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Evidence for a Genetic Duplication involving Alcohol Dehydrogenase Genes in *Drosophila buzzatii* and Related Species

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Abstract

Drosophila buzzatii has two closely linked loci ($0{\cdot}4 \pm 0{\cdot}3$ cM apart) encoding NAD^+-dependent alcohol dehydrogenase. These two loci produce complex, non-overlapping electrophoretic phenotypes and differ in their ontogenetic expression, that of *Adh-1* relative to *Adh-2* being less in adults than in third-instar larvae. Isozymes produced by the two *Adh-2* alleles analysed have similar *in vitro* ADH activity levels, substrate specificities and thermostabilities, whereas the isozymes of the two *Adh-1* alleles examined differ in ADH activity and specificity for primary *versus* secondary alcohol substrates. *D. buzzatii* is a member of the *mulleri* subgroup of the *repleta* group of *Drosophila* species and the two-locus electrophoretic phenotype for ADH is widespread within the *mulleri* subgroup and possibly the *hydei* subgroup but rare in the other three subgroups of *repleta*.

Introduction

Although gene duplication has long been suggested as an important element in evolution, it is only recently that suitable biochemical techniques have become available to search for evidence of its occurrence. Some examples have been inferred from pairs of functionally similar, closely linked loci encoding globins and trypsins in mammals (Fitch and Margoliash 1970; Ohno 1970), phosphoglucose isomerase in bony fishes (Avise and Kitto 1973) and amylases, peptidases, vitellogenins and heat-shock proteins in *Drosophila* spp. (Dickinson and Sullivan 1975; Corces *et al.* 1980; Postlethwait and Jowett 1980). This paper provides evidence suggesting the recent origin of a duplicated alcohol dehydrogenase locus in some *Drosophila* species.

Drosophila species in the *immigrans*, *melanogaster*, *nasuta*, *obscura* and *willistoni* groups each have a single alcohol dehydrogenase (ADH, EC 1.1.1.1). Electrophoretic, presumed genetic, variation has been reported in several of these species (Stone *et al.* 1968; Johnson *et al.* 1969; Ayala *et al.* 1972; Yang *et al.* 1972; Anderson *et al.* 1977; Chambers *et al.* 1978; Eisses *et al.* 1979) but only for *D. melanogaster* has such a polymorphism been thoroughly characterized. Differences in *in vitro* activity levels, substrate specificity and thermostability have been described between the two common electrophoretic variants, ADH-F and ADH-S, and between these and a rarer, thermostability variant, ADH-FCh.D. (Wilks *et al.* 1980). Furthermore there are correlations between ADH activity and fitness when the different genotypes are exposed to the alcohols found in the rotting fruits on which this species lives (Oakeshott *et al.* 1980).

Eight cactophilic species (*D. aldrichi*, *D. arizonensis*, *D. longicornis*, *D. mojavensis*, *D. mulleri*, *D. pachuca*, *D. propachuca* and *D. tira*) in the *mulleri* subgroup of the *repleta* group also show phenotypes indicative of a single *Adh* locus in adults (Richardson and Smouse 1976; Richardson *et al.* 1977; Starmer *et al.* 1977). However, electrophoresis of larvae of *D. aldrichi* and *D. buzzatii* (also a cactophilic species in this subgroup) reveal additional bands of ADH activity and the electrophoretic pattern in *D. buzzatii* varies in a manner inconsistent with single-locus inheritance (Mulley 1975). Therefore, Mulley (1975) proposed two structural loci in this species, *Adh-1* and *Adh-2*, with a total of five alleles, $Adh\text{-}1^{a,b,c}$ and $Adh\text{-}2^{a,b}$ (named in decreasing order of anodal mobility).

Only $Adh\text{-}1^{b}$ and $Adh\text{-}1^{c}$ consistently occur at polymorphic frequencies in natural populations and these two are also the only ones so far analysed genetically in laboratory crosses; they segregate as a single-gene difference (Barker and Mulley 1976). Evidence from geographic variation in gene frequencies and perturbation experiments also suggests the action of selection on $Adh\text{-}1^{b}$ and $Adh\text{-}1^{c}$ (Mulley *et al.* 1979; Barker and East 1980) and it is possible that alcohols are relevant to this selection (Starmer *et al.* 1977), since they are important constituents of the rotting cladodes of *Opuntia* on which *D. buzzatii* live (Barker and East, unpublished data).

