FREQUENCY DISTRIBUTION OF ESTERASE-5 ALLELES IN TWO POPULATIONS OF *DROSOPHILA PSEUDOOBSCURA*

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ABSTRACT

Statistical tests comparing allele frequencies in natural populations with those predicted by various theories of genic variation depend critically on the accurate enumeration of alleles. This study used a series of five sequential electrophoretic conditions to characterize the allele frequency distributions of esterase-5 in two large population samples of *Drosophila pseudoobscura* from California. In Standard chromosome lines 12 electromorphs were discriminated using a single electrophoretic condition. When four additional criteria were used, the number of electromorphs increased to 41, 33 in one population and 22 in the other. Both populations had the same two alleles in high frequency, with other alleles present in frequencies of 6% or less. Although each population had a number of unique alleles, a χ^2 contingency test demonstrated no significant genetic divergence between them. A statistical comparison of allele frequencies in both populations with that predicted by neutral models suggests that the individual and combined distributions deviate from neutrality in the direction of purifying selection.——Sex-Ratio chromosomes differed markedly from Standard chromosomes in both allelic content and diversity. In 32 Sex-Ratio chromosomes from one population only three alleles were found, all of which were detected under the initial "standard" electrophoretic conditions. Moreover, none of these alleles was found in the Standard chromosome lines.

ALLELE frequency distributions in natural populations (determined primarily by gel electrophoresis) have been used to test various theories of genic polymorphism. Comparisons of allele frequencies in different geographic populations can suggest whether selection or genetic drift is responsible for observed similarities or divergence. Empirical allele frequencies in natural populations can also be compared with those predicted by the theory of selective neutrality (KIMURA and CROW 1964). Both forms of testing can be, and indeed have been, seriously compromised by undetected heterogeneity within electrophoretic classes.

The introduction of electrophoresis into population genetics (HARRIS 1966; HUBBY and LEWONTIN 1966) was accompanied by a warning that electrophoretic alleles were a heterogeneous collection of genetic variants (LEWONTIN and HUBBY 1966). KING and OHTA (1975) proposed that the term "electromorphs" be used for electrophoretic variants to emphasize that they are phenotypes that may contain additional alleles. The use of additional experimental techniques in the past 10 years has indeed shown how serious the heterogeneity of elec-

tromorph classes can be. Heat denaturation (BERNSTEIN, THROCKMORTON and HUBBY 1973; SINGH, HUBBY and LEWONTIN 1974; SINGH, HUBBY and THROCKMORTON 1975), urea denaturation (LOUKAS, VERGINI and KRIMBAS 1981), gel sieving (JOHNSON 1977) and sequential electrophoresis (COYNE 1976; SINGH, LEWONTIN and FELTON 1976) (which repeatedly tests electromorphs under a series of electrophoretic conditions) have all demonstrated a large increase in the number of alleles detected at some enzyme loci. For example, in *Drosophila pseudoobscura* the number of observed electromorphs of esterase-5 (*Est-5*) increased from 13 at a single electrophoretic condition to 32 following four electrophoretic conditions (SINGH 1979). Other loci including octanol dehydrogenase (COYNE and FELTON 1977) and hexokinase (BECKENBACH and PRAKASH 1977) revealed little additional variation. A review of the more rigorous electrophoretic techniques and their results is provided by COYNE (1982).

Beyond this simple increase in the number of alleles at some enzyme loci, increased divergence between species was observed following the use of more rigorous techniques (BERNSTEIN, THROCKMORTON and HUBBY 1973; SINGH, HUBBY and THROCKMORTON 1975; COYNE 1976; SINGH, LEWONTIN and FELTON 1976; COYNE *et al.* 1979).

In addition, some genic similarities between populations within species proved to be illusory. Early studies using a single electrophoretic condition observed a striking uniformity of electromorph frequencies in *D. pseudoobscura* for many enzyme loci throughout the North American species range and including the isolated population of Bogotá, Colombia (PRAKASH, LEWONTIN and HUBBY 1969; LEWONTIN 1974). When these populations were reexamined using sequential electrophoretic techniques, the Bogotá population was clearly differentiated from the rest of the populations, being polymorphic at several loci for alleles unique to Bogotá (SINGH, LEWONTIN and FELTON 1976; COYNE and FELTON 1977; SINGH 1979). Except for alleles in low frequency, the North American populations remained fairly homogeneous for all loci examined except *Est-5* (SINGH 1979) for which there was some evidence that the populations may not all share the same high frequency alleles.

There is also evidence at both the xanthine dehydrogenase (*Xdh*) (SINGH, LEWONTIN and FELTON 1976) and *Est-5* (MCDOWELL and PRAKASH 1976) loci in *D. pseudoobscura* that some electromorphs found in high frequency and common to different populations in the early surveys may in fact harbor several additional alleles, none of which is in high frequency. Instead of having two high frequency alleles, the distribution became J-shaped with one allele common and the remainder rare.

The more rigorous techniques have thus resulted in qualitatively different conclusions about species and population similarities and have revealed substantially altered frequency distributions. However, is there still a large fraction of undetected variation that even the additional techniques are missing?

To determine the discriminatory power of sequential electrophoresis, RAMSHAW, COYNE and LEWONTIN (1979) screened various human hemoglobin variants with known amino acid sequences and found that sequential electrophoresis could detect a high percentage of amino acid substitutions, including

charge equivalent substitutions at the same site and identical substitutions in different parts of the molecule.

Therefore, if the hemoglobin results are general, sequential electrophoresis is an effective method of discriminating a high proportion of the extant variation. However, studies using this technique have not analyzed samples of sufficient size to obtain accurate allele frequency distributions for individual populations. All of the previous sequential surveys revealed low frequency variants that were unique to single populations. However, the sample sizes of the individual populations in these studies were small. Thus, it is impossible to determine whether the newly detected variants described in single population samples are truly unique genetic differences between populations or whether they are present in low frequencies in *all* populations.

The present study used sequential electrophoresis to determine the allele frequency distribution at the highly polymorphic *X*-linked locus *Est-5* in two large population samples of *D. pseudoobscura*. The allele frequency distributions were compared with each other and with that predicted by EWENS' (1972) sampling theory for selectively neutral alleles.

MATERIALS AND METHODS

Drosophila stocks

Isofemale lines of *D. pseudoobscura* were collected in May, 1979, from two locations: the James Reserve in the San Jacinto Mountains in southern California (elevation 1646 meters) and in a woodland near the Gundlach-Bundschu Winery in Vineburg in the Sonoma Valley of northern California (elevation 30 meters). One F_1 male from each isofemale line was crossed to several virgin females from a Delta/Bare^{Inv} balancer stock homozygous for a null esterase-5 (*Est-5*^{null}) allele.

The right arm of the *X* chromosome of *D. pseudoobscura* has two gene arrangements, Standard (ST) and Sex-Ratio (SR). The SR arrangement differs from ST by three nonoverlapping inversions (DOBZHANSKY and EPLING 1944). Males having the SR arrangement produce at least 95% female offspring. Since *Est-5* is in linkage disequilibrium with the SR inversions (PRAKASH 1974; CURTSINGER and FELDMAN 1980), its allelic variation was studied in both ST and SR chromosomes.

