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The genotype frequencies of one-year-old oyster spat (Crassostrea gigas) from parents of known genotype, are compared to the frequencies expected with Mendelian inheritance. The genotypes are characterized at three enzyme loci, Aspartate aminotransferase (AAT), Phosphohexose isomerase (PHI) and Leucine aminopeptidase (LAP), and a general protein locus. In this study expression of these loci as histochemically stained patterns on a gel following electrophoresis is controlled by two codominant alleles at each locus.

Frequencies of the genotypes at all four loci, in all offspring groups investigated, are not statistically different from those expected with Mendelian inheritance, supporting the hypothesis that this is the method of inheritance. It is further concluded that there are no large amounts of selection connected with any of these loci.

An additional observation, that there is no statistical difference between genotype ratios of siblings in two different growing areas,

is discussed in terms of G. Williams' (1975) "elm-oyster" model. It is suggested that the search for selection at single loci is probably not a generally useful one for the oyster breeder.

A Mendelian Study of *Crassostrea gigas*

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INTRODUCTION

With the increased use of aquatic organisms as quasi-domesticated "crops" has come the realization that genetic research is not only a means to improve economically important species, but also necessary if the vigor of stocks is to be maintained.

The oyster has lately become of importance as an experimental animal, for example as an indicator of environmental quality in bio-assays (Woelke, 1972), although it continues to be sought after primarily as a food item. Oysters have been cultured, in some areas quite intensively, for at least a century. Within historical times, in some areas oyster populations have been severely diminished through overfishing (Fasten, 1931) or disease (Sprague, 1971), while other areas have had exotic species introduced. The close resemblances and hybridizing abilities of the geographically remote Japanese and Portugese oysters may be a result of man's early transplanting activities (Menzel, 1974). Today, with the proliferation of oyster hatcheries and artificial populations of oysters, it is time to take a harder look at the relationship between farming oysters and the genetic structure of the species.

The most intense and long-range investigations of oyster genetics originated in an attempt to discover disease-resistant

strains of the American oyster, Crassostrea virginica (Longwell et al., 1967). Cytogenetic research at the Milford Research Laboratories of Connecticut has demonstrated a diploid chromosome number of 20, with "normal mitosis and meiosis, the latter showing some microscopic evidence of translocations." Later, breeding studies were performed, including interspecific hybridization. Selective breeding studies resulted in a breeding scheme for maximum vigor realized by crossing different strains of oyster (Longwell and Stiles, 1973).

Some similar investigations have involved Crassostrea gigas. The same diploid number of 20 has been verified for C. gigas, as for all species of oysters studied (Ahmed and Sparks, 1967). Hybridization of different species has met with varying degrees of success (Imai and Saki, 1961; Menzel, 1967). Self-fertilization has been demonstrated as a potential tool for inbreeding studies (Lannan, 1971). Heritability studies of several economically important traits has shown that they can be exploited by fairly simple breeding methods (Lannan, 1972).

The physiology of the Pacific oyster is such that on most of the West Coast of North America only limited spawning can occur, requiring oyster growers to import spat or use hatcheries as a source of "seed." As in the artificial propagation of any species, hatchery production has both disadvantages and advantages. The potentially negative aspect is that an undesirable artificial selection may be

inherent in hatchery practices. There is statistical evidence that larval mortalities in the hatchery are non-random, suggesting the possibility of such selection (Lannan, 1972). Yet the same hatchery techniques enable the genetical manipulation of oysters in order to generate lines of oysters with desired economic traits.

To be able to use methods of breeding genetics on oyster populations with confidence, it must first be confirmed that the breeding system of the oyster is of a Mendelian sort. In other words, independent segregation of chromosomes during gametogenesis must occur.

The hypothesis to be tested here is that the numbers of offspring of several single-locus genotypes in a group of siblings will not be different from those predicted by Mendelian assortment of genetic information. Departures from such ratios would imply either some other form of inheritance, or selection. "Selection occurs when one genotype leaves a different number of progeny than another. This may happen because of differences in survival, in mating, or fertility" (Crow and Kimura, 1970, p. 173). Thus if selection affects a locus, a departure from the Mendelian distribution of genotype numbers would be observed; for example, after 10% selection against the larvae of a certain genotype, the proportions of individuals of that genotype surviving would only be 90% of that expected without such selective mortality. The alternatives to the hypothesis are, therefore,

that either some other form of inheritance occurs in oysters, or that selection significantly affects the loci under observation. If selection is demonstrated, as manifest by significant excesses or deficits of one of the genotypes at any of the loci, some insight may be gained into the nature of the non-random larval survival in the hatchery system.

Selection may occur at several times during the process of mating oysters and raising their progeny to harvestable size. First, mating itself may not be completely random; there is variability in the timing of conditioning for spawning in adult oysters, a trait that may be under genetic control, so that each adult may not have an equal probability of mating with every other oyster of the opposite sex.

The day following successful fertilization of the egg is a critical one in larval survival. Metamorphosis through the blastula and gastrula stages should result in the 24-hour "D-shaped" larva. A considerable proportion of zygotes fail to undergo this transformation successfully. The second critical period is about four weeks later and involves metamorphosis of the veliger larvae in preparation for "setting" or attachment to the substrate. This complex process is also accompanied by considerable larval mortality.

Mortality of the successfully established spat is usually low in comparison to the earlier stages. Nonetheless, the potential for selective mortality exists. As methods for growing spat vary, the

young oysters may be exposed to different selective forces according to their environment. A secondary hypothesis to be tested is that the selective forces affecting survival of spat in one environment will not be significantly different from those in another. The test of this will involve comparing genotype frequencies between bottom-cultured oysters in one bay and their raft-cultured siblings in another.

In this study single-locus genotypes are determined by electrophoretic evaluations of several proteins. Assuming the one gene-one polypeptide hypothesis, the patterns revealed by electrophoresis and histochemical staining of oyster tissue may be interpreted as several forms of a protein resulting from the expression of one or more gene loci. Codominant alleles at a locus result in patterns predictable from knowledge of the protein's behavior in an electric field, the different forms of the protein being known as allozymes. The proteins investigated in this study had been previously analyzed as producing electrophoretic patterns which appeared to be compatible with a one-locus genetic system having two or more codominant alleles (Buroker, 1975). Observation of the offspring of two heterozygous individuals demonstrates whether the alleles are indeed codominent or whether different loci are involved; in the latter case only the parental genotype would be observed. Observation of the offspring of individuals of several known genotypes provides data to test the hypothesis of Mendelian inheritance.

MATERIALS AND METHODS

Experimental Animals

Breeding animals, mature Pacific oysters (Crassostrea gigas Thunberg) were obtained from a stock maintained in Netarts Bay, Oregon, by Oregon State University, and from Fowler's Oyster Company in Yaquina Bay, Oregon. These were brought into the laboratory and conditioned at 18°C in running sea water (Loosanoff and Davis, 1963).

