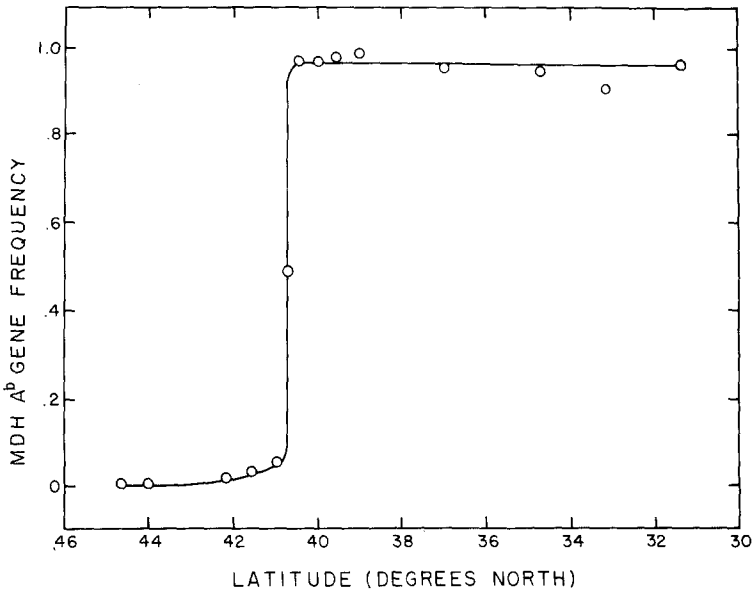

Fig. 3. Geographical variation in the *Mdh-A*^{b} gene frequency of *F. heteroclitus*.

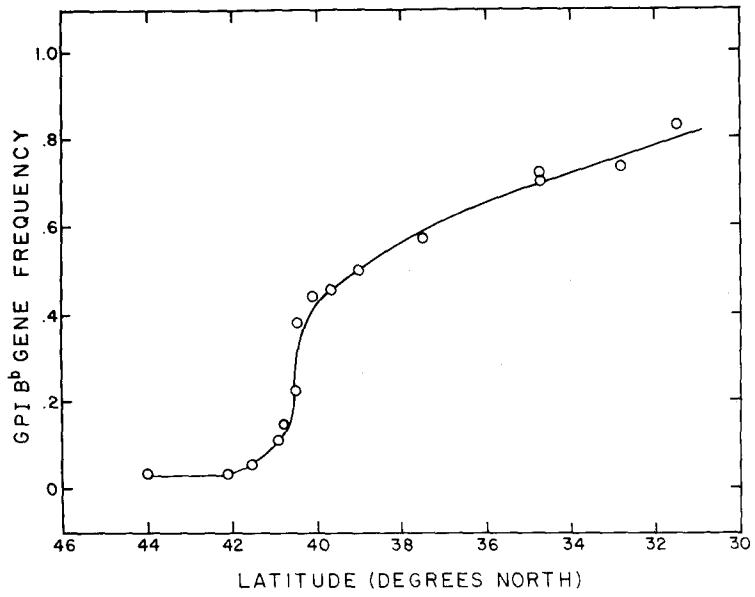

Fig. 4. Geographical variation in the *Gpi-B*^{b} gene frequency of *F. heteroclitus*.

