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The genetics of *Dacus oleae*

III. Amount of variation at two esterase loci in a Greek population

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1. INTRODUCTION

In the last few years several cases of esterase loci with impressively high numbers of electrophoretically distinct forms (alleles) have been reported. Burns & Johnson (1967) reported 13 alleles for the EstE in the butterfly *Colias eurytheme*, Ohba & Sasaki (1968) found five alleles for an esterase in *D. virilis*, while in *D. pseudobscura* (Prakash, Lewontin & Hubby, 1969) twelve alleles have been reported segregating at the Est5 locus. In a previous paper (Zouros, Tsakas & Krimbas, 1968) we reported on the genetics of two unlinked antosomal esterase loci, designated A and B, in the olive fruit fly *Dacus oleae* (Gmel). It was shown that eight alleles were segregating for the A locus and seven for the B. In all of the above cases a 'silent' allele—that is, an allele which does not give any band on the zymogram—was found to be segregating.

Thus among allozyme systems showing enzymic activity the esterase loci contain the greatest number of alleles in a frequency high enough to be found in samples of relatively small size. Another characteristic, the presence of 'silent' allele(s), common to esterase loci seems to be also common for loci with phosphatase activity (Johnson, 1966; Hubby & Lewontin, 1966; Zouros *et al.*, unpublished results). These two well-established features of esterases raise the following questions: (1) What accounts for the large number of alleles present in natural populations? (2) Are the 'silent' alleles equivalent selectively to others or do they confer some deleterious effect on the individuals bearing them?

D. oleae seemed to us to offer good material to answer these questions. Two highly polymorphic esterase loci, each with a 'silent' allele, can be studied simultaneously. Because *D. oleae* is the main pest of the olive fruit in the Mediterranean, it had been the subject of extensive research work. A great deal of its ecology and ethology is well known. In particular there exists information which permits, as we will discuss later, a rough estimate of the effective population size per orchard. Also considerable work has been done on the dispersion of this fly. This information facilitates population studies of the insect to a certain extent. We will show in this paper that the polymorphism at both esterase loci within a single population is much higher than previously described and will propose high mutation rate as the most reasonable explanation for the maintenance of these polymorphisms. We will

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also present data suggesting that silent alleles do not show any deleterious effect under natural conditions.

2. MATERIAL AND METHODS

Live flies were collected from an olive-tree orchard 40 km from Athens. This orchard, 2·5 km long and 0·5 km in width, was selected because it was separated from other olive-tree orchards and was never subjected to sprays by organophosphate insecticides. This was of importance since organophosphate compounds may effect esterase electrophoretic patterns of *D. oleae*, as they do in *Musca domestica* (Van Asperen & Mazijk, 1965).

Flies were trapped in special glass traps which contained a plastic sponge embedded in a lure solution of the following composition: 100 cc water + 5 cc hydrolysed protein (Staley no. 7) + 4 g ammonium sulphate (pH = 4·4).

The captured flies were electrophorized promptly as has been described in the previous paper (Zouros *et al.* 1968). In each gel a fly from a strain segregating only for the A_2, B_3 and B_4 alleles was inserted as a mobility standard.

3. RESULTS

About 500 flies were examined: 456 for both loci, plus 18 for the A locus and 13 for the B locus only (in some individuals we could not read clearly either system A or B). Thirteen active alleles plus one 'silent' for the A locus and 11 active alleles plus one 'silent' for the B locus were found in this sample. To be sure that each of these bands represents an allele of the loci mentioned, we attempted to get as much information as possible on the segregation of these genes. We did not attempt, however, a genetic analysis of all the alleles by deriving and crossing homozygous lines, or analysing a very large number of pair matings, in order to obtain segregation data for the most rare alleles. This would be very difficult since *D. oleae* larvae do not easily develop on any artificial media except fresh olive fruits, which besides being expensive are not available throughout the year. Instead we used females inseminated in nature and their progeny produced in the laboratory. The number of matings of these 'wild' females was unknown.

The data presented in Table 1 are derived from such 'half families', in which the segregation pattern at both loci suggested that only one fertile mating had taken place. For each of these 'half families' a χ^2 test was performed to measure the deviation from Mendelian expectation. In no case was the value of χ^2 significant at the 5% level. All chi-squares were consolidated and are summed values for each locus given in Table 1.

