Heredity **59** (1987) 259–264

Received 18 November 1986

High resolution electrophoretic variation at the esterase-6 locus in a natural population of *Drosophila melanogaster*

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One hundred and fifty-seven lines isoallelic for esterase-6 were extracted from a natural population of *D. melanogaster*. The relative electrophoretic mobilities of *Est6* allozymes in the different lines were determined by repeated pair-wise comparisons on high resolution cellulose acetate plates. Ten allozymic classes were resolved among the lines where only four, *Est6-VF*, *Est6-F'*, *Est6-F* and *Est6-S*, had been evident using standard electrophoretic procedures. The additional variation resulted from the subdivision of *Est6-F* and *Est6-S* into three and five classes respectively. Genetic analyses confirmed that the additional classes mapped at or near the *Est6* locus. Two of the additional classes occurring at low population frequencies were associated with significantly higher third chromosome viabilities. The most frequent class within *Est6-F* was in significant gametic disequilibrium with the linked polymorphic inversion *In(3L)P*. The same high resolution procedures were applied to 13 lines of *D.melanogaster* representing six *Est6* thermostability variants. Different thermostability variants did not group consistently according to electrophoretic classes. This implies that the two criteria are detecting protein variation independently from each other and that much greater variation occurs than is detected by either criterion alone.

INTRODUCTION

Knowledge of the extent of amino acid polymorphism is critical to an understanding of the evolutionary processes operating on proteins (Lewontin) 1974; Kimura, 1983). The first applications of electrophoretic procedures to this issue revealed unexpectedly high levels of polymorphism and the recent use of more sensitive electrophoretic procedures has revealed even higher levels (Nevo, 1978; Coyne, 1982, for review). For example, the xanthine dehydrogenase and esterase-5 polymorphisms of *Drosophila pseudoobscura* have now been resolved into 21 and 41 electrophoretic classes respectively (Keith, 1983; Keith *et al.*, 1985). It is now clearly important to test the generality of such high levels of polymorphism and to assess their effects on fitness.

The esterase-6 (E.C. 3.1.1.1) gene-enzyme system of *D. melanogaster* is well suited to such purposes, because much is known of its physiological function and standard electrophoretic procedures have already revealed polymorphic *Est6* allozymes whose geographic distributions strongly suggest the action of natural selection (c.f. *Est5* above, Yamazaki *et al.*, 1983). *Est6* is produced in the anterior ejaculatory duct of adult males, from where it is transferred to the female during copulation (Sheehan *et al.*, 1979; Richmond and Senior 1981). The presence of transferred *Est6* in the females stimulates sperm utilisation and oviposition and affects her latency to remating (Gilbert, 1981; Scott, 1986). *D. melanogaster* and its sibling species *D. simulans* are both polymorphic for the same two major *Est6* allozymes (Cabrera *et al.*, 1982 and references therein), and the action of natural selection between these allozymes is suggested by parallel latitudinal clines in gene frequencies in both species and on all three continents for which data have been collected (Oakeshott *et al.*, 1981; Anderson and Oakeshott, 1984).

Here, we report the application of more sensitive electrophoretic procedures to survey *Est6* variation in an Australian population of *D. melanogaster*. We have used a technique based on cellulose acetate electrophoresis, the resolving power of which we first calibrated against 25 *Est5* variants in *D. pseudoobscura* distinguised by sequential polyacrylamide gel electrophoresis by

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Keith (1983). Progeny from test crosses were analysed in order to check that the additional *Est6* variants detected mapped to the *Est6* locus. *Est6* variants were also tested for gametic disequilibrium with third chromosome viability modifiers and inversion polymorphism. Finally, lines from another *D. melanogaster* population representing six *Est6* alleles differentiated by thermostability criteria (Cochrane, 1976; Cochrane and Richmond, 1979) were analysed with the cellulose acetate system to determine whether the two criteria resolve the same variation.

MATERIALS AND METHODS

One hundred and fifty-seven individual third chromosomes were isolated from a population at Coffs Harbour, N.S.W., using a routine backcrossing program to a standard balancer third chromosome, TM3 (Lindsley and Grell, 1968). Forty-four wild chromosomes were sufficiently viable that they could be isolated and maintained as homozygous lines. One hundred and thirteen chromosomes which were lethal or semi-lethal as homozygotes were crossed to a stock heterozygous for TM3 and a *Df(3)vin*^{7} chromosome deleted for the *Est6* region (cytological locations of the *Df(3)vin*^{7} deletion breakpoints are 68C8-11 to 69B4-5, while *Est6* is at 69A1; Akam *et al.*, 1978). Progeny heterozygous for the wild and *Df(3)vin*^{7} third chromosomes were then used as parents to produce lines hemizygous for the wild type *Est6* alleles. This rescue of the lethal and deleterious wild chromosomes meant that the population was sampled randomly rather than biased in favour of homozygous viable third chromosomes.