Further work on the ADH's of *D. buzzatii* is important for two reasons. Firstly, it could add to the evidence for evolution by duplication. Secondly, it could test the model relationship between biochemical and fitness differences developed with ADH in *D. melanogaster*, using a species with very different genetic architecture for ADH and very different ecology. Accordingly, this paper presents a genetic analysis of $Adh\text{-}1^{b}$ and $Adh\text{-}1^{c}$ and the putative $Adh\text{-}2^{a}$ and $Adh\text{-}2^{b}$ alleles of *D. buzzatii*, a biochemical analysis of the allozymes produced by these genes, and a phylogenetic analysis of the two-locus electrophoretic pattern in species of the *repleta* group.

Materials and Methods

Details of the *D. buzzatii* stocks analysed are summarized in the following tabulation:

Origin	No. of lines	Phenotype	Presumptive genotype
Brazil	8	*1b2a*	$Adh\text{-}1^{b}/Adh\text{-}1^{b}$, $Adh\text{-}2^{a}/Adh\text{-}2^{a}$
Queensland			
Hemmant	12	*1b2a*	$Adh\text{-}1^{b}/Adh\text{-}1^{b}$, $Adh\text{-}2^{a}/Adh\text{-}2^{a}$
Hemmant	12	*1c2a*	$Adh\text{-}1^{c}/Adh\text{-}1^{c}$, $Adh\text{-}2^{a}/Adh\text{-}2^{a}$
Charleville and Dixalea	3	*1b2b*	$Adh\text{-}1^{b}/Adh\text{-}1^{b}$, $Adh\text{-}2^{b}/Adh\text{-}2^{b}$

The Brazilian *D. buzzatii* lines were provided by Dr F. de M. Sene (Universidad de Sao Paulo, Brazil) and the *D. hydei* line by Prof. L. H. Throckmorton (University of Chicago, Illinois) while the four *D. aldrichi* lines were derived from Hemmant in southern Queensland. Stocks of 18 other *repleta* group species were obtained from the National Drosophila Resource Center, Austin, Texas (stock numbers available from J.G.O. on request). The *D. melanogaster* control stocks were isoallelic for *Adh* (Wilks *et al.* 1980), as were the *Adh-1* alleles of the Hemmant *1b2a* and *1c2a* lines. The recipe for the food medium for *D. buzzatii*, *D. aldrichi* and *D. melanogaster* was: 100 g dead yeast, 60 g sucrose, 7 g agar, 5 ml propionic acid and 1 litre water. For all other species it was: 15 g dead yeast, 72 ml corn syrup, 37 ml malt, 72 g cornmeal, 5 g soya flour, 7 g agar, 2 peeled, homogenized bananas, 5 ml propionic acid and 1 litre water. All cultures were kept at $22 \pm 2°C$.

ADH electrophoretic mobilities were determined on cellulose acetate sheets or starch gels using a technique of Barker and Mulley (1976). For *D. buzzatii*, *D. aldrichi* and *D. melanogaster*, single individuals were ground in 20 μl of electrophoresis buffer, and for all other species, in 5 μl. The gels were stained according to the method of Chambers *et al.* (1978), and all ADH bands were shown

to represent true ADH activity by demonstrating their dependence on both NAD^+ as cofactor and 2-propanol as substrate.

To prepare extracts for spectrophotometric assays of ADH, 20 third-instar larvae were taken from each culture, weighed, and homogenized in 100 mM ice-cold glycine–sodium hydroxide buffer, pH 9·5, at 20 mg live weight per millilitre. The homogenates were centrifuged at about 12 000 *g* for 30 min and the supernatant collected and stored on ice until assayed (always within 6 h of preparation). The standard assay mixture contained 100 µl 50% (v/v) 2-propanol, 100 µl 28 mM NAD^+, 600–700 µl 100 mM glycine–sodium hydroxide buffer, pH 9·5, and 100–200 µl of the enzyme extract, giving a final volume of 1 ml. The conversion of NAD^+ to NADH was monitored at 340 nm in a Pye-Unicam SP1800 spectrophotometer at 25°C. One unit of activity was defined as an increase in absorbance of 0·001 per minute and ADH activity was expressed as units per milligram live weight.

Substrate specificity was investigated by substituting the 2-propanol in the assay mixture with the same volume of 50% (v/v) ethanol, 1-propanol, 1-butanol or 1-pentanol. ADH thermostability was investigated by sealing 500-µl samples of the extracts in small glass test tubes and immersing these in a 40°C water-bath for 5 min. The treatment was terminated by plunging the tubes into ice-cold water, and ADH activity was assayed as above with 2-propanol as substrate.