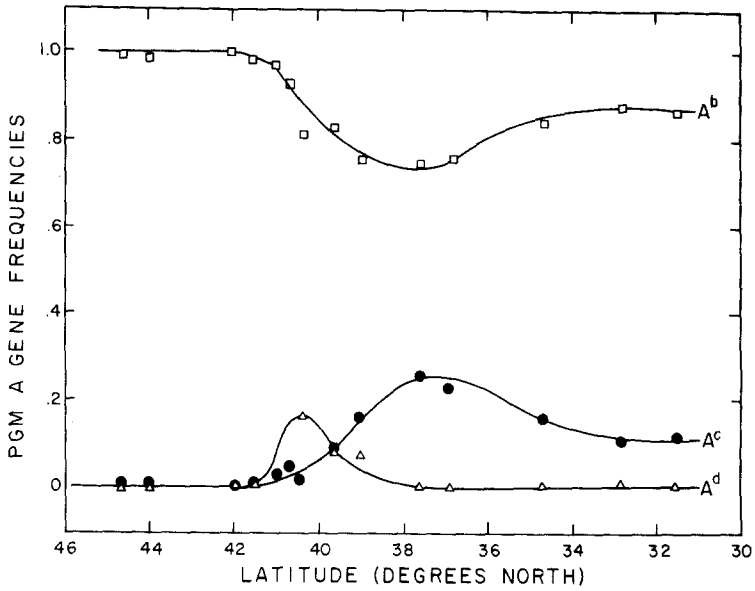

Fig. 5. Geographical variation in the *Pgm-A* frequencies of *F. heteroclitus*.

Table II. Temporal Pattern in Gene Frequencies

Locality	Year	*Ldh-B*[a]	N^a	*Mdh-A*[a]	N^a	*Gpi B*[b]	N^a	*Pgm A*[b]	N^a
Woods Hole,									
Mass.	1969[b]	0.269	245	0.978	245	—	—	—	—
	1972	0.231	78	0.981	78	0.075	114	0.982	114
	1973	0.261	161	0.979	96	0.108	65	0.977	65
Stony Brook,									
N.Y.	1971[c]	0.212	308	0.972	308	0.113	309	0.969	308
	1972[c]	0.242	306	0.951	286	0.101	306	0.979	306
	1972	0.250	60	0.952	280	0.105	280	0.970	280
Wachapreague,									
Va.	1972	0.782	174	0.020	174	0.573	48	0.717	60
	1973	0.773	99	0.071	99	—	—	—	—
	1975	0.780	100	0.035	100	0.589	90	0.800	30
Beaufort, N.C.									
	1972	0.883	98	0.020	98	0.692	52	0.833	63
	1973	0.893	131	0.058	52	0.722	72	0.868	38
	1975	0.879	99	0.050	80	0.723	99	0.830	106

[a] *N*, Sample size.
[b] Whitt (1970).
[c] Mitton and Koehn (1975).

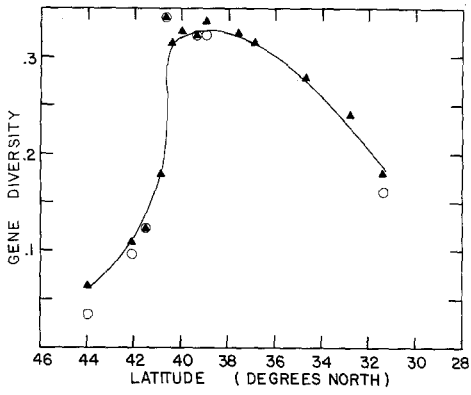

Fig. 6. Relationship between gene diversity (average heterozygosity) for the four polymorphic loci and latitude (degrees north). Gene diversity is defined as according to Nei (1975): $H=\sum_{l=1} h_l/r$, where h_l is the heterozygosity for the lth locus and r is the number of loci. Calculations of these estimates are based only on the four polymorphic loci described. A more accurate measure of genic variation for these populations would include the five monomorphic loci found for these enzyme systems; thus $\bar{H}_{exp}$ (▲) and $\bar{H}_{obs}$ (○) would be lower by a factor of 5.

Linkage disequilibria as measured by the maximum likelihood estimation of Hill (1974) showed that the Stone Harbor, New Jersey, population (sample site 13) did not deviate significantly ($\chi^2_{[1.\ 0.01]}=6.64$) from the null hypothesis of equilibrium for the four polymorphisms. This agrees with the findings of Mitton and Koehn (1975) for a Long Island, New York, population. This lack of linkage disequilibria may be the result of pooling age and sex classes.

Temporal Pattern of Variation

Table II summarizes the data on the short-term temporal stabilities of the allelic frequencies at these four polymorphic loci. No significant variation was found.

Since the age of sexual maturity is 1 year and the life expectancy 4 years for *F. heteroclitus* (Valiela *et al.*, 1977), the temporal study should reflect the complete turnover of a population.