The original crosses that produced nonaberrant ratios of male and female offspring were maintained as shown in Figure 1. If the parental male from the original cross was still alive, several virgin female offspring were backcrossed to increase the frequency of the paternal *Est-5* allele. The remaining females were frozen and stored at −70° until used for electrophoresis. If the original F_1 male died before the backcross, some female offspring were frozen and the remainder left to sib-mate. A total of 237 lines, 116 from the James Reserve and 121 from Gundlach-Bundschu were maintained as holding stocks segregating for null and wild *Est-5* alleles. These holding stocks permitted repeated electrophoretic examination of a single active *Est-5* allele in each line without the necessity of creating isogenic lines. When a new electromorph was found, one line from each population in which it was present was made isogenic for subsequent genetic analysis.

Because there are no dominant sex-linked markers in *D. pseudoobscura*, representative isogenic lines for each electromorph were constructed as shown in the bottom half of Figure 1. In no case were mobilities of isogenic electromorphs different from those observed in frozen female flies heterozygous for a wild and null allele.

Maintenance of lines having the SR arrangement requires repeated outcrossing and single pair matings. To eliminate the additional labor of multiple single pair matings, some of which will have the SR inversion and others the ST arrangement, the recessive marker *short* (having the ST *X* chromosome arrangement) was introduced into the lines. A visual inspection of males then revealed those possessing the SR arrangement. SR lines were maintained using two alternate crosses as shown in Figure 2.

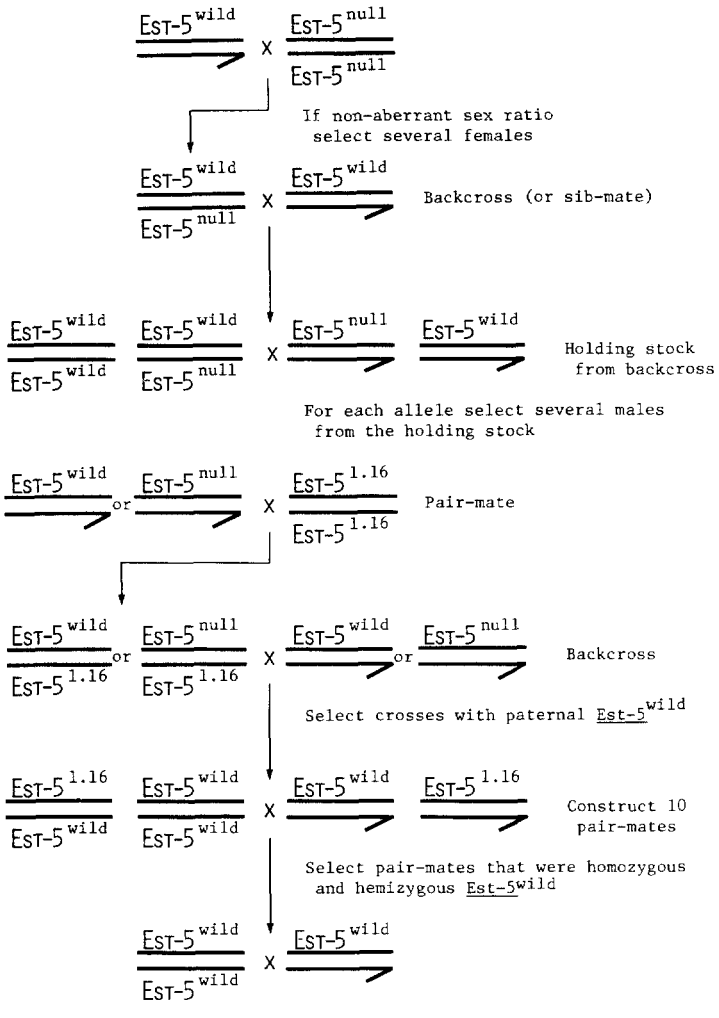

FIGURE 1.—Construction of the esterase-5 holding stocks and representative isogenic lines.

Electrophoretic techniques

Polyacrylamide slab gels were run and stained according to HUBBY and LEWONTIN (1966). The first four sequential electrophoretic criteria were similar to those of COYNE, FELTON and LEWONTIN (1978). A total of five different electrophoretic conditions were used, consisting of 5 and 8% gels and three buffers; these conditions are shown in Table 1. The ratio of bis-acrylamide to acrylamide was held constant at 5%.

Est-5 standard alleles from previous studies (PRAKASH, LEWONTIN and HUBBY 1969; COYNE, FELTON and LEWONTIN 1978) were run on all gels. Lines were originally classified relative to these standards by their mobility under electrophoretic condition 1 [5% gel, Tris-borate-EDTA (TBE), pH 8.9]. Electrophoretic mobilities were judged by visual pairwise comparisons of electromorphs. Two lines were considered to belong to the same electromorph class when they had identical mobilities in adjacent pockets on a gel or when run adjacent to another line of the same mobility.

According to this method lines are regrouped until all lines of a specific electromorph class have been tested in adjacent pockets. Although this technique necessitates running repeated samples from each line, the process of side-by-side comparison of electromorph mobilities eliminates

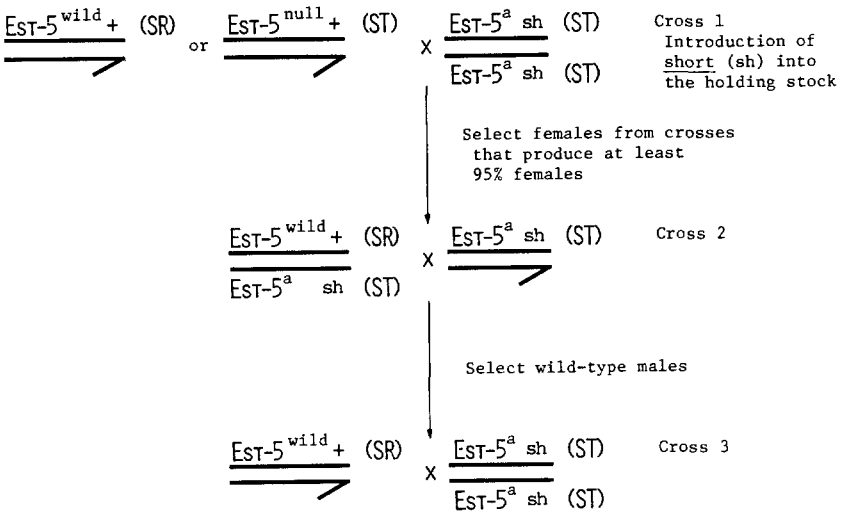

FIGURE 2.—Maintenance of the SR chromosome lines by the introduction of short (sh), with the ST chromosome arrangement, into the holding stocks.

the problem of comparing measured mobilities both within and between gels (RAMSHAW, COYNE and LEWONTIN (1979). It is essential in the accurate identification of electromorphs with small mobility differences. All lines that were classified in the same electromorph class under standard conditions were subsequently compared as a group under each additional electrophoretic condition. Lines were again regrouped until those with identical electrophoretic mobility were run in adjacent pockets. The string of mobilities for a given electromorph represents its mobility under each of the five criteria relative to the standard alleles of COYNE, FELTON and LEWONTIN (1978). The order of the mobilities corresponds to the order of the electrophoretic conditions shown in Table 1. For example, the third mobility in the string is the mobility of an electromorph in a 5% gel, pH 7.1, measured relative to a standard allele. Therefore the *Est-5*$^{0.95/1.00/0.96/0.95/1.00}$ allele migrates less than the standard *Est-5*$^{0.95/1.00/1.00/1.00/1.00}$ allele under both conditions 3 and 4 which correspond, respectively, to a 5 and 8% gel of pH 7.1.