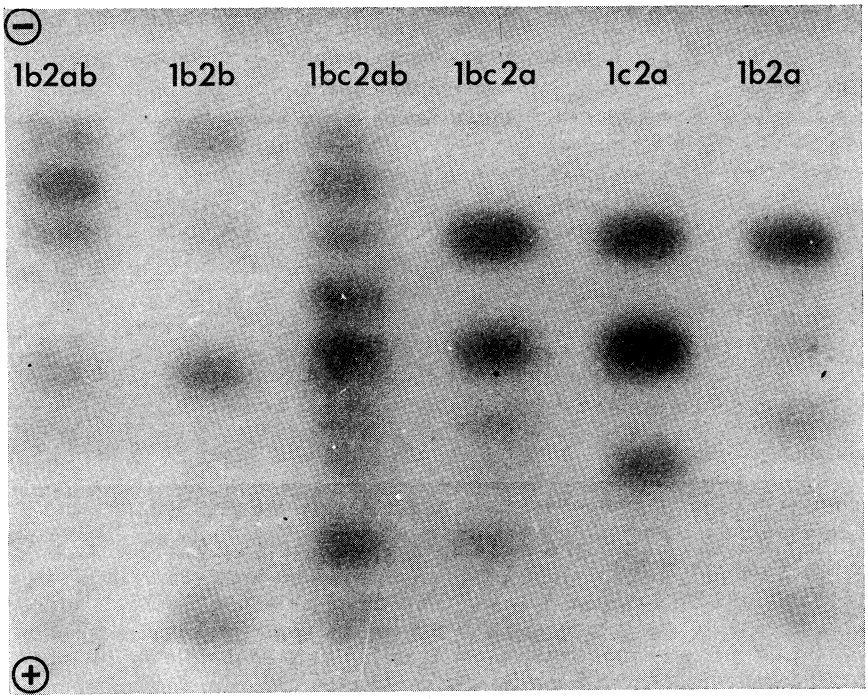

Fig. 1. Photograph of a starch gel showing various ADH phenotypes in *D. buzzatii* larvae.

Results

Genetic Analyses

Fig. 1 is a photograph of a starch gel showing various ADH phenotypes seen in third-instar *D. buzzatii* larvae. To explain this complex banding pattern two *Adh* loci were proposed, with three relatively anodal bands being products of *Adh-1* alleles, three relatively cathodal bands being products of *Adh-2* alleles, and four intermediate bands being products of interlocus hybridization. [As in other *Drosophila* species (Chambers *et al.* 1978), there are also some minor bands, probably breakdown

bands—*viz.* the second most cathodal bands in the four-banded *1b2b* and *1b2a* the most anodal of the four bands in *1c2a*.] This genetic interpretation is elaborated in Fig. 2.

Two lines of evidence confirmed the existence of the *Adh-2* locus. First, a single-factor cross was made between the *1c2a* and *1c2b* phenotypes, and the F_1 showed a *1c2ab* phenotype. The F_2 segregation of 54 *1c2a*, 110 *1c2ab* and 47 *1c2b* does not differ significantly from the 1 : 2 : 1 ratio expected for the F_2 segregation of a single gene difference ($\chi^2_2 = 0{\cdot}85$, $P > 0{\cdot}05$).