Gene Diversity

The gene diversity parameter ($\bar{H}_{exp}$) for 14 localities in which gene frequencies for all four loci were available is plotted as a function of latitude (degrees north) in Fig. 6. The general pattern is a concave downward curve with maximum gene diversity at the mid latitudes of the species' distribution. Selected values for the observed average heterozygosity ($\bar{H}_{obs}$) are also plotted in Fig. 6. Both $\bar{H}_{exp}$ and $\bar{H}_{obs}$ show similar patterns in relation to latitude.

DISCUSSION

Natural populations of both animals and plants harbor a wealth of genetic

variability (for review, see Powell, 1975). The forces responsible for maintaining this diversity are still largely unresolved (Lewontin, 1974). Are the *majority* of protein polymorphisms adaptive or selectively neutral?

One approach to answering this question has been to demonstrate an association between the genetic attributes of a population and some aspect of the environment (Hedrick *et al.*, 1976). Clinal variations along environmental gradients are often used as evidence for the adaptive significance of protein polymorphisms in aquatic organisms (e.g., Koehn, 1969; Schopf and Gooch, 1971; Johnson, 1971, 1977; Powers and Powers, 1975). However, the force of such arguments is difficult to evaluate because of the possible role of migration (Kimura and Ohta, 1971), linkage (Ohta and Kimura, 1970), and hybridization of populations which have diverged genetically (Clarke, 1973), because of a random drift, selection, or a combination of the two. With these considerations in mind, the spatial patterns in gene frequency described for *F. heteroclitus* will now be analyzed.

Genetic Drift and Selective Neutrality

Random genetic drift of selectively neutral genotypes could have given rise to the genetic diversity observed in this species. For example, if northern and southern populations were previously isolated, it is conceivable that isolates could have become relatively homozygous by the random fixation of selectively neutral alleles. After the establishment of gene flow, geographical patterns of variation (i.e., clines) could have arisen from migration and gene flow between the genetical dissimilar populations. Normally, a requisite for the random fixation of neutral alleles is a small effective breeding population size. Estimates of *F. heteroclitus* populations obtained for three salt marshes are 50,000–70,000 (Bissel Cove, Rhode Island; Nixon and Oviatt, 1973), 18,000–25,000 (Great Sippewissett, West Falmouth, Massachusetts; Valiela *et al.*, 1977), and 19,000–23,000 (Lewes, Delaware; Lotrich, 1975). From the data of Valiela *et al.* (1977), we can estimate the effective breeding population size (N_e, Crow and Kimura, 1970) to be 18,000–25,000. Thus present-day populations appear to be relatively large, which is inconsistent with the random fixation of neutral alleles. Unless the breeding population of an isolate was much smaller, it seems improbable that genetic divergence could occur via the random fixation of neutral alleles.

In order for clines to form by migration and genetic drift of selectively neutral mutations, the criteria described by Kimura and Maruyama (1971) must be met. For a one-dimensional stepping-stone model, these are either

$$\sqrt{m/\mu} < < k$$

or

$$N_e m < < N_t \mu k$$

where m is the migration rate per generation, μ is the neutral mutation rate, N_e is the effective size of each colony, k is the number of colonies, and $N_t = kN_e$. Since the latter criterion can be derived from the former, we need only consider the former.

Although a precise estimate for the migration rate (m) of *F. heteroclitus* does not exist, Lotrich (1975) does provide an approximation. His study showed that individuals exhibit a home range of only 36 m and that a continuum of individuals existed throughout the study area. Since a 1% per day migration rate for the 60-day period of study would have altered his data remarkably, Lotrich (1975) concurred with Bigelow and Schroeder (1953) that *Fundulus* is one of the most stationary of all marine fish.

If we assume that these findings can be extended to the entire year, then an additional calculation shows that a 0.01% per day migration rate throughout the year would not alter his capture–recapture data significantly. This would put an upper limit on m of 500–1000 individuals per generation.

