

# Restriction endonuclease variation in the region of the alcohol dehydrogenase gene: a comparison of null and normal alleles from natural populations of *Drosophila melanogaster*

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The restriction endonuclease variation in the 12 kb region surrounding twelve *Adh* null alleles extracted from three Tasmanian populations has been compared with normal alleles from the same populations. Each of the null alleles had the same haplotype as revealed by digestions with eight hexanucleotide restriction enzymes. This haplotype also occurred in 4 of the 46 chromosomes bearing normal alleles which were tested; these four chromosomes with the null allele haplotype carried the *Adh*<sup>S</sup> allele. The data suggest that the *Adh* null alleles from geographically separate populations share a common ancestry and are derived from the same mutation in an *Adh*<sup>S</sup> allele.

## INTRODUCTION

The average frequencies of null activity alleles at loci encoding enzymes in natural populations are generally reported to be less than 0.3 per cent (Voelker *et al.*, 1980; Langley *et al.*, 1981; Allendorf *et al.*, 1981) except where the loci are duplicated and then very much higher frequencies are commonplace (Freeth, 1986). The discovery of unexpectedly high frequencies (up to 3.9 per cent) of null alleles at the single alcohol dehydrogenase locus (*Adh*) in some Australian populations of *Drosophila melanogaster* (Freeth and Gibson, 1985) prompted questions about the origins of the alleles and their maintenance in natural populations.

The *Adh* null alleles were found in geographically separate populations and persisted over years within populations (Freeth and Gibson, 1985). Analyses of the biochemical properties of 20 *Adh* null alleles extracted from four Tasmanian populations showed that, although the *Adh* gene was intact, they did not produce alcohol dehydrogenase (ADH) activity and, of those tested, all were CRM negative (Freeth, Gibson and de Couet, 1986). The biochemical data did not provide any evidence for heterogeneity in properties between

separately extracted *Adh* null alleles, although the homozygous viabilities of second chromosomes bearing the alleles varied from lethal to normal.

Any attempt to explain the relatively high frequencies of null alleles must take account of the number of different alleles that are present in the populations. Whilst the molecular identities of alleles with similar properties can be gauged from their DNA sequences, variation in the molecular landscape surrounding a locus can be assessed with restriction endonuclease mapping and used to derive the ancestry of specific gene mutations. This approach has been successfully applied in human populations to trace the origins of  $\beta$ -thalassaemia variants (Antonarakis *et al.*, 1982; Orlein *et al.*, 1982), sickle cell haemoglobin genes (Pagnier *et al.*, 1984), serum albumin variation (Murray *et al.*, 1984) and  $\alpha$ -antitrypsin deficiency alleles (Cox *et al.*, 1985).

To provide some indication of the heterogeneity and relatedness of the *Adh* null alleles we have used restriction endonucleases to map a 12 kb region including the *Adh* locus in samples of second chromosomes bearing null and normal alleles. These data have been used to test whether the molecular landscape surrounding the *Adh* null activity alleles differs from that of normal

alleles and to compare null alleles extracted from different populations, and in different years, to assess their molecular similarities.

## MATERIALS AND METHODS

### *Populations sampled and extraction of second chromosomes*

The 58 second chromosomes bearing *Adh* alleles used in these experiments were extracted from three of the Tasmanian populations of *D. melanogaster* described by Freeth and Gibson (1985). Two of the populations, Cygnet and Huonville II (Avondale Farm) are about 13 km apart in the south of the island and the third, Tamar (Marian's Vineyard in the Tamar valley), is 300 km to the north. Between the northern and southern fruit growing regions of Tasmania there are few natural *Drosophila* habitats and attempts to collect *D. melanogaster* in the central region of the island were unsuccessful, although populations were expected to occur associated with human settlements.

The Cygnet and Tamar populations were sampled in 1984 by setting up single female lines (75 for Cygnet and 82 for Tamar) and extracting second chromosomes bearing normal *Adh* alleles (one from each line) using *CyO* (Lindsley and Grell, 1968) as a balancer (see Freeth and Gibson, 1985). The Huonville II population was sampled in 1985 and the second chromosomes were extracted

by crossing a male from a single female line to females heterozygous for *CyO* and a deficiency covering the *Adh* locus, *Df(2L)64j*, (Lindsley and Grell, 1968), then backcrossing a single *+/Df(2L)64j* male to *CyO/Df(2L)64j* females to establish a *+/Df(2L)64j* line.