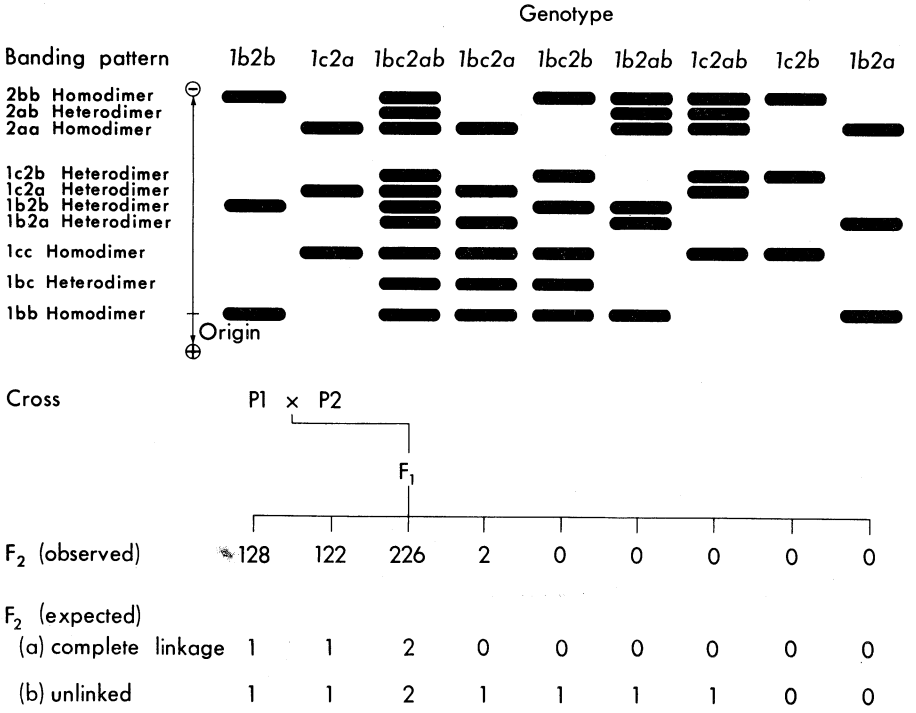

Fig. 2. Diagram of an ADH gel showing the nine possible larval phenotypes produced by *Adh-1*^{b}, *Adh-1*^{c}, *Adh-2*^{a} and *Adh-2*^{b} in *D. buzzatii*. Apart from breakdown bands (which are not shown), a total of 10 different ADH bands can occur; the three fastest are ADH-1 dimers, the three slowest ADH-2 dimers and the four intermediate inter-locus heterodimers. (Note that in Fig. 1 the 1c2a and 1b2b heterodimers are almost indistinguishable.) The figure also shows observed and expected F_2 segregation ratios of the nine phenotypes after crossing the *1b2b* and *1c2a* genotypes.

Secondly, a two-factor cross was made between the *1b2b* and *1c2a* phenotypes and the F_1 showed a *1bc2ab* phenotype. Fig. 2 shows the phenotypes and their numbers observed in the F_2 and it is clear that *Adh-1* and *Adh-2* must be closely linked. Only four phenotypes were observed in the F_2—namely the two doubly homozygous parental types, the doubly heterozygous F_1 type and the singly heterozygous *1bc2a* type. The latter, recombinant, type only occurred in 2 out of 478 F_2 individuals. Given that genetic recombination is absent in males (Ashburner and Novitski 1976), this represents a recombination fraction of $0{\cdot}4 \pm 0{\cdot}3$ cM.

When only the segregation of the ADH-2 variants in the F_2 is considered, a ratio of 124 *2a*, 226 *2ab* and 128 *2b* is obtained, which concurs with the 1 : 2 : 1 ratio observed in the previous single-factor cross for *Adh-2* ($\chi^2_2 = 1{\cdot}48$, $P > 0{\cdot}05$).

Phylogenetic Analyses

In an attempt to determine how widespread the two-locus genetic architecture for *Adh* is in the *repleta* group, ADH electrophoretic patterns were examined in 20 species other than *D. buzzatii* distributed across all five subgroups of the *repleta* group (Wasserman 1963). Ground preparations of several individuals of each species were subjected to electrophoresis and, for those which could be bred, both third-instar larvae and adults were examined. Given the electrophoretic patterns described by Richardson and Smouse (1976), Richardson *et al.* (1977) and Starmer *et al.* (1977), and the results in Figs 1 and 2, the essential phenotypic properties of the *buzzatii*-type two-locus system were defined as the presence of at least three bands per larva and at least partial loss of the faster (more anodal) bands in adults. The essential phenotypic properties of the *melanogaster*-type one-locus system were defined as two or fewer bands similar in expression in both larvae and adults. [Under different conditions some authors have observed a faster, much fainter, third band in each *D. melanogaster* homozygote (Chambers *et al.* 1978)—this did not develop under our conditions.]