Taking a neutral mutation rate of 10^{-8} (presumably a minimal estimate) and the estimate of m given above, we see that k, the number of colonies, must be of the order of 10^6 or greater. It is currently impossible to adequately evaluate the significance of this estimate for k without censusing the total number of marsh habitats separated by greater than 36 m. Therefore, formation of the clines via genetic drift of selectively neutral alleles and migration is a viable hypothesis.

Selection

In response to selection, the genetic divergence found for *F. heteroclitus* could have originated by two quite different mechanisms. The first would require a historical subdivision of the species, similar to that described for neutral alleles, into selectively different environments (e.g., cold north vs. warm south) with directional selection of the isolates. The clines would then arise via migration and gene flow after the removal of the isolating "barriers." Even though the origin of genetic divergence in this case is different, the clines for this selective mechanism would be established in the same manner as that proposed for selectively neutral alleles described above (i.e., migration and gene flow). The second mechanism would require that clines originated in response to selective pressures imposed by an ecological gradient. In other words, the relative fitness for each genotype changed in concert with the environmental gradient.

Since the possibility of previous isolation is not testable, we have addressed our research efforts to the selective mechanisms by studying the structural and functional characteristics of the allozymes in relation to environmental variables (Powers, 1972; Powers and Powers, 1975; Place and

Powers, 1977). In the present study, we have established the existence of a series of latitudinal clines in gene frequencies for *F. heteroclitus*. A host of physical (e.g., temperature, salinity, humidity, tides, and dissolved oxygen) and ecological (e.g., predators, food sources, and primary productivity) parameters vary with latitude along the East Coast of the United States. Among these parameters, mean water temperature is the most conspicuous. Figure 7 illustrates the steep thermal gradient in mean water temperature found along this region. Clearly, water temperature is highly correlated ($r=0.991$) with latitude, demonstrating a 1 C change per degree latitude.

Poikilotherms, such as fish, modify their metabolic rate in response to environmental temperature (for review, see Prosser, 1967, 1973). Since metabolic rate is, in turn, a function of a complex, interconnected series of temperature-sensitive enzymes, we hypothesized that temperature is a contributing factor responsible for the establishment and/or maintenance of the clines.

The justification for selecting temperature as a possible selective force relies on several sources. Correlations between gene frequency and environmental temperature have been described in *Drosophila* (Johnson and Schaffer, 1973), bryozoans (Schopf and Gooch, 1971), and fish (Koehn and Ras-1967; Koehn, 1969; Johnson, 1974, 1977; Powers and Powers, 1975; Hjorth and Simonsen, 1975). The associations between the allozymes and temperature have been extended in some cases to the demonstration of kinetic differences between phenotypes (Koehn, 1969; Koehn *et al.*, 1971; Merritt, 1972; Powers, 1972; Powers and Powers, 1975; Miller *et al.*, 1975). Johnson (1971) has found correlations between larvae survival and lactate dehydrogenase genotype in *Anoplarchus purpurescens*. There are three lines of evidence

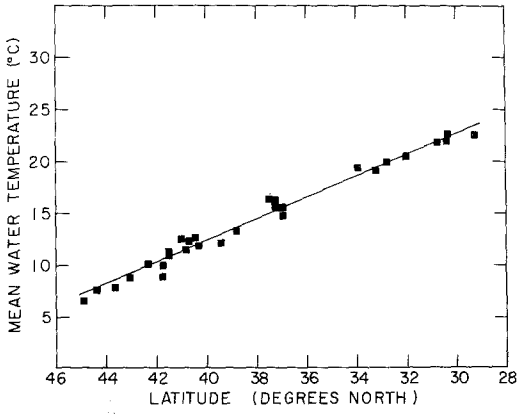

Fig. 7. Mean water temperature (C) vs. latitude (degrees north).

concerning the influence of temperature on gene frequencies in *F. heteroclitus*. Mitton and Koehn (1975) found that a χ^2 test for homogeneity in gene frequency between populations inside and outside a New York power plant thermal effluent suggested that *Ldh-B* was responding to some component of the effluent environment. Furthermore, gene frequencies for *Mdh-A* in the thermal outfall are more similar to those of the southern populations we have described than to those characteristic of New York populations. And, lastly, biochemical data on the LDH-B allozymes indicate a possible selective advantage for the *Ldh-B*^{b}*B*^{b} allotype in cold water and for *Ldh-B*^{a}*B*^{a} in warm habitats (Powers, 1972; Powers and Powers, 1975; Place and Powers, 1977).