At the same time, a reference population of 300 oysters was selected randomly from the Netarts Bay stock, and established in two fiberglass trays which remained anchored on the mud flats in Netarts Bay. At approximately two-month intervals (dependent on convenient low tides), a sample of 30 oysters was randomly drawn from these trays. These were brought to the laboratory and sacrificed immediately prior to electrophoretic characterization.

Accomplishment of Matings

Gametes were obtained either by "stripping" the conditioned oysters of their sex products with a syringe, or the stimulation of spawning by raising the water temperature to 30°C. The sperm and eggs thus obtained were mixed to produce matings. The crosses were accomplished on a diallel format in order to produce as many

combinations of parental matings as possible and ensure a large variety of parental genotypes. The allozyme genotypes of the parental oysters were determined after each successful mating.

Larval Culture

The culture of the larvae involved a small-scale simulation of the practices and regimes of the Oregon State University oyster hatchery (Breese and Malouf, 1975). Ten-liter polypropylene buckets were used as rearing vessels in place of the 400-liter tanks employed in the production hatchery.

The eggs of each female oyster were divided evenly among a number of buckets corresponding to the number of spawned male oysters (not exceeding two million eggs per bucket). A few drops of turbid sperm and seawater suspension were added to each bucket and the contents of the buckets gently stirred to aid fertilization. After five minutes, the volume was adjusted to 10 liters. Two days after fertilization the larvae were redistributed into additional buckets such that the density of the larvae was no more than 50,000 per bucket. All buckets were kept in a dark room at 25 $\pm$ 1^{o}C. All water employed in the larval culture regime was sand-filtered, ultraviolet irradiated sea water brought to ambient temperature (25 $\pm$ 1^{o}C). The water was changed twice a week. Each bucket was emptied, the larvae being retained on screens of appropriate mesh size, then refilled with

treated sea water and the larvae were returned to the buckets. At this time 5 ml of a 5% solution of Sulmet (sulfumethazine) was added to each bucket for prophylaxis.

The algal food supply was grown in a small-scale simulation of the oyster hatchery's culture system (Breese and Malouf, 1975). Batch feeding involved the introduction of enough algae, Pseudo-isochrysis paradoxa, to each bucket to obtain 40,000 cells/ml sea water. The first week the larvae were fed once a day, and twice a day for the remainder of their pre-metamorphosis period.

At the third week about 40 washed oyster shells (cultch) were added to each bucket. All successfully metamorphosed larvae attached themselves to the cultch, where they were then counted.

The cultch from each bucket was strung on heavy cord, each string being tagged with a plastic tag to identify the mating involved. These remained in the sea water system of the laboratory until it was possible to take them to their growing areas. In most cases, the spat-bearing cultch from each mating was divided into two groups. The first group was transferred to Netarts Bay at six weeks of age, where the strings of cultch were tied at both ends to trays resting on the intertidal mud flats. The second group was suspended from a raft in Yaquina Bay at 10 weeks of age.

Sampling of Offspring

After a year's growth, 41 samples of 30 oysters were taken.

Table 1. Larval survival statistics.

Female	Male	Eggs produced	"D" shaped larvae ($\times 10^4$)	Small spat		Year old spat[a, b]	
				No.	Survival (% eggs $\times 10^{-3}$)	Yaquina	Netarts
1	1	6.4×10^5	4.5	20	0.02	-	43
1	26	"	0.6	0	0	-	-
1	30	"	24.8	2776	4.3	416	103, 72
1	78	"	16.0	3727	5.8	208	47, 21
1	82	"	14.6	3158	4.9	184	67
1	W43	"	11.6	169	2.6	112	43
1	G55	"	10.5	4488	7.0	527	79
47	1	1.3×10^6	17.8	1450	1.1	161	64
47	26	"	4.3	632	0.49	59	37
47	30	"	12.2	2960	2.3	152	83, 51
47	78	"	27.9	6981	5.4	318, 202	67, 49
47	82	"	17.4	4543	3.5	211	53, 84
47	W43	"	15.9	2440	1.7	336	74
47	G55	"	14.2	758	0.58	63	34
G55	1	1.7×10^6	1.4	31	0.02	35	-
G55	26	"	0.4	18	0.01	42	-
2	1	13.6×10^6	35.0	33	0.002	38	-
2	26	"	108.0	362	0.03	86	-
2	W43	"	70.0	195	0.01	78	-
2	82	"	123.0	633	0.05	118, 67	55, 49

[a]Numbers are larger than "small spat" numbers due to difficulties of correctly counting newly-set spat.

[b]Each number represents one cultch string.

Each sample was drawn from one string of cultch. In some cases a single mating had resulted in enough offspring to warrant several strings of cultch. Three other matings had fewer than 30 surviving spat and therefore were not used.

Data for larval survival in each mating are summarized in Table 1.

Preparation of Samples for Electrophoresis

Subsequent to harvesting gametes, each adult oyster was pre-prepared as follows. After the shell was opened, the adductor muscle was removed and about one gram of tissue placed in a 100 x 15 mm heavy-walled test tube on ice. Two milliliters of cold 2% phenoxy-ethanol in twice-distilled water were added and the tissue was homogenized for about 30 seconds with an electric tissue macerator. The sample was then clarified by centrifugation at low speeds for about three minutes. The supernatant was decanted into 75 x 10 mm test tubes. A sample of approximately 0.1 ml was drawn into each of 10 capillary tubes. The small test tubes with capillary tubes intact were then frozen at -20°C so that the capillary tubes could be removed individually without having to repeatedly thaw the entire sample and risk loss of enzyme activity.

Tissue extracts from the progeny were prepared in the same manner after first freezing the entire animal to facilitate removal of the adductor muscle.

Starch-gel electrophoresis was accomplished by the method of Kristjannsson (1963). The gel consisted of a 12% solution of electrostarch (Otto Hiller lot 303) in a modified Schiff (1972) gel buffer. The procedure was as follows: distilled water (320 ml) was added to 80 ml of the concentrated (5x strength) buffer to produce 400 ml of gel buffer. Starch (51 g) was then dissolved in 100 ml of the buffer, while the remainder of the buffer was heated to the boiling point. Next, 275 ml of the hot buffer was added to the dissolved starch solution to produce a 12% weight/volume solution. This was shaken in a flask over a flame until the desired opacity was observed. The solution was then degassed using an aspirator, and poured into a vertical mold. The gel mold consisted of two pieces of plate glass separated by strips of 9 mm thick plexiglas clamped together to form a rectangle of dimensions 140 x 225 x 9 mm. After the gel cooled to room temperature the mold was removed from the vertical position and the upper glass plate and all plexiglas strips were removed. A slice was then cut in the gel parallel to one of its 225 mm edges and about 40 mm from it.