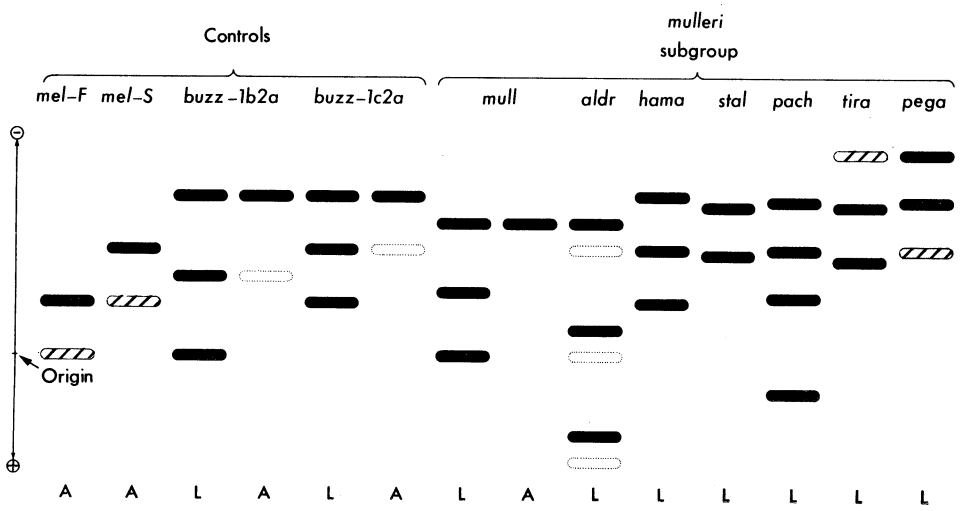

Fig. 3. Diagram of an ADH gel. L, larvae; A, adults. The numbers of larvae and adults scored were 14 and 13 for *D. mulleri*, 30 and 0 for *D. aldrichi*, 8 and 0 for *D. hamotophila*, 5 and 0 for *D. stalkeri*, 6 and 0 for *D. pachuca*, 5 and 0 for *D. tira*, and 5 and 0 for *D. pegasa*. The patterns for the *D. melanogaster* controls were the same in larvae as in adults. The various shading intensities shown correspond approximately to the relative band intensities observed on the gels (hatching = intermediate intensity).

Fig. 3 summarizes the ADH patterns observed for seven species other than *D. buzzatii* in the *mulleri* subgroup. Larvae of all the species (except *D. stalkeri*) show at least three ADH bands and adults of *D. mulleri*, the only species of the seven for which adults were assayed, show only the slowest band. Richardson and Smouse (1976) also have reported a single-banded ADH phenotype for *D. mulleri* and *D. tira* adults (as well as adults of five other *mulleri* subgroup species not included in this study). This suggests that many species in this subgroup may have a *buzzatii*-like two-locus ADH system in which the locus controlling the faster migrating band is apparently expressed less in adults than in third-instar larvae.

Fig. 4 summarizes the patterns observed for 13 *repleta* group species outside the *mulleri* subgroup. The four *melanopalpa*, two *fasciola* and two *mercatorum* subgroup species all show only two ADH bands in both larvae and adults, so on the criteria above are unlikely to have two structural *Adh* loci. However, the five *hydei* subgroup species have three-banded ADH patterns in larvae, and the fastest band is absent in the adults of *D. nigrohydei*, *D. eohydei*, *D. bifurca* and *D. hydei*, but not *D. neohydei*. If the fastest band in the first four of these five *hydei* subgroup species is not just a breakdown band, then the patterns observed would suggest that these four species may have a two-locus ADH system analogous to that in *D. buzzatii*.

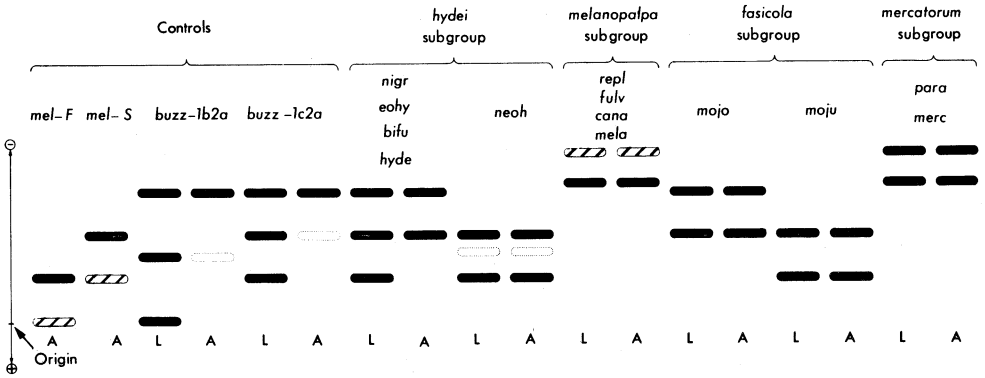

Fig 4. Diagram of an ADH gel. L, larvae; A, adults. The numbers of larvae and adults scored were 6 and 8 for *D. nigrohydei*, 5 and 5 for *D. eohydei*, 7 and 6 for *D. bifurca*, 2 and 2 for *D. hydei*, 11 and 14 for *D. neohydei*, 4 and 4 for *D. repleta*, 7 and 5 for *D. fulvimaculoides*, 5 and 3 for *D. canapalpa*, 3 and 1 for *D. melanopalpa*, 6 and 6 for *D. mojuoides*, 3 and 2 for *D. moju*, 5 and 5 for *D. paranaensis*, and 6 and 6 for *D. mercatorum*.