In this way we were able to collect segregation data for 12 alleles of the A locus and for nine alleles for the B locus. For three alleles of the A locus, namely A_{00}, A_0 and A_{01} no segregation data are available, but the staining and the appearance of their bands were the same as for the other active alleles of the A locus studied. For the locus B we have no segregation data for the alleles B_{45}, B_{566} and B_8, but

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their heterozygotes form hybrid enzymes with the well-known alleles of the B locus. The A_9 allele, which was segregating in one of these 'half families' was not found in the population sample. We conclude that at least 15 alleles for the A locus and 12 for the B locus were present in the population. Figure 1 in the previous paper (Zouros *et al.* 1968) gives the symbols and the respective positions of all alleles.

Tables 2 and 3 indicate the number of individuals belonging to different pheno-

Table 1. *Data on the segregation of the A and B loci*

Locus	No. of 'half families' studied	Total no. of progeny analysed	No. of alleles tested	Consolidated χ^2	D.F.	P
A	28	461	12	38·00	33	0·20–0·30
B	46	721	9	15·60	25	0·90–0·95

Table 2. *The numbers of individuals with different biochemical phenotypes at the A locus in the population sample*

Phenotype	No.	Phenotype	No.	Phenotype	No.
$A_{00}-$	1	$A_{12}-$	12	A_3-	5
A_0A_1	5	$A_{12}A_2$	17	A_3A_4	1
A_0A_{12}	1	$A_{12}A_3$	2	A_3A_5	3
A_0A_2	4	$A_{12}A_5$	1	A_3A_7	5
A_0A_7	2	$A_{12}A_7$	4	A_3A_8	1
$A_{01}A_2$	1	$A_{12}A_8$	1	A_4-	2
A_1-	21	A_2-	139	A_4A_7	4
A_1A_{12}	7	A_2A_{23}	2	A_5-	2
A_1A_2	61	A_2A_3	30	A_5A_6	1
A_1A_{23}	2	A_2A_4	11	A_5A_7	4
A_1A_3	4	A_2A_5	10	A_6A_7	2
A_1A_4	6	A_2A_6	3	A_7-	7
A_1A_5	2	A_2A_7	34	A_7A_8	9
A_1A_6	2	A_2A_8	11	A_8-	2
A_1A_7	8	$A_{23}-$	1	A_8A_8	13
A_1A_8	5	$A_{23}A_8$	3	Total	474

Table 3. *The numbers of individuals with different biochemical phenotypes at the B locus in the population sample*

Phenotype	No.	Phenotype	No.	Phenotype	No.
B_1B_4	1	B_3B_6	12	$B_{45}B_6$	3
B_2-	2	B_3B_7	2	$B_{45}B_7$	3
B_2B_4	22	B_4-	203	B_5-	2
B_2B_{45}	2	B_4B_{45}	20	B_5B_6	4
B_2B_5	2	B_4B_5	22	B_5B_7	3
B_2B_{56}	2	B_4B_{56}	10	B_6-	8
B_2B_{566}	1	B_4B_{566}	3	B_6B_7	1
B_2B_6	7	B_4B_6	42	B_7-	9
B_2B_7	6	B_4B_7	65	B_7B_8	1
B_3-	4	B_4B_8	2	B_8B_8	2
B_3B_4	11	$B_{45}B_5$	2	Total	469

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types found in our sample for A and B locus. Forty-seven different phenotypes were detected for the A locus, 32 for the B locus and 172 for both loci.

A problem arose in the estimation of allele frequencies due to the presence of silent alleles. Gene frequency estimation cannot be based on a simple counting of genes since the genotypes A_iA_i and A_iA_s are not distinguishable (where A_i is any active and A_s is the 'silent' allele). The same problem arises for the B locus. This difficulty is directly connected with our major problem on the behaviour of 'silent' alleles in natural populations. Since heterozygotes for A_s and B_s are not distinguishable the only way to detect any deleterious effect of the 'silent' alleles is to compare the number of homozygotes A_sA_s and B_sB_s observed with that expected in a random mating population, where no selective forces are operating. For this purpose our estimation must not be based at all on the A_sA_s or B_sB_s classes. For the same reason we prefer to underestimate the frequency of the 'silent' alleles rather than to overestimate it, since any overestimate would lead to the conclusion that in nature A_sA_s and B_sB_s individuals are in lower than expected frequency.