The methods used to detect putative *Adh* null alleles in the Cygnet and Tamar samples followed the technique of Voelker *et al.* (1980) modified to avoid dysgenic phenomena (Freeth and Gibson, 1985). From the flies collected at Huonville II in 1985, 185 single female lines were screened by classifying their progenies into ADH phenotypes (see Freeth and Gibson, 1985). Abnormal segregation patterns in seven of the lines suggested the presence of null alleles and these were isolated. In addition two null alleles (H36 and H41) had been extracted from the Huonville II population sampled in 1984. The frequencies of *Adh* null alleles in the three populations were 0.4 per cent (Cygnet, 1984), 3.2 per cent (Huonville II, 1984), 1.88 per cent (Huonville II, 1985) and 0.5 per cent (Tamar, 1984). The 12 null alleles used in this study (one from Cygnet, three from Tamar, two from Huonville II in 1984 and six in 1985) were isolated either by using *CyO* as a balancer or by exposing the F2 progenies of single female lines to 1-penten-3-ol vapour (Sofer and Hatkoff, 1972) and isolating the surviving flies, which are homozygous for an *Adh* null allele.

The numbers of normal *Adh* alleles investigated from each population are given in tables 1,

**Table 1** Restriction endonuclease map haplotypes in the Tamar population. In this and the following two tables the extracted chromosomes are ordered according to the ADH electrophoretic phenotype to aid comparisons

Line	<i>Bam</i> HI -7.2	$\Delta$ 280	<i>Hpa</i> I -6.9	<i>Hind</i> III -3.0	$\nabla$ 200	<i>Adh</i> +0.2	<i>Pst</i> I +1.0	<i>Xho</i> I +1.2	<i>Pst</i> I +1.3	$\Delta$ 400	<i>Bam</i> HI +3.9	<i>Eco</i> RI +9.0
Tamar												
1	-	-	+	-	-	S	-	+	+	+	+	-
17	-	-	+	-	-	S	-	+	-	-	+	-
19	+	-	-	-	-	S	-	-	-	-	-	+
33	+	-	-	-	-	S	-	+	-	-	-	+
50	+	-	-	-	-	S	-	+	-	-	-	-
12	-	-	-	-	-	F	-	+	-	-	-	-
29	-	+	+	-	-	F	-	+	-	-	-	-
30	-	-	-	-	-	F	-	+	-	-	-	+
32	-	-	-	-	-	F	-	+	-	-	-	-
40*	-	-	+	-	+	F	+	+	-	+	+	-
44	-	-	-	-	-	F	+	+	-	+	-	-
54	-	-	-	-	-	F	-	+	-	-	-	-
56	+	-	-	-	-	F	-	+	-	-	-	-
60	-	-	-	-	-	F	-	+	-	-	-	-
73	+	-	-	-	-	F	-	+	-	-	-	-
240	+	-	-	-	-	null	-	+	-	-	-	+
265	+	-	-	-	-	null	-	+	-	-	-	+
340	+	-	-	-	-	null	-	+	-	-	-	+

\* The Tamar 40 chromosome has a complex structure, see p. 104.

**Table 2** Restriction endonuclease map haplotypes in the Cygnet population

Line	<i>Bam</i> HI -7.2	$\Delta$ 280	<i>Hpa</i> I -6.9	<i>Hind</i> III -3.0	$\nabla$ 200	<i>Adh</i> +0.2	<i>Pst</i> I +1.0	<i>Xho</i> I +1.2	<i>Pst</i> I +1.3	$\Delta$ 400	<i>Bam</i> HI +3.9	<i>Eco</i> RI +9.0
Cygnet												
3	+	+	-	-	-	S	-	+	-	-	-	-
8	-	-	-	-	-	S	-	-	+	-	-	+
19	+	-	-	-	-	S	-	+	-	-	-	+
25	+	-	-	-	-	S	-	+	-	-	-	+
28	-	-	+	-	-	S	-	+	-	-	-	-
45	-	-	-	-	-	S	-	+	-	-	-	-
61	+	-	-	-	-	S	-	-	-	-	-	-
72	-	-	-	-	-	S	-	+	-	-	-	-
5	+	-	-	+	+	F	-	+	-	-	-	-
11	+	-	-	-	-	F	-	+	-	-	-	-
20	-	-	-	-	-	F	-	+	-	-	-	-
29	-	-	-	+	+	F	-	+	-	-	-	-
33	-	-	-	-	+	F	-	+	-	+	-	-
35	-	-	-	+	+	F	-	+	-	-	-	-
51	-	-	-	-	-	F	-	+	-	-	-	+
95	+	-	-	-	-	null	-	+	-	-	-	+

2 and 3; the alleles were taken at random from those available from the chromosome extractions.