The resolving power of cellulose acetate plate electrophoresis was calibrated against that of the sequential polyacrylamide gel electrophoresis (4 *p*H's and 2 gel concentrations) which Keith (1983) used to distinguish mobility variants at the *Est5* locus of *D. pseudoobscura*. Lines representing 25 randomly chosen *Est5* variants were tested on our cellulose acetate system in a blind trial using four buffers differing in *p*H (*p*H 6·2, 7·0, 8·5 and 10·4). A combination of any two of these buffer systems could detect all 25 electrophoretic classes, while 23 could be resolved using the *p*H 8·5 buffer alone. Only the *p*H 8·5 buffer was used in the analysis of the *Est6* system of *D. melanogaster* as other buffers did not provide such sensitive resolution.

Cellulose acetate plate electrophoresis was carried out on adult male flies using weight-standardised mass homogenates to minimise variation in staining intensity. Approximately 70 mg of males were weighed out from each line. Six μl of running buffer (80 mM TRIS-boric acid, 1·3 mM EDTA, *p*H 8·5) per mg fly were added and the flies thoroughly homogenised and centrifuged for 5 min. The supernatant was then stored at −20°C. Single fly extracts for the genetic analyses were prepared in a similar way using 10 μl of running buffer per fly.

Electrophoresis was carried out on Titan III cellulose acetate plates (#3024, Helena Laboratories, Beaumont, Texas). Sample homogenates were centrifuged a second time and the supernatants loaded onto the plates, which had been previously immersed in running buffer for 20 min. The samples were electrophoresed for 110 min at 250 V (6 mA/plate) at 4°C and stained with an agar overlay consisting of 5 ml 0·1 M phosphate buffer *p*H 6·0, 300 μl 1 per cent β-naphthyl-acetate (Sigma) in acetone, 7 mg fast garnet GBC salt (Sigma) and 5 ml 3 per cent agar. Plates were destained in 15 percent ethanol and 5 percent glacial acetic acid.

An initial unreplicated screen of all lines was used to construct a tentative order of electrophoretic mobility classes among lines. Each line was then tested repeatedly in pair-wise comparisons against all lines of the same or adjacent putative mobility classes until a consistent classification was obtained. The pair-wise comparison of lines was carried out by running only two lines on each plate, alternating the two lines several times across the plate (fig. 1(a)). Three generations of flies were analysed to ensure consistency of classification across homogenates and generations.

RESULTS

Ten electrophoretic classes of *Est6* were found among the 157 isoallelic lines (fig. 1(b)). This variation could be divided into four major mobility groups between which there were relatively large mobility differences. Three of the major groups are likely to correspond to the *Est6-VF*, *Est6-F*, and *Est6-S* variants of Rodino and Danieli (1972), Costa *et al.* (1982) and Lindsley and Zimm (1985), while the fourth major group, *Est6-F′* lay between *Est6-VF* and *Est6-F*. The *Est6-VF* and *Est6-F′* groups contained only one class each (classes 1 and 2), the anodal mobilities of which were measured as 1·18 and 1·12 respectively, relative to the most common class (8) within the *Est6-S* group, the mobility of which was defined as 1·00 (table 1). Classes 3, 4 and 5, within the *Est6-F*