To ensure that gels of each condition were of uniform quality, electromorph standards that were only detected using a specific criterion were run on all gels of that condition. For example, the standard *Est-5*$^{0.89/1.02/1.00/1.00/1.00}$ allele was run side-by-side with the standard *Est-5*$^{0.89/1.00/1.00/1.00/1.00}$ allele on all gels of condition 2 (8%, pH 8.9). If the mobility differences of the standards were not detected, the gels were not used.

Molecular weight determination

The gel sieving method of HEDRICK and SMITH (1968) was used to determine the molecular weights of the monomeric alleles. The following molecular weight standards were used: horse heart myoglobin (M_r 16,890), the monomer of bovine serum albumin (M_r 68,500), the *Est-5* dimer (M_r 105,000) and the trimer of bovine serum albumin (M_r 205,500). The standards and three test alleles were run in gels of 4.5, 6, 8 and 10% acrylamide. All mobilities were measured relative to the *Est-5*$^{0.98/1.24/1.07/1.15/1.00}$ allele.

RESULTS

Number of ST chromosome alleles

A sample of 237 ST *X* chromosome lines (116 from the James Reserve and 121 from Gundlach-Bundschu) were screened for *Est-5* mobility. Standard electrophoretic conditions (condition 1) revealed 12 electromorphs, including a

TABLE 1

Sequential electrophoretic conditions

Condition	Acrylamide concentration (%)	Buffer	pH	Field strength (V/cm gel)	Running time (hr)
1	5	TBE	8.9	18	3.5
2	8	TBE	8.9	18	8.5
3	5	TBE	7.1	10	7.5
4	8	TBE	7.1	10	14.0
5	5	Glycine-NaOH	10.4	12	6.0

Buffers: (1) TBE, pH 8.9 = 0.08 M Tris, 0.01 M boric acid, 0.001 M EDTA tetrasodium salt; (2) TBE, pH 7.1 = 0.08 M Tris, 0.24 M boric acid, 0.001 M EDTA tetrasodium salt; (3) glycine-NaOH, pH 10.4 = 0.05 M glycine, 0.04 N NaOH. Buffers 1 and 2 were brought to the appropriate pH using concentrated HCl. When buffer 3 was used, the gels were preincubated in 0.5 M boric acid for 30 min prior to staining.

null allele. When examined electrophoretically the null allele showed no activity at any stage of the life cycle. Nor did it form active heterodimers when females heterozygous for *Est-5*^{null} and either the *Est-5*$^{0.85/1.00/1.00/1.00/1.00}$ or the *Est-5*$^{1.16/1.00/1.00/1.00/1.00}$ allele were tested by electrophoresis. Four additional criteria increased the number of electromorphs to 41, a 3.4-fold increase over standard conditions (Table 2). These variants behave as allelic forms of the *Est-5* locus (see *Genetic analysis*).

Figure 3 shows the mobilities of three variants under two electrophoretic conditions, one in which the variants are indistinguishable and a second in which mobility differences are discriminated. The variants were deliberately chosen to demonstrate one of the smallest and also a larger mobility difference detected for the *Est-5* alleles in this study. The gel photographs demonstrate the importance of repeated side-by-side comparisons of electromorph mobilities in several replicate gels to reliably detect small mobility differences. In this study the scoring was conservative; only those variants with consistently different mobilities in replicate gels were classified as distinct. Certainly a single gel would not have been sufficient to discriminate the *Est-5*$^{0.95/1.00/1.00/1.00/1.00}$ and the *Est-5*$^{0.95/1.00/1.00/1.02/1.00}$ allele shown in Figure 3 because the mobility differences are so small. However, when repeated within and between gel comparisons consistently showed the same mobility differences which were confirmed by genetic analysis, the two were classified as separate variants.

Monomeric and dimeric forms of *Est-5* have been shown to be in dynamic equilibrium with the dimer relatively unstable under physiological conditions (HUBBY and NARISE 1967). Apparently, the dimer is most prevalent under standard electrophoretic conditions since monomer bands are rarely observed (COBBS 1976). This study showed two unusual phenotypes. The first of these, represented by three alleles (*Est-5*$^{0.98/1.22/1.07/1.13/1.00}$, *Est-5*$^{0.98/1.24/1.07/1.15/1.00}$ and *Est-5*$^{1.06/1.16/1.00/1.32/1.02}$, had very fast mobilities in 8% gels. When females heterozygous for one of these alleles and either the *Est-5*$^{0.85/1.00/1.00/1.00/1.00}$ or the *Est-5*$^{1.16/1.00/1.00/1.00/1.00}$ allele were run in 8% gels, no heterodimers were observed (Figure 4).

TABLE 2

Allele frequency distributions in ST chromosomes

Allele	No. of lines		
	James Reserve	Gundlach-Bundschu	Total
Null	1	0	1
0.85/1.00/1.00/1.00/1.00	1	2	3
0.89/1.00/1.00/1.00/1.00	1	0	1
0.89/1.02/1.00/1.00/1.02	3	0	3
0.89/1.02/1.02/1.02/1.02	2	0	2
0.95/1.00/1.00/1.00/1.00	2	6	8
0.95/1.00/1.00/1.02/1.00	1	0	1
0.95/1.00/0.96/0.95/1.00	0	1	1
0.95/1.00/0.97/0.97/1.04	0	1	1
0.98/0.98/0.98/0.98/0.98	1	0	1
0.98/1.00/1.00/1.00/1.00	0	1	1
0.98/1.00/1.07/1.07/1.04	0	1	1
0.98/1.00/1.08/1.09/1.00	1	2	3
0.98/1.02/1.08/1.09/1.03	1	0	1
0.98/1.02/1.08/1.09/1.04	2	0	2
0.98/M-D/1.07/M-D/1.00	2	0	2
0.98/1.22/1.07/1.13/1.00	0	1	1
0.98/1.24/1.07/1.15/1.00	1	2	3
1.00/1.00/0.96/0.96/0.96	0	1	1
1.00/1.00/0.98/0.98/1.00	5	5	10
1.00/1.00/1.00/1.00/1.00	40	44	84
1.00/1.00/1.01/1.00/1.00	1	6	7
1.00/1.00/1.00/1.00/1.02	1	3	4
1.04/0.98/1.01/1.01/0.97	1	0	1
1.06/0.99/0.94/0.96/1.00	1	0	1
1.06/0.99/0.96/0.98/1.00	2	0	2
1.06/0.99/1.00/0.98/1.00	1	1	2
1.06/1.00/0.98/0.98/1.00	4	3	7
1.06/1.00/1.00/1.00/1.00	24	26	50
1.06/1.16/1.00/1.32/1.02	0	2	2
1.06/M-D/0.99/M-D/1.02	2	0	2
1.07/1.00/1.00/1.00/1.00	1	0	1
1.09/1.00/1.00/1.00/1.00	3	4	7
1.09/1.00/1.02/1.02/1.02	1	0	1
1.12/0.99/1.02/1.00/0.98	1	1	2
1.12/0.99/1.02/1.00/0.99	0	1	1
1.12/0.99/1.02/1.02/0.99	1	0	1
1.12/1.00/1.00/1.00/1.00	1	0	1
1.12/1.00/1.02/1.02/1.00	5	7	12
1.16/1.00/1.00/1.00/1.00	1	0	1
1.16/1.00/1.00/1.02/1.02	1	0	1
Total	33 alleles (19 unique) 116 genes	22 alleles (8 unique) 121 genes	41 alleles 237 genes