The gel was then placed in a refrigerator at 12^{o}C resting upon two trays containing a Ridgway (1970) electrode buffer which was conducted to the gel ends by wicks made from folded Handiwipes. Seventy-five milliamps of current, supplied by a DC power source (Heathkit Model Sp17-A) was conducted through the gel at 200 volts

for 10 minutes. The gel was then removed and filter paper strips 2 mm wide soaked in the oyster extract were inserted along the slice, up to 35 per gel. One additional strip contained a marker dye, bromophenol blue.

Current was again applied to the gel, for 15 minutes, at which time the filter paper strips were removed and the surface of the gel covered with plastic wrap to prevent dehydration. Voltage was again applied across the gel. Electrophoresis was terminated after approximately four hours, or when the dye marker had reached the anodal wick. The gel was sliced horizontally into three 3-mm thick replicate layers which were immediately stained for enzyme activities.

The histochemical staining methods for localizing the enzyme activities, and the formulae for the gel and electrode buffers are summarized in Table 2.

Enzyme Systems

The essential problem in determining which enzyme systems to study was to find ones which could be consistently resolved, easily readable and interpretable. While other investigations involving electrophoresis of oyster tissues were in process (Schaal and Anderson, 1974; Buroker, 1975), their procedures were not always successful in resolving all systems, whether due to the use of a different species of oyster (in the former case), or simply due to

Table 2. Formulae for buffers and stains.

Gel buffer system (5x concentration, pH 8.0)

TRIS	9.57 g
Citric acid	4.80
Lithium hydroxide	2.30
Boric acid	13.05

} 1 liter

Tray buffer system (5x, pH 8.1)

Lithium hydroxide	12.6 g
Boric acid	92.7

} 1 liter

Enzyme system	Abbreviation	Reference	Modification
Aspartate aminotransferase	AAT	Johnson et al. (1972)	none
Leucine aminopeptidase	LAP	Wall (1968)	Fast garnet GBC salt substituted for Black K salt
Muscle protein (general protein)	GP	Weber and Osburn (1969)	none
Phosphohexose isomerase	PHI	Shaw and Prasad (1970)	Buffer is pH 8.0, not pH 7.1. Omit $MgCl_2$
Sorbitol dehydrogenase	SDH	Shaw and Prasad (1970)	Use 0.5 M Tris-HCl, pH 7.1, not 0.05 M Tris-HCl pH 8.0

variation in technique, source of chemicals, and degree of skill of the technician. As Buroker's study had shown some enzyme systems to be polymorphic in Crassostrea gigas, these were investigated until four systems were consistently resolvable in the adult oyster. These were Aspartate aminotransferase (AAT), Leucine aminopeptidase (LAP), Sorbitol dehydrogenase (SDH), and General protein (GP). Later Phosphohexose isomerase (PHI) was worked out for the year-old oysters; however, SDH activity could not be resolved for these small oysters.

The five systems investigated include one structural protein and four enzymes which play very different roles in the metabolism of the oyster and could therefore be subject to different sorts of selective forces.

The structural protein of the adductor muscle is apparently composed of many polypeptides, judging from the number of observed electrophoretic bands. These are probably produced by several loci, only one of which (that producing the most anodally migrating protein bands) showed any polymorphism. The two-banded heterozygote form suggested a monomeric polypeptide under control of two codominant alleles at the locus.

Leucine aminopeptidase is in the class of enzymes known as proteases, and is a rather non-specific exopeptidase which hydrolyzes most NH_2-terminal amino acids of peptides. It is found in most

tissues of the oyster (Buroker, 1975, p. 7) but a more exact role is not known. Of at least two polymorphic loci, the most consistently resolved locus appears to have several alleles, of which only two were observed in this study. The double-banded heterozygote pattern is typical of an enzyme which is a monomer.

Aspartate aminotransferase belongs to the category of enzymes which catalyze reactions exchanging keto and amino functions. In the mitochondrial matrix is found a specific aspartate-glutamic transaminase, while a more general cytosol AAT is involved in the urea cycle, reversibly catalyzing glutamic acid and oxaloacetic acid to α-ketoglutaric acid and aspartic acid. The extraction process makes it unlikely that any mitochondrial AAT was observed in this experiment.

In a study of variation of enzyme activities in the American oyster, C. virginica, Chambers et al. (1975) found a significantly lower mean specific activity for supernatant AAT during the oyster's two seasonal reproductive peaks. An earlier study (Hammen, 1968) suggested that AAT activity in bivalves regulated amino acid balance and thus osmotic balance. The high AAT level found in Crassostrea suggests an atypical metabolism in which reactions involving oxaloacetate are more important than those involving pyruvate.

The functional AAT molecule is a dimer. Only two alleles were observed at the locus examined in this study.

Sorbitol dehydrogenase reduces D-glucose (in the presence of H_2 and metal) to sorbitol, a sugar alcohol. More exact function in the oyster is not known, nor is it known why it could not be resolved in the young oysters. Its subunit composition appears to be tetrameric, although all five bands of the heterozygote were not always clear. Two alleles at one locus were observed.

Phosphohexose isomerase is an important enzyme of the glycolytic pathway. It catalyzes the conversion of fructose-6-phosphate to glucose-6-phosphate, which in turn may be involved in several different pathways. It is found in most tissues of the oyster (Buroker, 1975) and is a dimer. Although up to five alleles have been observed at the one locus, only two were observed in this study. Although the staining procedure for this system was not worked out at the time of preparation of the parental oysters, judging from the genotypes of the offspring, none of the adults were heterozygous.

Statistical Tests

Chi-square was used as a test statistic to compare the genotype numbers observed in a sample of the progeny to the numbers expected to result from the parental genotypes, assuming Mendelian segregation (Snedecor and Cochran, 1967).

Examination of the bimonthly sample data involved searching for any significant changes in genotype frequencies throughout the

year. The data were examined using a Chi-square test for homogeneity in a 3 x 6 array with three rows (three possible genotypes) and six columns (the six seasonal samples). SDH was not included in the array because of small numbers of two of the genotypes. Observed and expected values were compared using the Chi-square test (Snedecor and Cochran, 1967, p. 250).

RESULTS AND DISCUSSION

Assortment of Genetic Information

The assortment of alleles for three allozyme systems is summarized in Tables 3 through 6. PHI is not included in these tables since only one genotype was present in the progeny of any one mating. Inspection of these tables reveals that of the four enzyme systems examined in 41 samples of sibling groups, only one (a sample for General Protein from one group of spat) showed any significant deviation from Mendelian expectation. When the significance level of rejection of the hypothesis is 5%, such a deviation could occur by chance once in 20 times. It may be concluded that these alleles segregate independently prior to mating in C. gigas.