Biochemical Analyses

Table 1 presents the data on ADH activity in third-instar larvae for the 35 *D. buzzatii* lines as well as the three *D. melanogaster* controls and the four *D. aldrichi* lines. All *D. buzzatii* lines have far less ADH activity than any of the *D. melanogaster* phenotypes, while on average the *D. aldrichi* lines have only about two-thirds the activity of *D. buzzatii* lines. However, there is also considerable variation between lines within both *D. aldrichi* and *D. buzzatii*, and within the latter, some of the variation reflects their ADH electrophoretic phenotype, although within *1b2a* apparently not their geographic origin.

Table 2 presents an analysis of variance of logarithmic transforms of the *D. buzzatii* data nested by electrophoretic phenotype and line. The overall differences between phenotypes and between lines within phenotypes are both highly significant, and those between *Adh* phenotypes are decomposed into two specific contrasts corresponding to differences at each of the two *Adh* loci. The contrast between the *1b2a* and *1c2a* phenotypes is highly significant, indicating that, overall, lines homozygous for $Adh\text{-}1^b$ differ significantly from those homozygous for $Adh\text{-}1^c$; $Adh\text{-}1^b$ lines only average about 80% of the activity of $Adh\text{-}1^c$ lines. The contrast between the *1b2a* and *1b2b* lines is not significant, suggesting that overall, $Adh\text{-}2^a$ and $Adh\text{-}2^b$ homozygotes do not differ substantially in ADH activity.

Table 3 compares the substrate specificities of the *D. melanogaster*, *D. aldrichi* and *D. buzzatii* phenotypes. The data for each phenotype are summarized in the table over all the constituent lines. Following Wilks *et al.* (1980), substrate specificity

Table 1. ADH activities (units per milligram liveweight) with 2-propanol as substrate of lines representing various *Adh* phenotypes of *D. aldrichi* and *D. buzzatii*

The mean activity and its standard error (with the number of cultures assayed in parenthesis) are presented for each line, phenotype and species. Values for the Australian and Brazilian *1b2a* lines of *D. buzzatii* are also given separately. For comparison, mean activities (±s.e.) using the same assay conditions for six cultures each of *D. melanogaster* ADH-F, ADH-S, and ADH-FCh.D. were 529±147, 269±49 and 221±42, giving an overall mean value for this species of 340±60

Line No.	*D. aldrichi*	*D. buzzatii*			
		1c2a	*1b2a* (Aust.)	*1b2a* (Brazil)	*1b2b*
1	54±1(3)	94±4(12)	89±4(18)	60±7(7)	74±3(16)
2	40±4(4)	83±3(25)	57±4(13)	78±3(18)	77±3(10)
3	60 (1)	94±5(5)	73±3(13)	72±4(22)	74±2(15)
4	34 (1)	98±5(17)	79±1(2)	81±6(6)	
5		94±5(15)	64±4(11)	63±3(19)	
6		80±5(10)	65±5(7)	62±3(8)	
7		78±7(10)	49±3(10)	51±6(7)	
8		86±5(9)	60±4(13)	59±2(25)	
9		67±3(7)	64±4(17)		
10		75±3(21)	50±3(14)		
11		63±3(20)	73±7(11)		
12		69±2(14)	72±3(20)		
Locality means			67±2(149)	66±2(112)	
Genotype means		81±2(165)	66±1(261)		75±1(41)
Species means	46±3(9)		72±1(467)		

Table 2. Analysis of variance on logarithmic transforms of the ADH activity data for *D. buzzatii* shown in Table 1

F' (d.f.) represents the *F* ratio and its degrees of freedom for cases in which unequal subclass sizes necessitated the use of recalculated mean squares and degrees of freedom (Sokal and Rohlf 1969). ** $P < 0{\cdot}01$. *** $P < 0{\cdot}001$. n.s., not significant

Source of variation	Degrees of freedom	Sum of squares	Mean square	*F* ratio	*F'* (d.f.)
Between phenotypes	2	4·328	2·164		6·28(2,24)**
1c2a versus *1b2a*	1	4·170	4·170		10·60(1,20)***
1b2a versus *1b2b*	1	0·732	0·732		2·13(1,17)n.s.
Between lines within all phenotypes	32	9·728	0·304	7·42***	
Between cultures within lines	432	17·712	0·041		

is expressed in terms of the ratio of activity with the secondary alcohol 2-propanol as substrate to that with the primary alcohol in question. All the ratios for *D. buzzatii* and *D. aldrichi* are smaller than the corresponding values for *D. melanogaster* and there are also differences between *1b2a* and *1c2a* in *D. buzzatii*. All four of the ratios

in *1b2a* are smaller than the corresponding values in *1c2a* and this difference reaches significance for the 2-propanol : 1-pentanol ratio ($F^1_{16} = 7{\cdot}90$, $P < 0{\cdot}05$). There are no significant substrate specificity differences between the two homozygotes at the *Adh-2* locus ($F^1_{14} = 0{\cdot}60$, $P > 0{\cdot}05$).