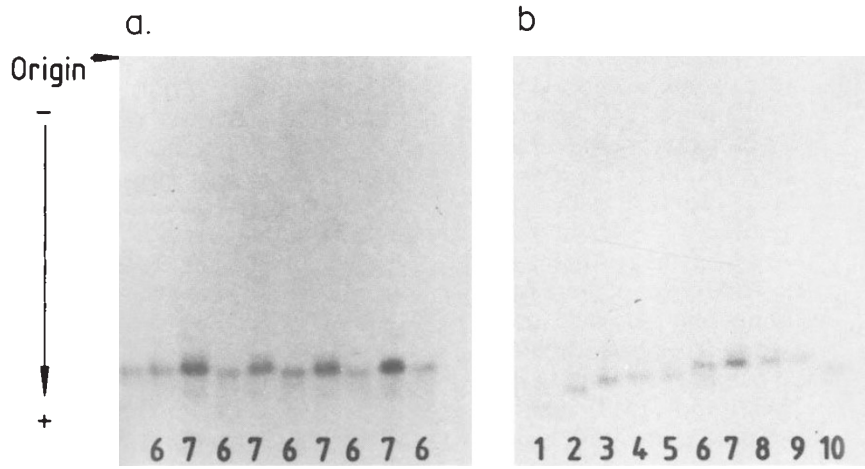

Figure 1 (a) An example of a pairwise comparison of two electrophoretic classes. Classes 6 and 7 were loaded on alternate lanes as indicated. (b) A consensus plate of *Est6* electrophoretic classes. Lane numbers are synonymous to electrophoretic class.

group, showed relative mobilities of 1·08, 1·07 and 1·06 respectively. Classes 6, 7, 8 and 9, within the *Est6-S* group, showed relative mobilities of 1·02, 1·01, 1·00 and 0·99 respectively. Class 10, also within the *Est6-S* group, consistently failed to show a clear band and had reduced staining intensity (fig. 1(b)), possibly due to rapid protein denaturation during electrophoresis.

Two classes were most frequent in the population, class 8, an *Est6-S* class, with a frequency of 55 percent, and class 4, an *Est6-F* class, with 22 per cent (table 1). Four classes (1, 5, 7 and 9) had frequencies of 3 per cent to 7 per cent, while the remaining four classes (2, 3, 6 and 10) had frequencies of 1 per cent or less. The combined frequencies of the *Est6-F* and *Est6-S* groups were 25 per cent and 70 per cent respectively, which agrees with previous frequency data (±3 per cent) from six nearby populations (Oakeshott *et al.*, 1981).

Table 1 Relative mobilities and frequencies of ten *Est6* electrophoretic classes from the Coffs Harbour population ($n = 157$). Classes are numbered in decreasing order of anodal relative mobility and their correspondence with the four classes detected by standard procedures are also indicated

Electrophoretic class		Relative mobility	Frequency
VF	1	1·18	0·038
F'	2	1·12	0·013
F	3	1·08	0·006
	4	1·07	0·217
	5	1·06	0·025
S	6	1·02	0·006
	7	1·01	0·070
	8	1·00	0·554
	9	0·99	0·064
	10	smear	0·006

Five crosses were set up using different combinations of the *Est6* electrophoretic classes in order to check that the additional variation mapped to the same locus as the *Est6-F/Est6-S* difference (III,36·8 on genetic maps; Wright, 1963). The five crosses, using a total of eight homozygous lines were; classes 1 × 2, 1 × 8, 1 × 9, 4 × 9, and 5 × 8. The phenotype of the F_1 progeny from these crosses comprised the parental bands only, showing that the variation was inherited codominantly and acted in *cis*. The F_1 progeny of each cross were then backcrossed to one of the parental stocks and 200 backcross progeny electrophoresed for each original cross. Only parental bands were seen among a total of 1000 backcross progeny analysed. The absence of recombination products among these progeny precludes the existence of effects due to modifier loci further than 0·30 to 0·46 centimorgans from the *Est6* locus ($P < 0·05$ and $P < 0·01$, respectively). This suggests that the additional variants map to the *Est6* locus, although it is admitted that classes 3, 6, 7 and 10 were not involved in the crosses as they were available as hemizygous lines only.

All four chromosomes containing class 5 *Est6* were homozygous viable; on the other hand, four

classes, 3, 6, 7 and 10, were only found in chromosomes cultured as hemizygotes (wild/*Df(3)vin*^{7}) because of their poor homozygous viability. These associations suggest the existence of gametic disequilibrium between particular *Est6* electrophoretic classes and detrimental genes on chromosome III. Such disequilibrium was confirmed by the segregation ratios in the backcross progeny for the cross of the wild chromosomes to the TM3 balancer. Analysis of these ratios revealed that two electrophoretic classes, 5 and 9, were each associated with higher mean homozygous third chromosome viabilities (relative to their viabilities as heterozygotes with TM3) than the other eight classes ($P = 0{\cdot}06$, and $P = 0{\cdot}03$, respectively, and see fig. 2).