The assumption of Mendelian inheritance is implicit in theories of breeding genetics; the fact that the Pacific oyster has such an inheritance mechanism supports the use of statistical methods of selective breeding. Given that genetic variability exists in the population, such methods may be used to evaluate lines and individuals for any of the quantitative traits considered important to the oyster grower (Lannan, 1974).

Selection, acting either at one of these four loci or at some nearby loci, might also cause a departure from the expected

Table 3. Summary of segregation of AAT alleles. Expected numbers of offspring genotypes in parentheses.

Tag no.	Parental genotypes		Offspring genotypes			χ^2	Probability of a greater χ^2 value
	Female	Male	SS	SF	FF		
	SF	FF	(0)	(15)	(15)		
Y085				12	18	0.83	.4
Y086			0	17	13	0.30	.6
Y087			0	17	13	0.30	.6
Y088				18	12	0.83	.4
Y089				17	13	0.30	.6
G49				16	14	0.03	.85
G47				18	12	0.83	.4
W82				17	13	0.30	.6
Y084				16	14	0.03	.85
G40				16	14	0.03	.85
	SF	SF	(7.5)	(15)	(7.5)		
Y095			6	13	11	2.19	.4
Y096			7	14	9	0.40	.8
Y090			8	14	8	0.13	.9
Y091			9	12	9	1.20	.5
Y092			9	13	8	0.60	.75
Y093			8	15	7	0.06	.95
Y094			10	14	6	1.19	.55
G82			7	14	9	0.40	.8
G42			7	14	9	0.40	.8
Y082			9	14	7	0.40	.8
Y083			6	15	9	0.60	.75
	SS	FF		(30)			
Y080				30			
W98				30			
W99				30			
G99				30			
G96				30			
W84				30			
W83				30			

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Table 3. (Continued)

Tag no.	Parental genotypes		Offspring genotypes			χ^2	Probability of a greater value
	Female	Male	SS	SF	FF		
	SS	SF	(15)	(15)			
W88			18	12		0.83	.4
W87			17	13		0.30	.6
W91			14	16		0.03	.85
W93			12	18		0.83	.4
W94			12	18		0.83	.4
W92			13	17		0.30	.6
W89			18	12		0.83	.4
W95			13	17		0.30	.6
W90			13	17		0.30	.6
	SS	SS	(30)				
W86			30				
W85			30				
	SF	SS	(15)	(15)			
Y098			13	17		0.30	.6
Y097			12	18		0.83	.4

Table 4. Summary of segregation of LAP alleles. Expected offspring genotypes in parentheses.

Tag no.	Parental genotypes		Offspring genotypes			χ^2	Probability of a greater value
	Female	Male	SS	SF	FF		
	SS	SF		(30)			
Y097				30			
Y098				30			
	SS	SF	(15)	(15)			
Y086			15	15		0.00	
Y087			11	19		1.63	.2
Y088			18	12		0.83	.4
Y089			15	15		0.00	
Y095			13	17		0.30	.6
Y096			15	15		0.00	
G49			12	18		0.83	.4
G47			13	17		0.30	.6
G42			13	17		0.30	.6
G82			15	15		0.00	
	SS	SS	(30)				
Y090			30			-	
Y091			30				
Y092			30				
Y093			30				
Y094			30				
Y084			30				
Y085			30				
G40			30				
Y082			30				
Y083			30				
	FF	SF		(15)	(15)		
W84				13	17	0.30	.6
	FF	SS		(30)			
W83				30			
W82				30			
	SF	FF		(15)	(15)		
W86				13	17	0.30	.6
W85				16	14	0.03	.85

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Table 4. (Continued)

Tag no.	Parental genotypes		Offspring genotypes			χ^2	Probability of a greater value
	Female	Male	SS	SF	FF		
	SF	SF	(7.5)	(15)	(7.5)		
Y080			10	12	8		
W98			9	13	8		
G96			8	12	10	1.46	
W88			8	11	11	2.73	
W87			7	14	9	0.40	
	SF	SS	(15)	(15)			
W99			12	18		0.83	.4
G99			14	16		0.03	.85
W94			17	13		0.30	.6
W93			12	18		0.83	.4
W92			14	16		0.03	.85
W91			18	12		0.82	.4
W95			13	17		0.30	.6
W90			14	16		0.30	.6
W89			17	13		0.30	.6

Table 5. Summary of segregation of GP alleles. Expected offspring genotypes in parentheses.

Tag no.	Parental genotypes		Offspring genotypes			χ^2	Probability of a greater χ^2 value
	Female	Male	SS	SF	FF		
	FF	FF			(30)		
W83					30		
W82					36		
	FF	SS		(30)			
W84				30			
	SF	FF		(15)	(15)		
Y098				14	16	0.03	.85
Y097				16	14	0.03	.85
W99				16	14	0.03	.85
G99				13	17	0.30	.60
W86				11	19	1.63	.20
W85				16	14	0.03	.85
	SF	SF	(7.5)	(15)	(7.5)		
Y092			5	16	9	1.20	.55
Y091			4	13	13	5.93	.05*
Y090			6	15	9	0.60	.75
W93			8	12	10	1.46	.5
W91			8	13	9	1.26	.55
W94			6	14	10	1.19	.55
W92			9	13	8	1.26	.55
	SS	FF		(30)			
Y084				30			
Y085				30			
G40				30			
	SS	SS	(30)				
G42			30				
G49			30				
G82			30				
G47			30				
	SF	SS	(15)	(15)			
Y086			13	17		0.30	.60
Y088			11	19		1.63	.20

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Table 5. (Continued)

Tag no.	Parental genotypes		Offspring genotypes			χ^2	Probability of a greater χ^2 value
	Female	Male	SS	SF	FF		
	SF	SS	(15)	(15)			
Y087			13	17		0.30	.60
Y089			12	18		0.83	.40
Y045			16	14		0.03	.85
Y096			12	18		0.83	.40
Y094			14	16		0.03	.85
Y093			18	12		0.83	.40
Y080			17	13		0.30	.60
W98			16	14		0.03	.85
W88			14	16		0.03	.85
W87			12	18		0.83	.40
W89			10	20		2.70	.10
W95			12	18		0.83	.40
G96			19	11		1.63	.20
W90			13	17		0.30	.60

Table 6. Summary of tests for departure from Mendelian inheritance patterns. X^2 values for individual strings of spat (subgroups of full-sib groups). Blank indicates all individuals of same genotype.