Table 3. Means and standard errors (with the number of cultures assayed in parenthesis) for the substrate specificity ratios of various *Adh* phenotypes of *D. aldrichi*, *D. buzzatii* and *D. melanogaster*

Substrate specificity ratio	*D. aldrichi*	*D. buzzatii*			*D. melanogaster*		
		1c2a	*1b2a*	*1b2b*	ADH-F	ADH-S	ADH-FCh.D.
2-propanol : ethanol	2·95 ±0·11 (2)	3·18 ±0·05 (77)	3·00 ±0·04 (145)	3·04 ±0·02 (41)	6·25 ±0·27 (6)	4·02 ±0·15 (6)	4·92 ±0·45 (6)
2-propanol : 1-propanol	2·25 ±0·23 (7)	2·25 ±0·03 (36)	2·21 ±0·03 (89)		4·87 ±0·05 (6)	3·20 ±0·09 (6)	3·06 ±0·05 (6)
2-propanol : 1-butanol		1·77 ±0·05 (25)	1·67 ±0·07 (34)		3·27 ±0·03 (6)	1·81 ±0·03 (6)	1·84 ±0·15 (6)
2-propanol : 1-pentanol		1·18 ±0·02 (25)	1·11 ±0·01 (35)				

Table 4 summarizes the *in vitro* ADH thermostabilities of the *D. melanogaster* and *D. buzzatii* phenotypes. The thermostabilities of all three *D. buzzatii* phenotypes are greater than those of the common, heat-sensitive ADH-F and ADH-S variants but less than that of the rare, thermostable ADH-FCh.D. variant of *D. melanogaster*. Analysis of variance confirms the similarity among the three *D. buzzatii* phenotypes ($F^2_3 = 0{\cdot}32$, $P > 0{\cdot}05$), and indicates highly significant differences in thermostability between lines within phenotypes ($F^{10}_{82} = 2{\cdot}45$, $P < 0{\cdot}01$).

Table 4. Means and standard errors (with the number of cultures assayed in parenthesis) for the ADH thermostabilities (percentage activity remaining after heat-treating extracts for 5 min at 40°C) of various *Adh* phenotypes of *D. buzzatii* and *D. melanogaster*

D. buzzatii			*D. melanogaster*[A]		
1c2a	*1b2a*	*1b2b*	ADH-F	ADH-S	ADH-FCh.D.
73·9 ±0·9 (33)	74·4 ±1·1 (31)	72·6 ±1·0 (31)	37·6 ±2·6	36·1 ±3·3	92·9 ±1·6

[A] Data for *D. melanogaster* adults taken from Gibson *et al.* (1980).

Discussion

The crossing experiments are consistent with the existence of a pair of closely linked loci (0·4±0·3 cM apart) each encoding an ADH. The two loci must differ significantly in their sequences because none of the electrophoretic variants at either locus overlaps the mobility of any variant at the other. Furthermore, at least one

of the ADH-1 variants must differ in ADH activity and substrate specificity, although not in thermostability, from the ADH-2 variants. (If the ADH-1 variants differ from each other, then obviously only one, and possibly neither, can be the same as the ADH-2 variants.) Finally, while the organ distribution of ADH-1 and ADH-2 appears to be identical in third-instar larvae, occurring almost entirely in the fat body (East, unpublished data), the expression of *Adh-1* relative to *Adh-2* apparently is less in adults than in third-instar larvae.

The close genetic linkage in *D. buzzatii* and the limited distribution of comparable electrophoretic patterns across the various *Drosophila* species subgroups strongly suggest that the two *Adh* loci do in fact represent a true gene duplication. This notion is supported by the apparent existence of interlocus hybridization. However, a recombination fraction of $0{\cdot}4 \pm 0{\cdot}3$ cM suggests that several genes other than *Adh* could be included in the duplicated chromosome region. These genes could well be regulators of *Adh* expression, which might explain the divergence in ontogenetic specificity between the two loci.