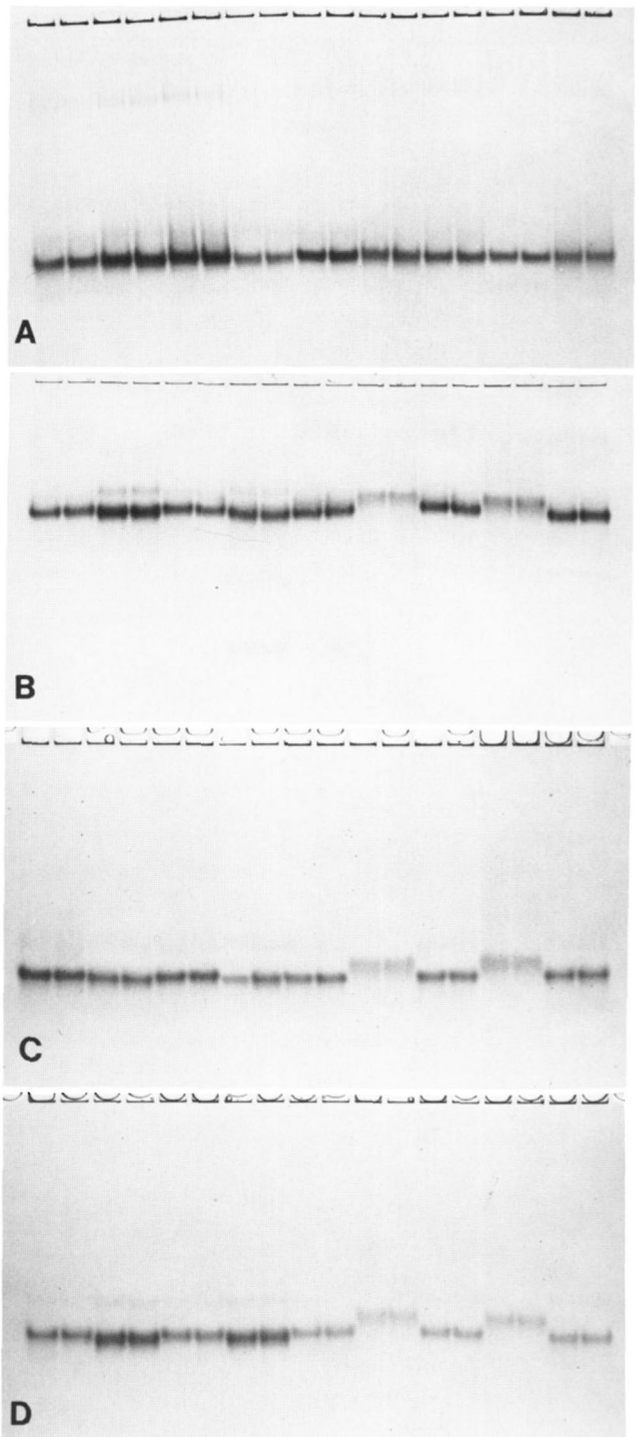

FIGURE 3.—Polyacrylamide gels showing the effect of different electrophoretic conditions on electrophoretic mobilities. Gel A, 5% gel of pH 8.9; gels B, C and D, 8% gels of pH 7.1. Pockets are numbered from left to right. Order of alleles is identical on all gels. Pockets 1, 2, 5, 6, 9, 10, 13, 14, 17 and 18 contain *Est-5*$^{0.95/1.00/1.00/1.00/1.00}$; pockets 3, 4, 7 and 8 contain *Est-5*$^{0.95/1.00/1.00/1.02/1.00}$; pockets 11, 12, 15 and 16 contain *Est-5*$^{0.95/1.00/0.96/0.95/1.00}$.

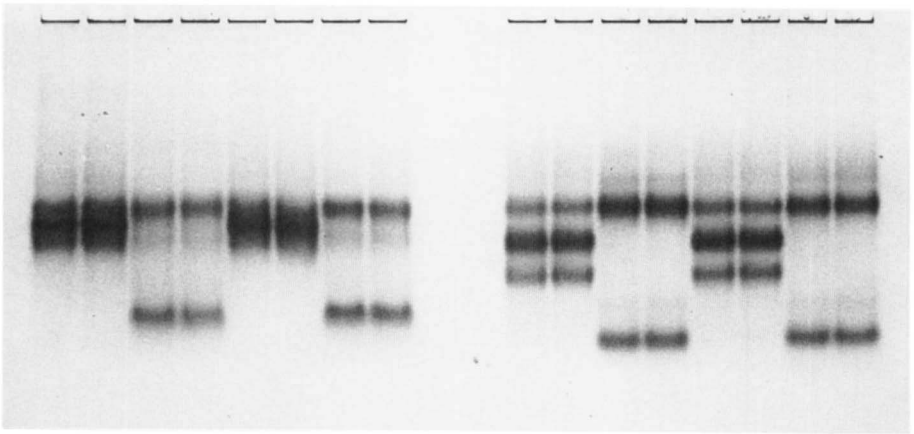

FIGURE 4.—An 8% gel of pH 7.1 showing the fast mobilities of two alleles and their inability to form heterodimers. All pockets contain heterozygous females, one fly per pocket. Pockets 1, 2, 5 and 6: *Est-5*$^{0.98/1.00/1.00/1.00/1.00}$/*Est-5*$^{0.85/1.00/1.00/1.00/1.00}$; pockets 3, 4, 7 and 8: *Est-5*$^{0.98/1.24/1.07/1.15/1.00}$/*Est-5*$^{0.85/1.00/1.00/1.00/1.00}$; pockets 11, 12, 15 and 16: *Est-5*$^{1.06/1.00/1.00/1.00/1.00}$/*Est-5*$^{0.85/1.00/1.00/1.00/1.00}$; pockets 13, 14, 17 and 18: *Est-5*$^{1.06/1.16/1.00/1.32/1.02}$/*Est-5*$^{0.85/1.00/1.00/1.00/1.00}$.