Spat string	AAT			LAP			GP		
	X^2	d.f.	prob.	X^2	d.f.	prob.	X^2	d.f.	prob.
Y087	0.30	1	.6	1.63	1	.2	0.30	1	.6
Y088	0.83	1	.4	0.83	1	.4	1.63	1	.2
Y089	0.30	1	.6	0.00	1		0.83	1	.4
G47	0.83	1	.4	0.30	1	.6	-	-	-
G49	0.03	1	.85	0.83	1	.4	-	-	-
W82	0.30	1	.6	-	-	-	-	-	-
Y084	0.03	1	.85	-	-	-	-	-	-
G40	0.03	1	.85	-	-	-	-	-	-
Y095	2.19	2	.4	0.30	1	.6	0.03	1	.85
Y096	0.40	2	.8	0.00	1		0.83	1	.4
Y090	0.13	2	.95	-	-	-	0.60	2	.75
Y091	1.20	2	.50	-	-	-	5.93	2	.05*
Y092	0.60	2	.75	-	-	-	1.20	2	.55
Y093	0.06	2	.95	-	-	-	0.83	1	.4
Y094	1.19	2	.55	-	-	-	0.03	1	.85
G82	0.40	2	.8	0.00	1		-	-	-
G42	0.40	2	.8	0.30	1	.6	-	-	-
Y082	0.40	2	.8	-	-	-	-	-	-
Y083	0.60	2	.75	-	-	-	-	-	-
Y086	0.30	1	.6	0.00	1		0.30	1	.6
Y080	-	-	-	1.46	2	.5	0.30	1	.6
W98	-	-	-	0.55	2	.75	0.03	1	.85
W99	-	-	-	0.83	1	.4	0.03	1	.85
G99	-	-	-	0.02	1	.85	0.30	1	.6
G96	-	-	-	1.46	2	.4	1.63	1	.2
W84	-	-	-	0.30	1	.6	-	-	-
W83	-	-	-	-	-	-	-	-	-
Y085	0.83	1	.4	-	-	-	-	-	-

(Continued on next page)

Table 6. (Continued)

Spat string	AAT			LAP			GP		
	X^2	d.f.	prob.	X^2	d.f.	prob.	X^2	d.f.	prob.
W88	0.83	1	.4	2.73	2	.25	0.03	1	.85
W87	0.30	1	.6	0.40	2	.85	0.83	1	.4
W91	0.05	1	.85	0.83	1	.4	1.26	2	.55
W93	0.83	1	.4	0.03	1	.85	1.46	2	.5
W94	0.83	1	.4	0.30	1	.6	1.19	2	.55
W92	0.30	1	.6	0.03	1	.85	1.26	2	.55
W89	0.83	1	.4	0.30	1	.6	2.70	1	.10
W95	0.30	1	.6	0.30	1	.6	0.83	1	.4
W90	0.30	1	.6	0.03	1	.85	0.83	1	.4
W86	-	-	-	0.30	1	.6	1.63	1	.2
W85	-	-	-	0.03	1	.85	0.03	1	.85
Y098	0.30	1	.6	-	-	-	0.03	1	.85
Y097	0.83	1	.4	-	-	-	0.03	1	.85

genotype-number distributions in the larvae even though the alleles had independently segregated prior to mating. For instance, in the case of a cross between heterozygous parents, if there were a selective advantage attendant on being heterozygous as opposed to being homozygous for either allele at a specific locus, an excess (significantly more than half) of the surviving offspring would be heterozygous. In this experiment, however, none of the matings resulted in genotype frequency distributions significantly different from those expected under Mendelian assortment. It must be noted, however, that the sample size of 30 could detect a selection magnitude no smaller than 33% against one allele at a locus. To be even 90% sure of a selection magnitude as low as 1% would require a sample size of 400,000 (Lewontin, 1974, p. 242), well beyond the scope of this or most experiments. A sample size of 120 would detect selection rates of at least 20% against one allele; such a sample was taken from a group of spat which in its sample of 30 had a comparatively high (although not significantly so) Chi-square value (Table 7). Again no departure from expected values was observed.

In a further attempt to observe small selection values all samples from all strings of spat from a single mating were pooled, resulting in sample sizes of 90 to 120 oysters. First, it was necessary to demonstrate that no significant difference existed between mating members kept in separate bays. A Chi-square test to

Table 7. Large-sample test for departure from Mendelian inheritance pattern. χ^2 values for three enzyme systems of 120 oysters (from one string of cultch). Mating W92.

	Genotype	Observed	Expected
AAT	FF	31	30
	SF	62	60
	SS	27	30
	$\chi^2 = .62$ Degrees of freedom = 2 p = .5		
LAP	SS	57	60
	SF	63	60
	$\chi^2 = .21$ Degrees of freedom = 1 p = .5		
GP	FF	36	30
	SF	64	60
	SS	27	30
	$\chi^2 = 1.77$ Degrees of freedom = 2 p = .25		

compare siblings from mating G47 x 78 showed no significant bay effect on the distributions of genotype frequencies (Table 8). Pooled Chi-square values for three loci (AAT, GP and LAP) were similar to those for the smaller samples (Table 9). No selection was detected.

The differences among the six bimonthly samples were not statistically significant (Table 10). The four enzyme systems (AAT, LAP, GP, and SDH) were not affected in their electrophoretic expression by seasonal environmental change. Such an assurance is necessary when characterizing genotypes by electrophoresis at different seasons of the year for parents and offspring. Since short-term acclimation may be accomplished by the individual producing different isozymes (Hazen and Prosser, 1974), it is possible that enzyme activities and electrophoretic patterns would be altered during the acclimation process. Observing individuals in different physiological states (at different seasons) could lead to erroneous results if the electrophoretic expression of the enzyme were not conserved during the year.

An interesting observation concerning the 120 oysters examined over the year's time was the small amount of variability at the polymorphic loci. Buroker's (1975) examination of naturally breeding populations in Puget Sound revealed five alleles at the LAP locus, three at the AAT, three at SDH, and five at PHI. In the Netarts Bay

Table 8. Test for bay effect on inheritance patterns. Numbers are pooled for mating G47 x 78.

Enzyme	Genotype SS	SF	FF		χ^2	df	Prob.
AAT	26	34		Yaquina			
	25	35		Netarts			
	25.5	34.5		Expected			
					.03	1	.75
GP	16	25	19	Yaquina			
	15	27	18	Netarts			
	15.5	26	18.5	Expected			
					.14	3	.95
LAP	30	30		Yaquina			
	31	29		Netarts			
	30.5	29.5		Expected			
					.02	1	.90

Table 9. Tests for departures from Mendelian inheritance patterns; X^2 values for pooled sibling-group data.