Gene Diversity and Environmental Variability

A concept first developed by Dobzhansky (1951) is useful in discussing the overall pattern of genetic variation for *F. heteroclitus* summarized in Fig. 6. He envisioned an association in which genetic variation was related to environmental variability. The concept was embodied into a theoretical formulation by Levins (1968). The basic tenet of this theory is that genetic variation may be regarded as an adaptation to environmental heterogeneity and uncertainty. Several studies have appeared which claim to support this assertion (Powell, 1971; Bryant, 1974; Nevo, 1976; Johnson, 1976). However, conflicting evidence and interpretations have also been presented (e.g., Somero and Soulé, 1974; Ayala and Valentine, 1974; Ayala, *et al.*, 1975; Powers *et al.*, in preparation). Figure 8 shows that the greatest thermal variability is found at

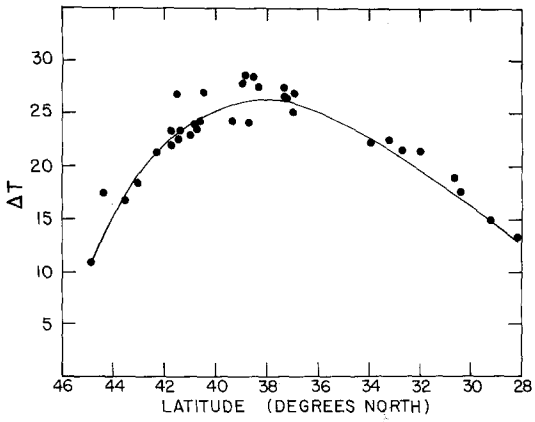

Fig. 8. Relationship between the annual thermal variation (ΔT) and latitude (degrees north). The parameter ΔT represents the mean maximal water temperature minus the mean minimal water temperature.

latitudes with the highest gene diversity (expected average heterozygosity). It is impossible at this time to ascertain whether this coincidence is adaptive or merely a trivial mathematical consequence of the clines.

Heterosis would presumably arise because of heterozygotic flexibility in the varying environment. If the variance in fitness of the heterozygote in a temporally and spatially varying environment is less than that of either homozygote, then a case of overdominance occurs. For example, if this *Ldh-*B^aB^b heterozygote is intermediate to both homozygotes (*Ldh-*B^aB^a and *Ldh-*B^bB^b), then selection for *Ldh* B^aB^a in warmer water and against *Ldh-*B^aB^a in colder waters would result in a net heterozygote (*Ldh-*B^aB^b) superiority in the changing thermal environment (Powers and Powers, 1975; Place and Powers, 1977).

CONCLUSION

Fundulus heteroclitus has been shown to possess extensive geographic variation in gene frequencies for four unlinked genetic polymorphisms. The patterns are stable temporally for at least 2–4 years. We postulate that these clines are being maintained by selective pressures imposed by the steep thermal gradient found along this animal's habitat. We cannot rule out the possibility that the clines occur from hybridization of two prior isolates which have diverged because of genetic drift and/or selection. We believe that insight can be gained regarding the possible adaptative nature of these protein polymorphisms by establishing the structural and functional properties of each enzyme in relation to *in situ* temperature regimes. However, demonstrating selection for one locus will not resolve the controversy between the balanced and neoclassical schools (Lewontin, 1974). Rigorous genetic, physiological, kinetic, and thermodynamic analyses must be applied again and again to as many enzyme loci as possible in order to assess how many loci are actually being affected by natural selection either directly or indirectly (e.g., via linkage or regulatory mechanisms) and how gene combinations interact in synergistic ways at the functional or regulatory level to increase relative fitness. Our long-term goal is to apply this approach to as many polymorphic loci as possible in the fish *F. heteroclitus*. The work presented here is one component of that effort.

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