When the molecular weights of these alleles were determined by gel sieving (slope of the regression line = -0.0007 ± 0.0001, $P < 0.05$), they were all found to be approximately half the 105,000 daltons determined for the dimeric *Est-5* molecule (NARISE and HUBBY 1966). The weights are approximately 52,000 for *Est-5*$^{0.98/1.24/1.07/1.15/1.00}$, 58,000 for *Est-5*$^{1.06/1.16/1.00/1.32/1.02}$ and 61,000 daltons for *Est-5*$^{0.98/1.22/1.07/1.13/1.00}$. The differences in the calculated weights could be due to conformational differences between the monomeric forms. Other monomeric alleles have previously been found by COBBS (1976) and COYNE, FELTON and LEWONTIN (1978). Alternatively, the three variants may differ in their monomer-dimer equilibrium state. Recent studies on several *Est-5* variants by E. ARNASON and G. CHAMBERS (personal communication) have shown not only that the monomer-dimer equilibrium is sensitive to temperature, but also that the temperature at which monomer *vs.* dimer predominates depends on the particular allele tested. It is, therefore, possible that, although the monomer is predominant for these three alleles at the running temperature of the gel (4°), they may have slight differences in their monomer-dimer (M-D) equilibria which may affect the mobilities.

Two additional alleles, *Est-5*$^{0.98/M\text{-}D/1.07/M\text{-}D/1.00}$ and *Est-5*$^{1.06/M\text{-}D/0.99/M\text{-}D/1.02}$ consistently had a broad area of streaking in 8% gels. A similar two-banded phenotype with streaking between the two bands was reported by COBBS (1976) and MCDOWELL and PRAKASH (1976). The two bands have been shown to correspond to the monomeric and dimeric forms of the enzyme (COBBS 1976). In this study distinct monomer and dimer bands could not be seen on all 8% gels. However, the streaking lay between the mobility of other monomer and dimer standards. They were, therefore, designated as showing both forms of the enzyme, M-D in 8% gels (COBBS 1976). When these alleles were crossed

to the standard *Est-5*$^{0.85/1.00/1.00/1.00/1.00}$ and *Est-5*$^{1.16/1.00/1.00/1.00/1.00}$ alleles, stable heterodimers were seen in both 5 and 8% gels (Figure 5).

SR chromosomes

In addition to the ST chromosome lines, 32 SR chromosome lines, all from the James Reserve, were sequentially examined for esterase-5 mobility. The SR chromosomes differed markedly from the ST chromosomes both in allelic content and diversity (Table 3). Only three alleles were found, all of which were seen under the initial standard electrophoretic conditions. This is in striking contrast to the 33 alleles found in the 116 ST chromosome lines from the James Reserve. The *Est-5*$^{1.04/1.00/1.00/1.00/1.00}$ was the most frequent allele in SR chromosomes, present in 72% of the lines. The *Est-5*$^{1.04}$ electromorph is predominant in all populations examined under standard conditions (PRAKASH and MERRITT 1972; PRAKASH 1974). The second most frequent allele, *Est-5*$^{0.97/1.00/1.00/1.00/1.00}$ was found in 22% of the lines and has been observed in many other populations. The final allele, *Est-5*$^{1.19/1.00/1.00/1.00/1.00}$, is unique to this study and is present in only two lines.

In addition to the lack of genic diversity in SR lines, the SR and ST arrangements have no alleles in common. Under standard conditions it appeared that the *Est-5*$^{1.04/1.00/1.00/1.00/1.00}$ allele was present in both chromosome arrangements. However, the mobilities of the SR and ST *Est-5*$^{1.04}$ electromorphs were clearly different under the sequential criteria.

Comparisons between populations

Table 2 shows electromorph frequencies in the individual populations, based solely on ST chromosome lines. After all five sequential criteria, the number

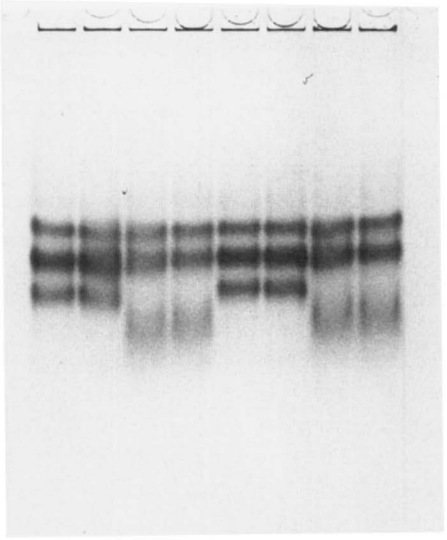

FIGURE 5.—An 8% gel of pH 7.1 showing the monomer-dimer smear of the *Est-5*$^{1.06/M\text{-}D/0.99/M\text{-}D/1.02}$ allele and its ability to form heterodimers with the *Est-5*$^{0.85/1.00/1.00/1.00/1.00}$ allele. All pockets contain heterozygous females, one fly per pocket. Pockets 1, 2, 5 and 6: *Est-5*$^{1.06/1.00/1.00/1.00/1.00}$/*Est-5*$^{0.85/1.00/1.00/1.00/1.00}$; pockets 3, 4, 7 and 8: *Est-5*$^{1.06/M\text{-}D/0.99/M\text{-}D/1.02}$/*Est-5*$^{0.85/1.00/1.00/1.00/1.00}$.

TABLE 3

Allele frequency distribution in SR chromosomes

Allele	No. of lines
SR *0.97/1.00/1.00/1.00/1.00*	7
SR *1.04/1.00/1.00/1.00/1.00*	23
SR *1.19/1.00/1.00/1.00/1.00*	2
Total	3 alleles 32 genes

of alleles detected increased in both population samples. In the James Reserve the 12 original electromorphs broke up into 33 allelic classes, whereas in Gundlach-Bundschu the original seven classes increased to 22. Although most of the new variants were present in low frequencies, the heterozygosity of each population increased somewhat from 0.736 under standard conditions to 0.829 at the James Reserve and from 0.678 to 0.808 at Gundlach-Bundschu.

NEI'S (1972) coefficient of similarity, I, was calculated from the electromorphs detected under standard conditions and after all five criteria. With the use of sequential techniques the similarity between the two populations decreased slightly from I = 0.984 to I = 0.980.

Under standard electrophoretic conditions two electromorphs were in high frequency in both populations. The most frequent electromorph, *Est-5*$^{1.00}$, was present in 41% of the lines from the James Reserve and in 49% from Gundlach-Bundschu. The second most common, *Est-5*$^{1.06}$, was found at a frequency of 29% at the James Reserve and 26% at Gundlach-Bundschu. These are the same two electromorphs that are most common in many populations throughout the species range (LEWONTIN and HUBBY 1966; PRAKASH, LEWONTIN and HUBBY 1969; LEWONTIN 1974). Although both of these common electromorphs contained a number of additional variants, the same alleles remained predominant in both populations in similar frequencies following sequential electrophoresis.