The crossing program to make the less viable lines hemizygous against the *Df(3)vin*^{7} deficiency provided a further viability test. The ratio of wild/*Df(3)vin*^{7} hemizygotes to TM3/*Df(3)vin*^{7} hemizygotes in the progeny of each cross could only be disturbed from 1 : 1 by the action of lethal or deleterious genes in the region around *Est6* uncovered by the deletion. Analysis of these ratios revealed one line from class 9 with a lethal gene in the *Est6* region while deleterious genes in the region (viabilities less than 45 percent of TM3 heterozygotes, which were significantly less than 100 percent at $P < 0{\cdot}05$) were identified in eight lines, five in class 8 and one each in classes 4, 7 and 9. However, the distribution of these nine lethal and deleterious genes across *Est6* electrophoretic classes was not significantly different from random ($\chi^2_8 = 2{\cdot}6$, $P > 0{\cdot}05$).

The *In(3L)P* inversion was found seven times among the 157 lines and all seven occurrences were in lines from class 4. This association between *Est6* class 4 and *In(3L)P* was highly significant ($\chi^2_1 = 26{\cdot}9$, $P < 0{\cdot}0005$).

Thirteen lines representing six of the seven *Est6* alleles which Cochrane (1976) and Cochrane and Richmond (1979) isolated by thermostability criteria were also electrophoresed. Two of these classes were slow variants designated *S*1 and *S*2 by the authors above, while four were fast variants designated *F*1, *F*2, *F*3 and *F*4. The single *S*1 line typed to our electrophoretic class 9, while two *S*2 lines typed to class 8. However, the other ten lines representing the four *Est6-F* thermostability classes clustered within two electrophoretic classes (4 and 5). In particular, both these electrophoretic classes were found among lines from each of the

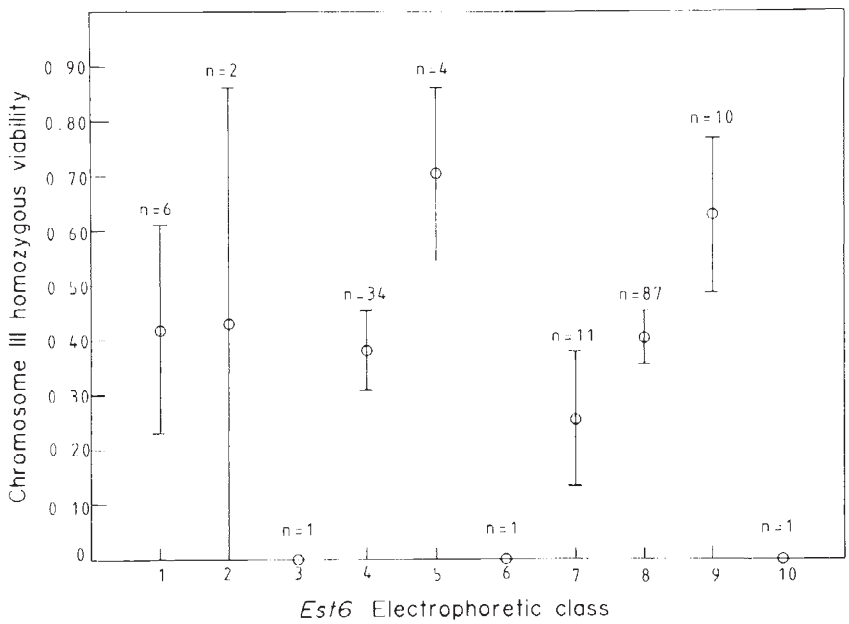

Figure 2 Mean chromosome III homozygous viabilities (±s.e.) derived from segregation ratios in the backcross progeny for the cross of the wild chromosome to the TM3 balancer. n = the number of lines in any given class.

Table 2 A comparison of electrophoretic and thermostability criteria for detecting variation at the *Est6* locus. Thirteen lines from six* thermostability classes were electrophoresed (lines and thermostability data from Cochrane and Richmond, 1979). The numbers in the body of the table represent the number of lines scored for any given category.

Electrophoretic class	Thermostability class *F*1	*F*2	*F*3	*F*4	*S*1	*S*2
4	1	2	1			
5	2		1	3		
8						2
9					1	

* Lines representing the seventh thermostability class (*S*3) were not available

thermostability classes *F*1 and *F*3 and, conversely, there were three thermostability classes in each of the electrophoretic classes 4 and 5 (table 2).

DISCUSSION

The application of high resolution cellulose acetate electrophoresis has resolved ten *Est6* electrophoretic classes among 157 isoallelic lines from a single population of *D. melanogaster* (table 1, fig. 1(b)). Standard electrophoretic techniques (Oakeshott *et al.*, 1981; Cabrera *et al.*, 1982; Anderson and Oakeshott, 1984) would have detected only four electrophoretic classes. Genetic test crosses were carried out among six of the ten classes and the results were consonant with the hypothesis that the additional variation mapped to the *Est6* structural locus.