Protein	Mating	Tag no.	No. in family	No. per genotype				X^2 (df=2)	Probability (greater X^2)
				ss	sf	ff			
General protein	G47 x 78 (sf x sf)	W93, 91 W94, W92	120	30	60	30	Expected	1.73	0.4
				31	52	37	Observed		
	1 x 30 (sf x ss)	Y088 Y087 Y089	90	45	45		Expected	3.22	0.2
				36	54		Observed		
Leucine amino peptidase	G47 x 1 (sf x sf)	Y080 W98 W97	90	22.5	45	22.5	Expected	2.31	0.3
				27	41	24	Observed		
	G47 x 78 (sf x ss)	W94, W93 W92, W91	120	60	60		Expected	0.08	0.8
				59	61		Observed		
	1 x 30 (ss x sf)	Y087 Y088 Y089	90	45	45		Expected	0.01	0.95
				46	44		Observed		
Aspartate amino transferase	1 x 30 (sf x ff)	Y087 Y088 Y089	90	45	45		Expected	0.90	0.4
				40	50		Observed		

Table 10. Bimonthly samples: Genotype distributions in 30-oyster samples.

Month	AAT			LAP			SDH			GP		
	Fast	Slow	Heter.	Fast	Slow	Heter.	Fast	Slow	Heter.	Fast	Slow	Heter.
Jan.	5	10	15	8	5	17	1	28	1	4	12	16
Mar.	7	15	8	7	5	18	0	27	3	2	16	12
May	7	8	15	7	4	19	2	26	2	4	11	15
July	8	12	10	7	6	17	2	25	3	5	13	12
Sept.	10	11	9	8	7	15	0	29	1	3	15	12
Nov.	9	12	9	7	9	14	1	28	1	5	13	12
X^2[a]	8.87					3.75		c			3.90	
P[b]	.5					.95					.95	

[a] Chi-square values are for tests of independence in 3 x 6 contingency tables; one for each enzyme system.

[b] P values are tabulated probabilities of the chance occurrence of greater X^2 values than these. None are significantly low.

[c] No X^2 is computed for the SDH system due to the infrequency of the fast allele.

group only two alleles were found at each locus. As this was a non-breeding group, some variability may have been lost randomly; it appears to be the most uncommon alleles that are missing. It seems more likely, however, that this lack of variability derives from the small number of parents used to create this group of oysters.

Linkage Study

As no categories of expected genotypes were missing in the five matings investigated, it was assumed that no tight linkage existed among AAT, LAP, GP, or PHI. Finding lesser degrees of linkage would involve proceeding in the manner illustrated in Table 11. Each mating must be examined and each individual scored as to genotype at all four loci. In Table 12 only three loci, AAT, LAP, and GP, were scored since all full-siblings shared the same PHI genotype (due to the coincidence that none of the parental oysters were heterozygous at the PHI locus).

The problem of sex linkage is an especially interesting one in the oyster, a protandrous hermaphrodite. No sex chromosomes have been identified to date (Longwell, 1973, p. 154). As the year-old oysters are expected to express the male sex, if any of the loci studied were located on a sex chromosome, only the genotype of the male parent might be observed. In no case did this occur; it can be concluded either that there is no sex chromosome, or that none of the

Table 11. Linkage study.[a]

Genotype no. (supra)	No. expected (no linkage)	No. observed	
		Mating Y091	
2	3.75	5	
20	3.75	6	
3	3.75	3	
12	3.75	5	$\chi^2 = 14.75$
11	1.875	3	8 degrees of freedom
19	3.75	2	$.1 < p < .05$
1	1.875	1	
21	7.5	4	
10	1.875	1	
		Mating W89	
3	3.75	7	
1	"	2	
7	"	2	$\chi^2 = 10.96$
21	"	7	7 degrees of freedom
9	"	4	$.25 < p < .1$
25	"	5	
27	"	2	
19	"	1	
		Mating W93	
1	1.875	3	
2	1.875	2	
3	3.750	1	
7	1.875	2	$\chi^2 = 8.93$
8	3.750	2	11 degrees of freedom
9	1.875	4	$.70 < p < .50$
19	1.875	1	
20	1.875	2	
21	3.750	4	
25	1.875	2	
26	1.875	3	
27	3.750	4	

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Table 11. (Continued)

Genotype no. (supra)	No. expected (no linkage)	No. observed	
	Mating Y086		
10	3.75	2	
16	"	2	
19	"	4	$\chi^2 = 4.15$
21	"	3	7 degrees of freedom
25	"	5	$.7 < p < .75$
27	"	5	
18	"	3	
12	"	6	
	Mating Y080		
1	1.875	1	
2	1.875	5	
3	3.750	3	
10	1.875	1	$\chi^2 = 11.07$
11	1.875	3	8 degrees of freedom
12	3.750	5	$.25 < p < .10$
19	3.750	2	
20	3.750	6	
21	7.500	4	

[a]Possible genotypes: A denotes AAT; L, LAP; G, GP; f, fast allele; s, slow allele; h, heterozygote.

1 AsLsGs	10 AfLsGs	19 AhLsGs
2 AsLsGf	11 AfLsGf	20 AhLsGf
3 AsLsGh	12 AfLsGh	21 AhLsGh
4 AsLfGs	13 AfLfGs	22 AhLfGs
5 AsLfGf	14 AfLfGf	23 AhLfGf
6 AsLfGh	15 AfLfGh	24 AhLfGh
7 AsLhGs	16 AfLhGs	25 AhLhGs
8 AsLhGf	17 AfLhGf	26 AhLhGf
9 AsLhGh	18 AfLhGh	27 AhLhGh

four protein systems are controlled by a gene located on such a chromosome.

Implications for Genetic Theory

A major debate in the field of genetics today centers around the importance of the amount of variability found in natural populations. One school of thought, referred to as the "balance" school, holds that each of the alleles at a locus has different selective value, the diversity of alleles being maintained by balancing selection, which often involves heterozygote superiority. The "neoclassical" or neutralist school suggests that the bulk of alleles are selectively neutral and are held in the population through the interaction of random drift and mutation. This experiment provided no strong support for either of these arguments.

Superficially, the lack of observable selection at any of the loci investigated would appear to support the arguments of the neoclassical school, which would suggest that such allelic variation has no effect on fitness. An unequivocal demonstration that some component of fitness is affected by the segregation of allozyme variants, specifically the selection for one or another allele at a polymorphic locus, would "contradict the fundamental premise of any neutralist theory" (Lewontin, 1974, p. 250). The lack of such a demonstration, however, does not automatically support the neoclassicists. Supporters of the balance school would point out that

experiments to measure single locus selection should be carried out using many independently derived electrophoretic alleles from nature, in order to minimize effects of linkage disequilibria. The parental oysters used in the present experiment were at least second generation hatchery products, in some cases close relatives.