After five sequential criteria were used the *Est-5*$^{1.00}$ electromorph broke up into five allelic classes, with *Est-5*$^{1.00/1.00/1.00/1.00/1.00}$ present at a frequency of 34% (40 of 116) in the James Reserve and 36% (44 of 121) at Gundlach-Bundschu. The remaining variants within the *Est-5*$^{1.00}$ electromorph ranged in frequency from 1 to 5% in the individual populations. The *Est-5*$^{1.06}$ electromorph harbored six additional alleles. Again, one allele, *Est-5*$^{1.06/1.00/1.00/1.00/1.00}$ was predominant at a frequency of 21% in both populations. None of the other variants in this class was present in a frequency higher than 3% in either population.

The remaining variants were present within electromorphs which were less frequent under standard conditions. In fact, many of these electromorph classes contained so many different alleles that all of the variants were rare. For example, the *Est-5*$^{0.98}$ electromorph, represented by eight lines in the James Reserve and seven at Gundlach-Bundschu, split into nine allelic variants, none of which had a frequency higher than 2% in either population.

Excluding the two most frequent classes, none of the alleles in the James Reserve was present in a frequency as high as 5%. At Gundlach-Bundschu only one allele, *Est-5*$^{1.12/1.00/1.02/1.02/1.00}$ was in a frequency as great as 6%. This allele was present in 4% of the lines in the James Reserve. Some of the rare alleles were present in both populations at very similar frequencies. For example the *Est-5*$^{1.00/1.00/0.98/0.98/1.00}$ allele was present in five lines in each population. Other low frequency alleles, such as *Est-5*$^{1.00/1.00/1.01/1.00/1.00}$, were found in both populations but in different frequencies. Each population sample had a number of alleles absent from the other. The James Reserve had 19 unique alleles, all in low frequencies. Thirteen unique alleles were present in only one line, five in two lines and one in three lines. A similar situation occurred at Gundlach-Bundschu. Eight alleles were unique, with seven found in only one line and one present in two lines.

The similarity of allele frequencies in the two populations was tested with a $2 \times n$ test of homogeneity which is robust to small expected numbers (LEWONTIN and FELSENSTEIN 1965). The populations were not significantly different when the two most frequent alleles were included ($\chi^2 = 43.37$, 40 d.f., $0.1 < P < 0.5$) or excluded ($\chi^2 = 42.48$, 38 d.f., $0.1 < P < 0.5$) from the analysis.

Genetic analysis

Since modifier loci have been reported that alter the mobility of alleles at structural loci (COCHRANE and RICHMOND 1979; FINNERTY and JOHNSON 1979), it is essential to verify whether the variants detected by sequential techniques behave as alleles. One method of determining whether the electrophoretic variants are the result of the locus itself or of *cis*-acting modifier loci is to show whether they segregate in a codominant fashion (COYNE, EANES and LEWONTIN 1979). If the variants are crossed to reference alleles of divergent mobilities, the electrophoretic mobilities of the homodimers will remain the same if the variation is due to the structural locus. If the mobility of the variant is due to a recessive modifier, in the heterozygote the altered mobility will be eliminated in the variant homodimer. If the altered mobility is due to a dominant modifier, in the heterozygote the homodimer of the reference allele will show the same mobility alteration as the variant homodimer.

In this study, males from 27 isogenic electrophoretic classes were crossed to virgin females homozygous for one of two different Est-5 standard alleles (*Est-5*$^{0.85/1.00/1.00/1.00/1.00}$ and *Est-5*$^{1.16/1.00/1.00/1.00/1.00}$). The F_1 heterozygous females were then examined. Two different standard alleles were used to test the suggestion that the particular reference allele chosen for the cross may not be susceptible to modification (COCHRANE and RICHMOND 1979; JOHNSON and FINNERTY 1979). Some variants were not examined because either there were no hidden variants in the class and, hence, no possible comparison or the electromorph class did not have a representative isogenic line because of many null alleles in the holding stocks or linked sterility and viability effects.

In adjacent gel pockets of the appropriate discriminatory criterion, female heterozygotes between each sequentially detected isogenic variant and the reference allele were run with heterozygotes between the standard allele and the reference allele. A representative 8% gel of pH 7.1 containing two electro-

morphs indistinguishable in 5% gels of pH 8.9 is shown in Figure 6. The mobility of the standard allele is unchanged while the mobility difference between the *Est-5*$^{1.06/1.00/1.00/1.00/1.00}$ and the *Est-5*$^{1.06/0.99/0.94/0.96/1.00}$ is maintained. The variants behaved in a similar codominant fashion in all 54 crosses.

Efficacy of sequential criteria

The use of sequential electrophoretic criteria has clearly increased the number of alleles detected. It is also useful to know which combination of criteria reveals the most variation. For example, can one detect more variation by a change in buffer pH, which will differentially change the charge of certain amino acids, or by a change in gel concentration, which will alter the sieving properties of the system? In previous studies using sequential techniques alleles were grouped hierarchically following their initial classification. When electromorphs were found to have different mobilities under one criterion they were never compared under subsequent conditions. It is, therefore, impossible to know whether other criteria would also have discriminated the variants.

To determine the efficacy of each electrophoretic condition in detecting variants, all lines within each electrophoretic class detected under standard conditions were subsequently compared side-by-side under *each* successive criterion. The design is not fully factorial since electromorphs differentiated under standard conditions (5%, pH 8.9) were not again compared. Nevertheless, it does permit the determination of which additional criteria, in conjunction with condition 1, are most discriminatory. Figure 7 shows the contribution of successive criteria. Twelve electromorphs were detected under condition 1. If only two criteria were used it would be best to use criteria 1 and 4 since they detect a total of 30 variants, whereas conditions 1 and 2, 1 and 3 or 1 and 5 detect a total of 22, 27 and 25 variants, respectively. Condition 2 did not detect any variants not seen by a combination of the other criteria. Therefore,

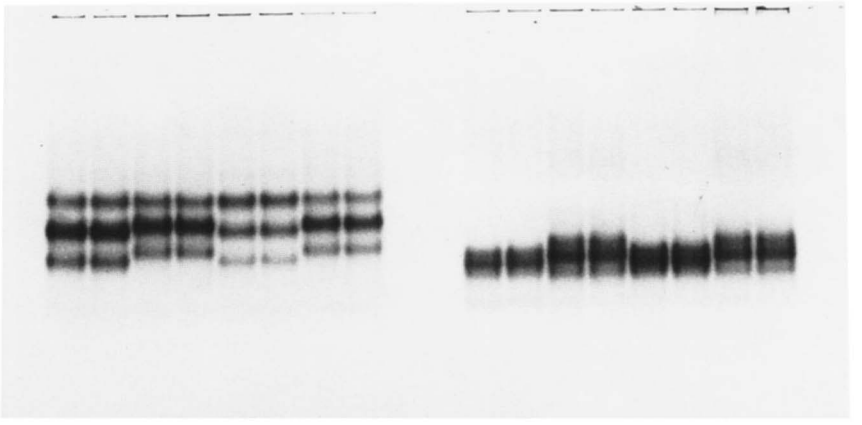

FIGURE 6.—Genetic analysis of a variant in an 8% gel of pH 7.1. All pockets contain heterozygous females, one fly per pocket. Pockets 1, 2, 5 and 6: *Est-5*$^{1.06/1.00/1.00/1.00/1.00}$/*Est-5*$^{0.85/1.00/1.00/1.00/1.00}$; pockets 3, 4, 7 and 8: *Est-5*$^{1.06/0.99/0.94/0.96/1.00}$/*Est-5*$^{0.85/1.00/1.00/1.00/1.00}$; pockets 11, 12, 15 and 16: *Est-5*$^{1.06/1.00/1.00/1.00/1.00}$/*Est-5*$^{1.16/1.00/1.00/1.00/1.00}$; pockets 13, 14, 17 and 18: *Est-5*$^{1.06/0.99/0.94/0.96/1.00}$/*Est-5*$^{1.16/1.00/1.00/1.00/1.00}$