The phylogenetic analysis strongly suggests that the duplication is widespread in the *mulleri* subgroup. All members of the subgroup investigated (except *D. stalkeri*) show comparable multibanded ADH electrophoretic patterns and the pattern recurs in species as widely separated in the subgroup as *D. mulleri* and *D. buzzatii* (Wasserman 1962, 1963). However, it is unclear whether the duplication exists beyond the *mulleri* subgroup. The species investigated in the *fasciola*, *melanopalpa* and *mercatorum* subgroups do not have comparable electrophoretic patterns to the *mulleri* subgroup species. There was some evidence for more than one *Adh* locus in some species of the *hydei* subgroup but without a thorough genetic analysis of ADH in at least one species of this subgroup, no definite conclusions can be reached on its mode of inheritance. On the basis of chromosomal analysis, the *hydei* are believed to be phylogenetically more distant from the *mulleri* than are the *fasciola* for example (Wasserman 1962, 1963), so any *Adh* duplication in the *hydei* may have arisen independently from that in the *mulleri*.

The duplicated *Adh* system in the *mulleri* subgroup seems to have developed further in evolutionary terms than the amylase (*Amy*) duplication in *D. melanogaster*, which is perhaps the most studied duplicated enzyme locus in *Drosophila*. The *Amy* duplication may not yet have become fixed in natural populations of *D. melanogaster* and there is little evidence that it exists in other *Drosophila* species (Dickinson and Sullivan, 1975). Furthermore, few of the non-allelic amylases in *D. melanogaster* are distinguishable electrophoretically and very strong linkage disequilibria exists between them in natural populations (de Jong *et al.* 1972). The observed differences in properties between the *Amy* and *Adh* duplications may be due to their different evolutionary 'ages', although the much tighter linkage between the *Amy* genes (about 0·01 cM, Bahn 1968) may also be relevant.

It is possible that the distribution of the *Adh* duplication through the *mulleri* subgroup is at least partly due to selection acting directly on the *Adh* locus, although it may also be acting on any other genes involved in the duplication. One simple model for the mode of action of the selection for the duplicated *Adh* locus derives from work on the *Amy* duplication. High levels of AMY activity are selectively advantageous in some starch environments (Hoorn and Scharloo 1978 and references therein) and may explain the apparent spread of the *Amy* duplication in *D. melanogaster*

populations. An analogous situation might apply to the *Adh* duplication since a selective advantage for high ADH levels has been demonstrated under at least some alcohol environments for *D. melanogaster* (Oakeshott *et al.* 1980). In fact there is suggestive evidence that the *mulleri* species do have higher ADH levels than closely related *repleta* group species without the duplication, since it was necessary to use four times more concentrated extracts of many *repleta* group species outside the *mulleri* subgroup to obtain ADH bands of equivalent staining intensity (see Materials and Methods). However, it remains to be tested how the alcohols in the environments of the cactophilic *mulleri* differ from those encountered by closely related non-cactophilic *repleta* species without the *Adh* duplication.

Furthermore, the relatively high levels of ADH activity in the admittedly very distantly related *D. melanogaster* demonstrate that high levels of ADH activity can be obtained without a duplicated *Adh* locus. Additionally, *D. aldrichi* and many isofemale lines within each *D. buzzatii* genotype have relatively low ADH activities. Yet *D. aldrichi* not only shares the two-locus *Adh* phenotype but also cohabits rotting *Opuntia* cladodes with *D. buzzatii*. Thus flies with a wide range of ADH activities are viable in this habitat.

The published data suggesting a selective maintenance of the *Adh* polymorphism in *D. buzzatii* also argue against any simple positive relationship between ADH activity and fitness among cactophilic *Drosophila*. For the *Adh-1* locus, polymorphism for the $Adh\text{-}1^b$ and $Adh\text{-}1^c$ alleles is apparently almost universal in natural populations, with $Adh\text{-}1^b$ generally more common (Barker and Mulley 1976). This and the direction of deviations from Hardy–Weinberg equilibrium in some collections (Barker 1981), suggest that the polymorphism might be maintained by heterozygote advantage, with $Adh\text{-}1^b/Adh\text{-}1^b$ being the fittest homozygote. There is no biochemical information for heterozygotes but the information on the homozygotes shows $Adh\text{-}1^b/Adh\text{-}1^b$ larvae have less activity than $Adh\text{-}1^c/Adh\text{-}1^c$ larvae. In addition, the $Adh\text{-}2^b$ variant has the same activity as $Adh\text{-}2^a$ but has been found in relatively few natural populations and then at very much lower frequencies ($<5\%$, Barker and Mulley 1976).

It therefore seems that any selection for the second *Adh* locus is more likely to be due to the advantage in having different types, rather than greater amounts, of ADH. The duplication provides the opportunity for the evolution of independent regulation of the *Adh* genes' expression and divergence in the properties of their products. The large activity differences between lines within *Adh* genotypes indicate that background modifiers of ADH activity levels are segregating in natural populations of *D. buzzatii*; selection on these modifiers would seem both more flexible and more efficient as a means of adapting to differing requirements for ADH activity levels.

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