These requirements are met by using the simple algebraic formula

$$(A_i-)+\sum_j (A_iA_j) = p_i^2+2p_i(1-p_i),$$

which is derived by assuming panmixia and makes use of the (A_i-) and (A_iA_j) classes (A_i and A_j are active alleles), which together constitute 97·3 % of the whole sample for the A locus and 99·5 % for the B locus. The calculated frequencies are indicated in Table 4. Alleles marked with an asterisk are calculated by a direct count. The frequency of A_s and B_s was calculated by subtracting the sum of the frequencies of all active alleles from unity.

Another estimation of the allelic frequencies was based upon maximum likelihood. As the algebraic computations were quite complicated we used a digital computer. The solution given for the A locus was very close to that calculated by the previous method. As far as the frequency of A_s is concerned the values are very close in both cases: 0·119 by the algebraic method, 0·123 by the computer.

The allelic frequencies at both loci show a similar pattern of distribution. One allele (A_2 or B_4) has a frequency close to 50 %, four alleles at the A locus and two at the B have frequencies betwen 5 % and 15 %, five alleles at A and six at B have frequencies between 1 % and 5 %, and finally four alleles at A and three at B have frequencies less than 1 %.

According to the calculated frequencies of A_s, the expected number of A_sA_s individuals in the sample is seven. We observed 13 such individuals, that is almost twice as many as expected. This is contrary to that expected if homozygotes for the 'silent' alleles had a lower fitness than homozygotes or heterozygotes for the active alleles. It is possible that a small number of individuals which did not show any band of the A system were not genotypically A_sA_s, since it has been observed that sometimes individuals bearing one active allele do not show a discernible band on the zymogram. The conclusion, however, remains that the number of observed A_sA_s flies were by no means lower than expected.

The calculated allelic frequencies were used in order to test the hypothesis that

both loci were independently in Hardy–Weinberg equilibrium. In these tests the sum of the expected numbers of genotypes A_iA_i and A_iA_s was compared with the observed number of the phenotype (A_i-). Classes with expected number lower than 5 were pooled together into one class. For the A locus $\chi^2 = 18{\cdot}38$ for 8 D.F. so that $0{\cdot}01 < P < 0{\cdot}02$. The deviation from Hardy–Weinberg equilibrium is mainly due to the fact that A_sA_s are more frequent than expected and contribute 5·14 units to the χ^2. Locus B does not depart from Hardy–Weinberg equilibrium: $\chi^2 = 4{\cdot}45$ for 2 D.F. so that $0{\cdot}05 < P < 0{\cdot}10$.

Table 4. *Gene frequencies in the sample of the natural population*

A locus		B locus	
$*A_{00}$	0·001	$*B_1$	0·001
$*A_0$	0·013	B_2	0·048
$*A_{01}$	0·001	B_3	0·020
A_1	0·139	B_4	0·622
A_{12}	0·049	$*B_{45}$	0·032
A_2	0·436	B_5	0·037
A_{23}	0·008	$*B_{56}$	0·013
A_3	0·055	$*B_{566}$	0·004
A_4	0·025	B_6	0·076
A_5	0·025	B_7	0·100
$*A_6$	0·008	$*B_8$	0·003
A_7	0·087	B_8	0·044
A_8	0·034	Total	1·000
A_9	—		
A_s	0·119		
Total	1·000		

4. DISCUSSION

The number of alleles of the two esterase loci reported here are certainly underestimated. In a sample of nearby 1000 genes two alleles (A_{00} and A_{01}) were found just once, while another allele (A_9) was not found at all. It might well be expected that a larger sample would reveal the presence of additional rather rare alleles. It must also be pointed out that our direct analysis of the sample gives a representative picture of the population at the time of sampling. This is essentially the only way to study multiallelic systems in natural populations, since any attempt to culture the sample would result in loss of alleles with low frequency and in subsequent alteration of the frequency of the remaining ones.

The problem of understanding the maintenance of abundant genic heterozygosity in natural populations is illustrated by numerous works on a variety of loci (Lewontin & Hubby, 1966; Harris, 1966; Johnson *et al.* 1966; O'Brien & McIntyre, 1969; Selander & Yang, 1969). But even at a single locus the evolution and maintenance in a single population of such a large number of alleles, many of them in a very low frequency, is not easily explained.