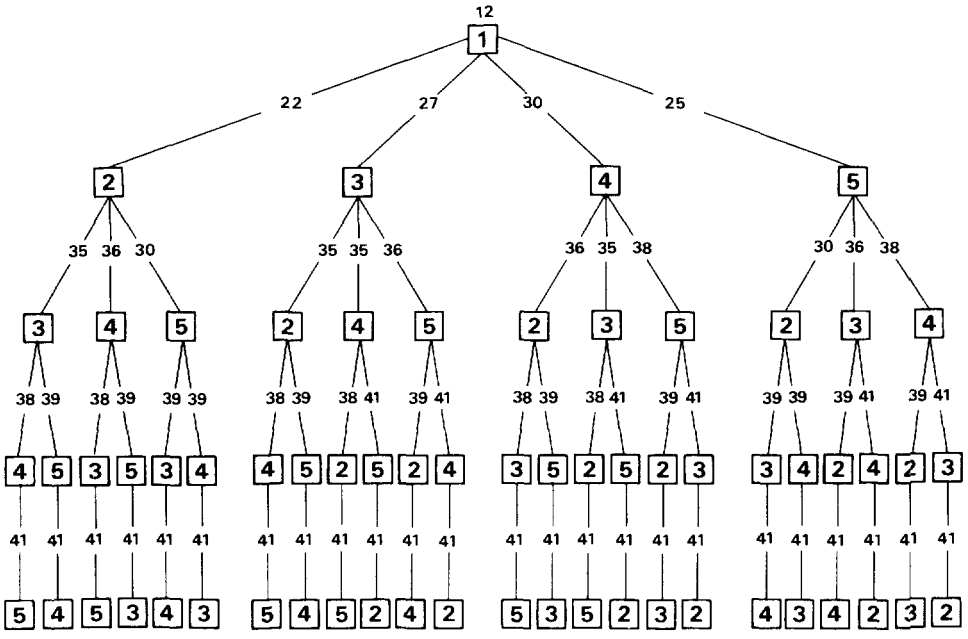

FIGURE 7.—Efficacy of sequential criteria. Numbers in boxes represent a specific electrophoretic condition and correspond to the sequential criteria in Table 1. Numbers on the lines connecting the boxes are the cumulative number of variants detected when each successive condition is applied.

the 8% gel of pH 8.9 is unnecessary for this enzyme if the other four conditions are used. Figure 7 also shows that the use of three different buffers revealed 88% of the variation. An additional five variants were only seen by increasing the gel concentration.

DISCUSSION

SR chromosomes

SR chromosomes were remarkably different from ST chromosomes in *Est-5* content and diversity. Only three SR electromorphs were found under condition 1; none harbored any additional variation. The striking lack of genetic diversity in the SR chromosomes is consistent with previous studies and is not restricted to *Est-5*. Both adult acid phosphatase-6 and octanol dehydrogenase-3 are fixed in SR chromosomes and polymorphic in ST chromosomes; two alleles at the phosphoglucomutase-1 locus are present in SR chromosomes (one being very rare), whereas in ST chromosomes both alleles are present in near-equal frequencies (PRAKASH 1974).

The lack of diversity in SR chromosomes could be the result of the initial monomorphism of the inversion and the time required to produce new alleles through recombination (NEI and LI 1980; STROBECK 1983) or mutation (NEI and LI 1975), to recurrent bottlenecks (NEI, MARUYAMA and CHAKRABORTY 1975; CHAKRABORTY and NEI 1977) or to selection.

In addition to the lack of genetic diversity in SR lines, the allelic content of *Est-5* in the two chromosome arrangements is completely nonoverlapping. Previous studies using a single electrophoretic condition have found that SR and ST chromosomes share some *Est-5* electromorphs but in very different frequencies. These observed similarities in allelic content could, however, be an artifact of analysis by a single electrophoretic condition. For example, NORMAN and PRAKASH (1980) have found that two electromorphs of amylase which were thought to be identical in several different third chromosome inversions were actually unique to different inversions following a series of electrophoretic conditions. Certainly the present study would have incorrectly classified both the ST and SR *Est-5*$^{1.04}$ electromorphs as identical if only the standard electrophoretic condition (1) was used.

Differences in allelic content between inversion and noninversion chromosomes can be explained by coadaptation of enzyme loci within the inversions (PRAKASH and LEWONTIN 1968; PRAKASH and MERRITT 1974; PRAKASH 1974), by the initial linkage disequilibrium created when the inversion was formed (NEI and LI 1975, 1980) or by the random drift of neutral alleles associated with the inversions (NEI and LI 1980; STROBECK 1983). Which of these theories accounts for the nonoverlapping allele frequencies in SR and ST chromosomes awaits some knowledge about the fitness of the *Est-5* alleles.

ST chromosomes

The number of alleles detected in ST chromosomes has increased dramatically, from the 12 seen under standard conditions to 41 after sequential electrophoresis. However, more significant than the mere increase in numbers is the fact that the two populations maintained very similar allele frequencies despite the presence of many unique alleles in low frequency. The *Est-5*$^{1.00/1.00/1.00/1.00/1.00}$ allele was found in 34% of the lines at the James Reserve and 36% at Gundlach-Bundschu, whereas the *Est-5*$^{1.06/1.00/1.00/1.00/1.00}$ allele was found in 21% of the lines in both. Therefore, although the most frequent classes under standard conditions did break up into additional classes, one allele remained in high frequency within each original electromorph. This suggests that, although the frequencies of the two common electromorphs may be somewhat reduced when more rigorous techniques are used, there may still be two very frequent alleles in all North American populations.

One purpose of the present study was to determine whether the unique alleles found in low frequency in small population samples would remain unique when larger samples were examined. If they remained restricted to individual populations they could alter the homogeneity in allele frequency previously observed in the North American populations of *D. pseudoobscura* (LEWONTIN 1974). Despite a large number of unique alleles in very low frequencies in both population samples, the two populations shared many variants in frequencies ranging from 1 to 6%. A χ^2 contingency test showed that the two populations did not differ significantly.