Proponents of the "balance" school would no doubt include the loci investigated here with those containing "functional variants." Such loci are characterized by having all the alleles competent, and the selection coefficient associated with each isoallele very small. Furthermore, as each biochemical pathway is catalyzed by products of several polymorphic loci, at each of which the contribution may not be additive, the (statistical) fitness would depend on the number of heterozygous loci in the pathway. However, as no significant excess of heterozygotes was found in any of the matings, no support is given to overdominance or heterosis as being driving forces in the maintenance of polymorphisms.

Based on the essential precepts of the "balance" school, that each of the many alleles at any locus produces gene products with different selective values, and that heterosis is closely correlated to fitness, Williams' (1975) "elm-oyster" model makes some predictions which have been to some extent tested by surveys of oyster populations in the wild (Buroker, 1975), by selection experiments (Lannan, 1974), and in the present investigation.

Williams begins with the same intuitive assumption that led to the search for selection in the oyster hatchery system: that the large amount of mortality occurring between zygote and adult stages of high-fecundity species such as the oyster may be nonrandom and involve the culling of less-viable genotypes. His hypothesis is that the intensity of selection in a population has a positive relation to its fecundity. Thus, out of the many genotypes of the zygotes produced from the spawning of adult oysters, only a few are the "fittest" (in Williams' terminology, "sisyphean") for a particular environment at a particular time. Although Williams allows some random mortality, he suggests that fairly strong selective forces work to eliminate less fit genotypes.

His first prediction, that considerable genetically controlled variation in adaptive performance will be observed, is strongly supported by even a superficial examination of oyster populations. The great variation in size and weight between individuals of one age-class group and even among siblings, is apparent from a glance at a string of spat. That this type of morphometric variation is to an extent genetically controlled has been established by analyzing the offspring of diallel crosses (Lannan, 1974). Traits with closer relationships to fitness such as fecundity also showed such genetic variability.

The second prediction, that different genotypes have very different likelihoods of surviving given episodes of stress or of exploiting

opportunities, has been supported here to the extent that the performance of certain mating pairs (in terms of proportionate offspring survival and growth) is better than others. The underlying reasons for this remain obscure, or may not be strictly genetic; no simple mechanism seems likely. As remarked before, my own data do not support the prediction of strong selection at least at a one-locus level. In view of Williams' prediction of very large amounts of selection, the argument that a small sample size is unable to detect the selection loses some of its force.

A related prediction is that the progeny of a pair of oysters raised in different environments should show markedly different gene and genotype frequencies at some loci after the high-mortality stages are passed. An experiment to test this should be fairly easy to perform. In my experiment, sibling groups were separated and raised for eight months in two different bays; the separation was subsequent to the high mortality stages, however, and although some mortality did occur after separation it was less than 5% of the total. This may explain why comparisons of the sibling groups show no significant differences between bays (see Table 11).

Williams' model also makes predictions about wild populations. These include marked shifts in gene frequencies between local areas, even those with great gene flow; close tracking of the environment by genetic change, resulting in marked departures from Hardy-Weinberg

equilibrium for some loci; and different gene and genotype frequencies in different year classes. While it is true that no native populations of C. gigas exist on the American seaboard, there are several areas in Washington in which natural spawning may occur yearly and has occurred in some cases for several generations. In a study of oyster populations in the Puget Sound area, Buroker (1975) has surveyed six genetic loci including those investigated in my study.

At five of six areas in Puget Sound, no departure from Hardy-Weinberg equilibrium was demonstrated for any of the six loci. One locus, LAP, of a high adult-mortality area, did show deviation. No geographical cline was present for any of the six loci. No significant differences were found between two year-classes at one location.

There was a significant difference at three loci (LAP, GP and GPI) between a first generation hatchery population and the other "wild" populations, which could be explained by a founder effect; i.e., since hatchery individuals are descended from a few recent ancestors, their gene frequencies do not reflect those of the total population.

Two populations on opposite sides of Hood Canal in Puget Sound did not have different allele frequencies at the PGI and PGM loci. However, this resulted from a deficiency of heterozygotes, often an artifact of two populations mixing in one area.

In summary, evidence from selection and electrophoretic studies does not generally support Williams' hypothesis. The reason for this may not lie in any basic error of his theory or of the "balance" school's theories, but in the obscurity surrounding what actually is the unit of selection. If there are few individual loci at which selection acts in a dramatic fashion, and each locus must be considered in the context of the entire genome, particularly including those other loci with which it is linked or acts epistatically, perhaps the search for single-locus selection is doomed from the start. This would be particularly true if the forces of selection themselves varied, for example seasonally, or with the degree of inter- and intra-population competition.

Implications for the Oyster Hatchery

The fact that electrophoretic variants are inherited in a straightforward Mendelian fashion suggests that it would be easy to select for other qualitative characters. Although these have not been identified in the oyster, other molluscs have characters such as shell coloration which may be of economic importance (Chanley, 1961). Once Mendelian inheritance has been demonstrated, lines having the desired characteristics may be established by proper selection of parents.

This series of experiments does not suggest that the process of determining oyster genotypes through electrophoresis is a particularly useful one for the oyster breeder. It may initially give some idea of the amount of variability present in the local or laboratory population; discovered loss of rare alleles or increased homozygosity may be an indication of a population that has been through a bottleneck or become inbred. The extent that the loss of variability at any individual locus is a dangerous one in terms of population fitness is difficult to assess. As long as wild populations exist from which individuals may be recruited it is probably not of immediate concern.

Although a "mating compatibility" effect has been demonstrated in oysters (Longwell and Stiles, 1970), in the sense that certain combinations of parents produce offspring whose performance is better than those resulting from other parents, it seems unlikely that the genotypes of such parents will be characterized at the enzyme level. In other words, it is doubtful that an enzyme marker linked to improved larval survival in the hatchery system will be found.

The primary reason for this is that there may be many genotypes of "fitter" offspring resulting from combinations of alleles at many separate loci. Different alleles at certain loci might affect certain parameters of fitness at varying stages of the life cycle, but be undistinguishable at least during the first year of life (since samples cannot be taken until the oyster is about one year old).

Additionally, if one assumes that selection against an allele at a single locus is on the order of 1%, sample sizes to detect this level are beyond the scope of any electrophoretic investigation.

The possibility that these single-locus enzymes are, in the context of the hatchery system, selectively neutral, implies that they could be used as markers if genetically superior lines could be established. However, a careful tagging and record-keeping system would accomplish the same purpose.

Additional parameters of interest to the oyster breeder include growth rate, body weight, shell shape, and disease resistance. There is a possibility that certain loci may be associated with gene complexes found to influence these traits. Discovery of such an allele would require the observation of a tremendous number of loci.

In conclusion, it is not likely that the examination of single loci will give much insight into the mechanisms maintaining the variability in oyster populations or the well-being of oyster stocks. Fortunately, such variability may be exploited, in terms of the economically important quantitative traits, without such specific knowledge.