By a series of experiments conducted by the Greek Ministry of Agriculture (unpublished) an attempt was made to estimate the size of *D. oleae* populations in olive-tree orchards at the time of their minimum density. It was found that the

number of flies per olive tree at that time is between one and ten, varying greatly, as expected, among orchards. These observations can provide a basis for a rough estimation of the effective size (N_e) of our population. *D. oleae* populations undergo great seasonal changes (Orphanidis & Soultanopoulos, 1962), but it is well known that N_e stands very close to the minimum value of the population size. The orchard where the flies were trapped contains approximately 2000 trees. The effective size then of the population can be estimated as between 2×10^3 and 2×10^4.

Another parameter we can calculate from our data is the effective number (n_e) of alleles at each locus. By applying the formula

$$n_e = \frac{1}{\sum_i p_i^2},$$

where p_i stands for the frequency of any allele, we found 4·2 for the effective number of alleles for the A locus and 2·4 for the B locus.

Kimura (1956) and Mandel (1959) discussed the conditions under which a multiallelic heterotic system can be maintained in stable equilibrium. Using the models provided by Wright (1966) one can arrive at the observed effective number of alleles making use of the mentioned values of N_e and giving reasonable values to mutation rate (u) and to selection coefficient (t). This selection coefficient represents in this case an averaged value over all the different homozygotes. It seems, however, unlikely that so many alleles, the majority of which have a frequency less than 5%, could be maintained by the effect of overdominance alone. Robertson (1962) has shown that in a finite population heterosis cannot easily account for the maintenance of alleles with an equilibrium frequency outside the range 0·2–0·8. In the case of overdominance the effect of random drift in eliminating alleles of low frequencies is more efficient than in the case of neutrality. Robertson's analysis was restricted to two alleles but one can expect the results to be more pronounced for more alleles (Kimura & Crow, 1964), since the effect of random drift increases with the number of alleles. Under such conditions one can hardly expect to find heterotic alleles in a population with an equilibrium frequency lower than 0·05, unless one assumes relatively large values for t.

The alternative hypothesis of selectively neutral or very nearly neutral isoalleles seems to provide a better explanation of the observed polymorphism at these two loci. In testing the Hardy–Weinberg hypothesis we did not find any excess of heterozygotes at either of the two loci. The hypothesis of neutrality can moreover perfectly account for the pattern of the allelic frequency distribution observed. In this case one expects one allele to be in high frequency and a few in intermediate frequencies, while many of them will be in low frequencies (Kimura, 1968). This is the pattern we found for both esterase loci of *D. oleae*, as well as Ohba & Sasaki (1968) for the Est2 of *D. virilis*. Additional observations exist suggesting that other esterase systems also are selectively neutral. This is the case for the Est6 locus of *D. melanogaster* and *D. simulans* (McIntyre & Wright, 1966), although Kojima & Yarbrough (1967) and Yarbrough & Kojima (1967) proposed frequency dependent selection for the same locus, with selective neutrality at equilibrium.

There exist some observations, however, which do not support the hypothesis of neutrality of esterase alleles in *D. oleae*. In the case of neutrality one expects to find the same allele in different frequencies, if looked at in different populations, merely due to random drift. In studying the genetics of different alleles (see crosses in Zouros *et al.* 1968) individuals coming from different parts of Greece were analysed. In almost all cases the A_2 and the B_4 alleles were the most frequent. We cannot, however, consider this fact as disproof of the hypothesis of neutral or nearly neutral isoalleles, since it might be a result of a small amount of migration. In fact, thinking in terms of long periods of time, not a great number of migrating individuals is required for such a result. There exists some information on the dispersion of this insect. Pelecassis (1962) and Orphanidis, Soultanopoulos & Karandinou (1962), using radioactive phosphorus as a label, found that *D. oleae* flies did not travel farther than a distance of 4·5 km during the period of their observations. The orchard from which our sample was taken is separated from other orchards by a distance greater than this. However, we must not forget that *D. oleae* is commensal with man, who possibly accounts for the dispersion of the insect (by olive-fruit transportation, etc.) more than the fly itself.