The lack of detectable divergence in allele frequencies between the James Reserve and Gundlach-Bundschu samples could be explained either by selec-

tion for the same alleles in both populations or by migration. Based on early estimates of gene flow using mutant orange-eyed flies (DOBZHANSKY and WRIGHT 1943, 1947), it appeared that there was little migration between populations that were more than a few kilometers distant. Later studies, which used wild-type flies marked with fluorescent dust (CRUMPACKER and WILLIAMS 1973; POWELL *et al.* 1976), found a threefold higher, but still relatively low, rate of dispersal.

JONES *et al.* (1981) and COYNE *et al.* (1982) have shown extensive dispersal over the unfavorable habitat of Death Valley, California, and conclude that populations separated by 15 km may have large amounts of migration between them. If the evidence for a high dispersal rate in *D. pseudoobscura* is confirmed, the genic similarity between the James Reserve and Gundlach-Bundschu populations separated by approximately 650 km is not inconsistent with homogenization by migration of neutral variants.

Tests of neutrality

To test statistically whether the migration of neutral variants could be responsible for the observed lack of genic differentiation in the two population samples, this study compared the empirical allele frequency distributions with those predicted by EWENS' (1972) sampling theory of selectively neutral alleles. When uniform selection coefficients for all alleles are assumed, this theory predicts allele frequencies too even if there is heterotic selection, and allele frequencies too uneven if there is purifying selection. In practice, EWENS' sampling theory is statistically not very powerful so that neutrality may be accepted even when selection is occurring (EWENS and FELDMAN 1976; EWENS 1977). In addition, rejection of the neutral theory by allele frequencies too even can be due to nonidentification of alleles, whereas allele frequencies too uneven can be due to nonstationary distributions (EWENS and GILLESPIE 1974).

With these possible ambiguities in mind the observed frequency distributions in the individual and combined populations were compared with those predicted by selective neutrality (EWENS 1972). This study used the homozygosity, $\hat{F}$, as the test statistic (WATTERSON 1977, 1978b) instead of the information measure (EWENS 1972) because the former is more efficient (WATTERSON 1977; EWENS 1979). WATTERSON (1978a) and EWENS (1979) give tables of simulated values of $\hat{F}$ for different numbers of alleles and sample sizes along with their probabilities under the neutral distribution. However, since the tables do not include as many alleles as observed in the James Reserve or combined populations, this study used the algorithm of STEWART (appendix to FUERST, CHAKRABORTY and NEI 1977) to generate 1000 neutral frequency distributions for the number of different alleles in each population sample. From these neutral distributions homozygosities were calculated and compared with those observed in the individual and combined populations (Table 4). All of the probabilities shown are for two-tailed tests. The allele frequency distributions of the individual and pooled populations are all uneven. Both the James Reserve and the combined population deviate significantly from neutrality with the Gundlach-Bundschu population almost significant ($P < 0.06$). The popu-

TABLE 4

Homozygosity test of neutrality

Population	Observed ($\hat{F}$)	Simulation		Probability (2-sided)
		Mean ($\hat{F}$)	Variance ($\hat{F}$)	
James Reserve	0.1712	0.0682	0.0002	<0.01
Gundlach-Bundschu	0.1924	0.1180	0.0011	<0.06
Total	0.1801	0.0685	0.0003	<0.01

lations, therefore, deviate from neutrality in the direction of purifying selection (deleterious alleles).

EWENS' sampling theory has shown significantly uneven allelic distributions when used to test allele frequencies from previous sequential electrophoretic studies (COYNE 1976; SINGH, LEWONTIN and FELTON 1976; COYNE and FELTON 1977; WATTERSON 1978a). This could, however, be due to pooling allele frequencies from disparate populations (SINGH, LEWONTIN and FELTON 1976; SLATKIN 1982).

In these previous studies the allele frequency distributions have been J-shaped with one common allele and the remainder in very low frequencies. In this study there are two frequent *Est-5* alleles with the remainder rare. The deviation from neutrality in the direction of deleterious alleles suggests that the low frequency variants are somewhat deleterious with the two common alleles favored or neutral. Since the two common alleles are found in near-equal frequencies in both populations, they may be maintained through balancing selection. Alternatively, sufficient migration of common neutral alleles could be responsible for the similar frequencies.

Since the *in vivo* function of *Est-5* is not known, attempts to determine whether the two common alleles are in fact maintained by balancing selection may depend on whether stable polymorphisms are established in population cage experiments. If the two common alleles are found to be maintained through balancing selection with the remaining low frequency variants deleterious, it would reconcile the problem of explaining large numbers of variants at a locus and balancing selection (LEWONTIN, GINZBURG and TULJAPURKAR 1978; ARNASON 1982).

Sequential electrophoretic studies have revealed what appear to be several classes of loci based on their allele frequency distributions. First are the nearly monomorphic loci which have harbored few, if any, additional variants. These include the octanol and malic dehydrogenase loci (COYNE and FELTON 1977) and the hexokinase locus (BECKENBACH and PRAKASH 1977) in *D. pseudoobscura* and *D. persimilis*. Second are the highly polymorphic loci with J-shaped allele frequency distributions. *Xdh* is included in this class with 27 electrophoretic alleles in *D. pseudoobscura* (SINGH, LEWONTIN and FELTON 1976) and 23 in *D. persimilis* (COYNE 1976). Another group of loci includes those with two common alleles. They may be highly polymorphic with large numbers of low frequency

variants as at *Est-5* in *D. pseudoobscura* or they may have few additional alleles as larval protein-8 in *D. pseudoobscura* (SINGH 1979) and α-glycerophosphate dehydrogenase (COYNE *et al.* 1979) and alcohol dehydrogenase (*Adh*) (KREITMAN 1980) in *D. melanogaster.*

Examination of variation in the DNA sequence of *Adh* in *D. melanogaster.* (KREITMAN 1983) has shown that selection can be discriminating enough to eliminate any deleterious amino acid substitution since (except for the amino acid substitution between Adh^{Fast} and Adh^{Slow}) only silent exon or intron sites were polymorphic.

Use of the sampling formula for selectively neutral alleles (EWENS 1972) has shown some evidence that both monomorphic and polymorphic loci with extremely J-shaped distributions deviate from neutrality in the direction of purifying selection. The results of the *Adh* analysis suggest that purifying selection can be discriminating enough to eliminate most variants from monomorphic loci. In the polymorphic J-shaped distributions, selection intensity may be somewhat reduced with a number of rare, slightly deleterious alleles segregating. The more detailed look at the *Est-5* locus has shown that the two common alleles may be maintained by balancing selection or may be selectively neutral with the low frequency variants slightly deleterious. The more thorough analysis may, therefore, contradict both the uniform overdominant theory, which suggests that all heterozygotes are more fit than homozygotes (WALLACE 1958), and the strict neutral theory, which suggests that all alleles are equivalent (KIMURA and CROW 1964). The departure from neutrality and the finding of two common alleles in both populations suggest the possibility that balancing selection or neutrality and purifying selection may be operating simultaneously on this locus. The results of this study suggest that more rigorous examination of allele frequencies on a locus-by-locus basis may support a multiplicity of evolutionary forces affecting protein polymorphisms.

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