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APPENDIX A

SPAT STRINGS; PARENTS AND LOCATIONS

Tag number	Female parent	Male parent	Location
Y087, Y089	1	30	N
Y088	1	30	Y
Y086	1	1	Y
Y096	1	W43	N
Y095	1	W43	Y
Y098	1	G55	Y
Y097	1	G55	N
Y091	1	78	Y
Y090	1	78	N
Y093, Y092	1	82	N
Y094	1	82	Y
Y080	G47	1	Y
W98	G47	1	N
W99	G47	26	Y
G99	G47	26	N
G96	G47	30	N
W88	G47	W43	Y
W87	G47	W43	N
W86	G47	G55	Y
W85	G47	G55	N
W93, W91	G47	78	Y
W94, W92	G47	78	N
W89	G47	82	Y
W95, W90	G47	82	N

Appendix A. (Continued)

Tag number	Female parent	Male parent	Location
W84	G55	1	Y
W83	G55	26	Y
W82	G55	26	N
G49	2	1	Y
G47	2	1	N
Y085	2	26	Y
Y084, G40	2	26	N
G42	2	W43	Y
G82	2	W43	N
Y082	2	82	Y
Y083	2	82	N

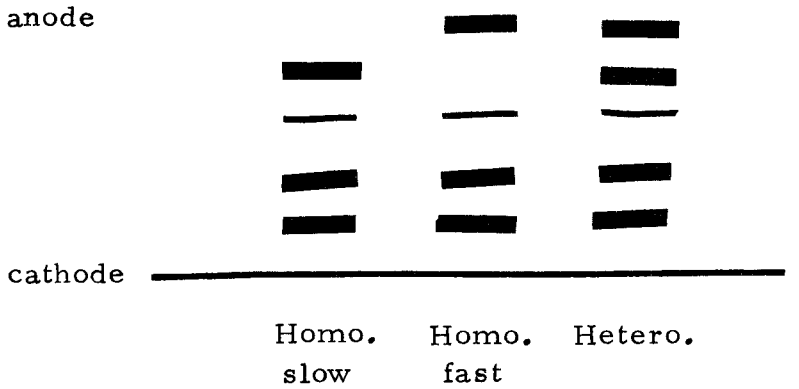
APPENDIX B

PARENTAL GENOTYPES

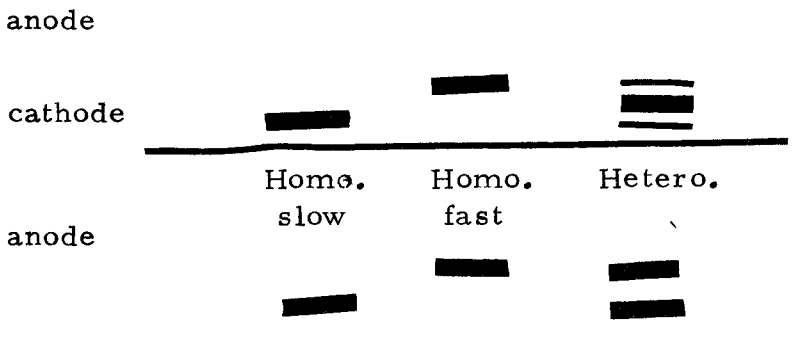
Parent	AAT	LAP	GP	GPI[a]
Female				
1	SF	SS	SF	SS
G47	SS	SF	SF	SS
G55	SS	FF	FF	SS
2	SF	SS	SS	FF
Male				
1	FF	SF	SS	SS
26	FF	SS	FF	FF
30	FF	SF	SS	FF
W43	SF	SF	SS	SS
G55	SS	FF	FF	SS
78	SF	SS	SF	SS
82	SF	SS	SS	SS

[a]GPI genotypes were not originally determined but are suggested by those found in the resulting matings.

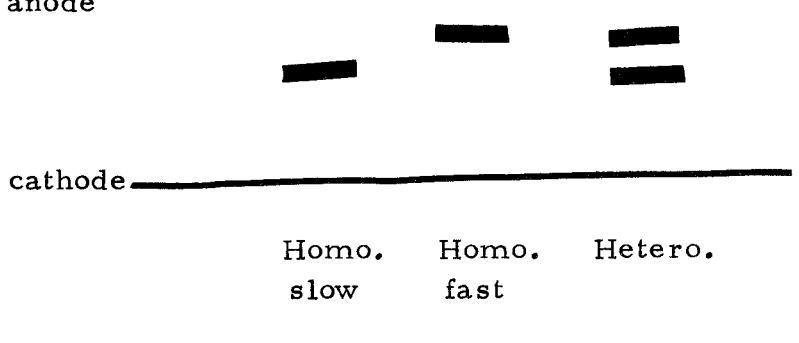
DIAGRAMS OF ELECTROPHORETIC PATTERNS



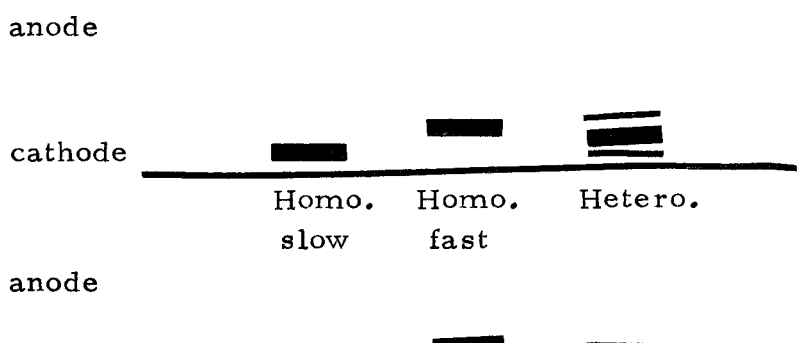
General protein



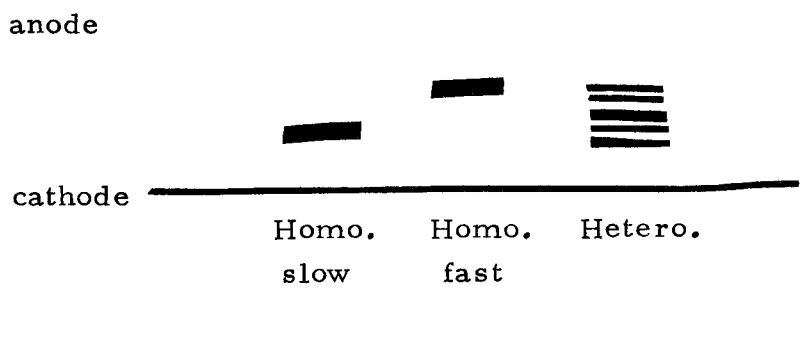
Aspartate aminostransferase



Leucine aminopeptidase



Phosphohexose isomerase



Sorbitol dehydrogenase