Two of the ten electrophoretic classes (5 and 9) were associated with greater than average chromosome III viability (fig. 2). Viability effects are known to be associated with inversions (Nassar, 1968; Watanabe and Yamazaki, 1976), but in this study lines carrying the *In(3L)P* inversion, which was in gametic disequilibrium with class 4, did not differ significantly in viability from other lines.

Voelker *et al.* (1978) reported a significant positive association between the *Est6-F* allele and the *In(3L)P* inversion in North American populations of *D. melanogaster*. In this study, *In(3L)P* was only found in lines bearing *Est6* class 4 (the most frequent *Est6-F* class), in which it occurred in seven out of 34 chromosomes. Thus, an association of *Est6-F* with *In(3L)P* has now been documented for two continents in different hemispheres and it would seem that the gametic disequilibrium between this inversion and *Est6-F* is widespread and relatively old. It is therefore possible that the population dynamics of *Est6* alleles may be at least in part determined by those of the larger chromosomal polymorphism. In particular, it could be argued that the latitudinal clines for the major *Est6-F* and *Est6-S* alleles (Anderson and Oakeshott, 1984) may be the outcome of their disequilibrium with *In(3L)P*, the frequencies of which also vary clinally with latitude (Knibb *et al.*, 1981). However, this latter argument is discounted by the fact that the same cline for *Est6* exists in *D. simulans*, a species with no known polymorphic inversions and the same major *Est6* variants (Ashburner and Lemeunier, 1976; Anderson and Oakeshott, 1984).

The finding of ten electrophoretic classes in this study compares with the 27 *Est6* electrophoretic classes reported for *D. simulans* by De Albuquerque and Napp (1981) from among over 2000 third chromosomes from two South American populations. However, in the latter study, *Est6* phenotypes were simply scored in wild/caught males whereas our classification was based on repeated pair-wise comparisons among extracted isoallelic lines. It is our experience, consonant with that of Keith (1983) and Keith *et al.* (1985), that the use of isoallelic lines and repeated pairwise testing is essential to confirm the classification of variants with small relative mobility differences.

It is possible that a small number of electrophoretic variants still remain undetected in the present sample since our cellulose acetate system only detected 23 of the 25 *Est5* variants in the *D. pseudoobscura* lines we tested (see materials and methods). Moreover, application of our system to 13 other lines of *D. melanogaster* representing six different *Est6* thermostability variants indicated that the variants detected by the two criteria are essentially uncorrelated (table 2). Electrophoresis may more readily detect substitutions on the outside of the *Est6* molecule affecting its surface charge. On the other hand, thermostability criteria may be more likely to detect internal substitutions affecting the stability of the tertiary protein structure. If it were presumed that the differences detected by the two tests are completely independent, then one might predict that a larger sample would reveal three or four thermostability variants within each of the ten electrophoretic classes at the *Est6* locus.

It is therefore clear that *Est6* is a highly variable locus and now joins two other loci showing similar degrees of polymorphism (*Est5* and *Xdh* of *D. pseudoobscura*: Coyne, 1982; Keith, 1983; Keith *et al.*, 1985). The results of many previous genetic analyses of *Est6* now require re-appraisal. With respect to its population genetics it is important

to determine which of the ten electrophoretic variants (or seven thermostability variants) underlies the *Est6-F/Est6-S* clines in both *D. melanogaster* and *D. simulans* (Anderson and Oakeshott, 1984). Given the highly variable nature of the *Est6* locus, it may well be that the major electrophoretic phenotypes showing the clinal variation are acting as markers for some other more physiologically relevant polymorphism in the gene. We are presently collecting comparative nucleotide sequence data in order to investigate this question, and to identify the amino acid substitutions distinguishing the major and minor electrophoretic differences, and the sites responsible for the thermostability variation.

Acknowledgements We thank Dr Tim P. Keith for the gift of the 25 lines of *D. pseudoobscura* typed for *Est5*. We are also indebted to Dr. Wayne R. Knibb for analysing the lines of *D. melanogaster* for chromosomal polymorphism. We thank Ann Y. Game, Jill Karotam, Elisabeth A. van Papenrecht, Marlene Saad, C. Lynne McIntyre, Robyn J. Russell and Steven W. McKechnie for stimulating discussions and comments on the manuscript.

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