In the absence of a large amount of migration and strong heterotic effect a relatively high mutation rate is required, in order to balance the effect of random drift in reducing the number of alleles. This effect of random drift increases greatly as the number of alleles increases, being roughly proportional to the square of the number of alleles (Kimura, 1955). Kimura & Crow (1964) pointed out that in neutral isoallelic systems, if the quantity $4N_e u$ is greater than unity more than half of the individuals in the population will be heterozygous. This is clearly the case for both of the esterase loci we studied in *D. oleae*. Using our conservative frequency estimate of A_8 we calculated that 359 out of 474 analysed flies, that is three-quarters of the sample, were heterozygous at the A locus. Even if all $(A_i -)$ flies were homozygous, which certainly is not the case, the number of heterozygotes (A_iA_j) would still be significantly greater than half of the sample (269 in 474). For the B locus we calculated that 277 out of 469 individuals were heterozygous, again significantly greater than one-half. Using the upper estimate of the effective population size—that is, 2×10^4—we conclude that the mutation rate has to be greater than $1{\cdot}25 \times 10^{-5}$ for both loci. By applying the formula $n_e = 4N_e u + 1$ and using this upper estimate of N_e we get $u = 4 \times 10^{-5}$ for locus A and $u = 1{\cdot}75 \times 10^{-5}$ for locus B. These values of u represent our *most conservative* estimates of the mutation rate at these esterase loci. With an effective population size of 2×10^3, which does not seem unreasonable, these values become ten times higher.

It seems therefore that for both EstA and EstB of *D. oleae* the mutation rate should be relatively high compared to the mutation rates for visible mutants, where it is considered to be about 10^{-6}. Whether it is true for other allozyme systems or not it is difficult to say, but one tends to believe that it could hold for the majority of esterase loci, at least as far as insects are concerned. It is possible that the molecular structure of esterases permits many amino acid changes without destroying the enzymic activity and without affecting the fitness of individuals

bearing them. For enzymes which are polymeric, as is EstB in our case, there might be less chance for non-deleterious mutations, since some changes could affect the ability of the subunits to combine into active polymeres. An alternative hypothesis would be that the configuration of the molecule of these esterases is such that single amino acid substitutions result in a change of the electrophoretic mobility more often than happens with other proteins. In both cases we are dealing with 'incomplete synonymous mutations' (Kimura, 1968), which happen to be electrophoretically distinct. Kimura emphasized the high frequency expected for such mutations in nature.

In EstE of *Colias eurytheme* a 'silent' allele is the most frequent one, but at most one homozygote for this allele was found in nature (Burns & Johnson, 1967). On the other hand every individual of *D. virilis* always turned out to be homozygous for the 'silent' allele for five out of its six esterase loci with the same substrate specificity. A strain homozygous for the 'silent' allele at all loci was obtained in the laboratory, however (Ohba & Sasaki, 1968). We have also obtained A_sA_s individuals of *D. oleae* in the laboratory. But A_sA_s and B_sB_s homozygotes were also found in the natural population studied. Their number was by no means lower than expected on the basis of the estimate of the A_s and B_s allele frequencies, so we can conclude that under natural conditions these 'silent' alleles do not have any detrimental effect on the individuals bearing them.

It is possible that in reality we are dealing more with a class of silent alleles rather than with a single allele. In most species studied a number of bands with esterase activity are present on the zymogram. It would seem possible that homozygotes for the silent alleles at one or more loci can survive because the individual carries several other genes having the same enzymic activity. From this point of view we would like to know if $A_sA_sB_sB_s$ individuals can survive. This genotype is, however, too rare to be found in a sample of 500 flies. It is also very difficult to obtain it in the laboratory since *D. oleae* is not a good species for pair mating cultures on artificial media.

SUMMARY

Two polymorphic esterase loci, EstA and EstB, of the olive-fruit fly *Dacus oleae* were studied in a natural population. The analysis of about 500 individuals revealed the presence of 15 alleles for EstA and 12 alleles for EstB. A 'silent' allele was found segregating at both loci. Segregation data for most of the alleles are presented. The allele frequency distribution follows the same pattern at both loci: one allele of each gene has a frequency of nearly 0·50, a few have frequencies between 0·05 and 0·15 and many are below 0·05. Two main hypotheses, those of overdominance and selective neutrality, were examined in order to explain these polymorphisms. We deduced that under both hypotheses a relatively high mutation rate is necessary to balance the result of random drift. This rate was estimated to be higher than 4×10^{-5} for the EstA locus. Since homozygotes for the 'silent' allele at the first or at the second locus were found in the population in expected frequencies, it was concluded that these alleles are not inferior to active ones under natural conditions.

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