*ADH electrophoresis*

The electrophoretic ADH phenotypes of flies homozygous or hemizygous for extracted *Adh* alleles were assessed on cellulose acetate mem-

branes stained for alcohol dehydrogenase activity as described by Wilks *et al.* (1980).

*DNA extraction*

DNA from the Cygnet and Tamar samples, and from all the null alleles, was extracted from adult flies following the method of Miklos (1984) except

**Table 3** Restriction endonuclease map haplotypes in the Huonville II population

Line	<i>Bam</i> HI -7.2	$\Delta$ 280	<i>Hpa</i> I -6.9	<i>Hind</i> III -3.0	$\nabla$ 200	<i>Adh</i> +0.2	<i>Pst</i> I +1.0	<i>Xho</i> I +1.2	<i>Pst</i> I +1.3	$\Delta$ 400	<i>Bam</i> HI +3.9	<i>Eco</i> RI +9.0
Huonville II												
8	-	-	-	-	-	S	-	+	-	-	-	-
10	-	-	-	-	-	S	-	+	-	-	-	-
13	-	-	-	-	-	S	-	+	-	-	-	-
18	+	-	-	-	-	S	-	+	-	-	-	-
16	+	-	-	-	-	S	-	+	-	-	-	+
14	-	-	-	-	-	S	-	+	-	-	-	-
3	-	-	-	-	+	F	-	+	-	-	-	+
4	-	-	-	-	-	F	-	+	-	-	-	-
5	-	-	-	+	+	F	-	+	-	-	-	-
6	-	-	-	+	+	F	-	+	-	-	-	-
7	-	-	-	-	-	F	-	+	-	-	-	-
9	-	-	-	+	+	F	-	+	-	-	-	-
12	-	-	-	-	-	F	-	+	-	-	-	+
15	-	-	-	+	+	F	-	+	-	-	-	-
17	-	-	-	-	-	F	-	+	-	-	-	+
19	-	-	-	+	+	F	-	+	-	-	-	-
98	+	-	-	-	-	null	-	+	-	-	-	+
70	+	-	-	-	-	null	-	+	-	-	-	+
144	+	-	-	-	-	null	-	+	-	-	-	+
9	+	-	-	-	-	null	-	+	-	-	-	+
77*	+	-	-	-	-	null	-	+	-	-	-	+
52†	+	-	-	-	-	null	-	+	-	-	-	+
36‡	+	-	-	-	-	null	-	+	-	-	-	+
41‡‡	+	-	-	-	-	null	-	+	-	-	-	+

\* Homozygous lethal chromosome

† Homozygotes sterile

‡ Extracted in 1984 whereas all other chromosomes in this population were extracted in 1985

that whole flies were used. The Huonville II DNA samples were also prepared from whole adults, but using the method of Chia *et al.* (1985). Adult flies for DNA extractions were aged for at least 4 days and then deyeasted for 2 hours prior to freezing in liquid nitrogen and storage at  $-70^{\circ}\text{C}$ .

#### Restriction endonuclease digests and electrophoresis of DNA

DNA prepared from stocks of each of the fifty-eight extracted *Adh* alleles was digested using hexanucleotide restriction endonucleases under buffer conditions suggested by the manufacturers (Amersham). The eight enzymes used in the survey were *Eco* RI, *Hind* III, *Sal* I, *Xho* I, *Pst* I, *Bam* HI, *Xba* I and *Hpa* I. These particular enzymes were chosen because each had previously been shown to reveal variation in the *Adh* region in other populations (Langley *et al.*, 1982; Birley, 1984). Electrophoresis of the digests was carried out at about 2V/cm for 16–19 hrs at room temperature in a horizontal gel apparatus with 1.0 per cent agarose gels prepared in buffer containing 0.04 M Tris HCl, pH 7.8, 5 mM sodium acetate and 1 mM EDTA.

#### Hybridisation

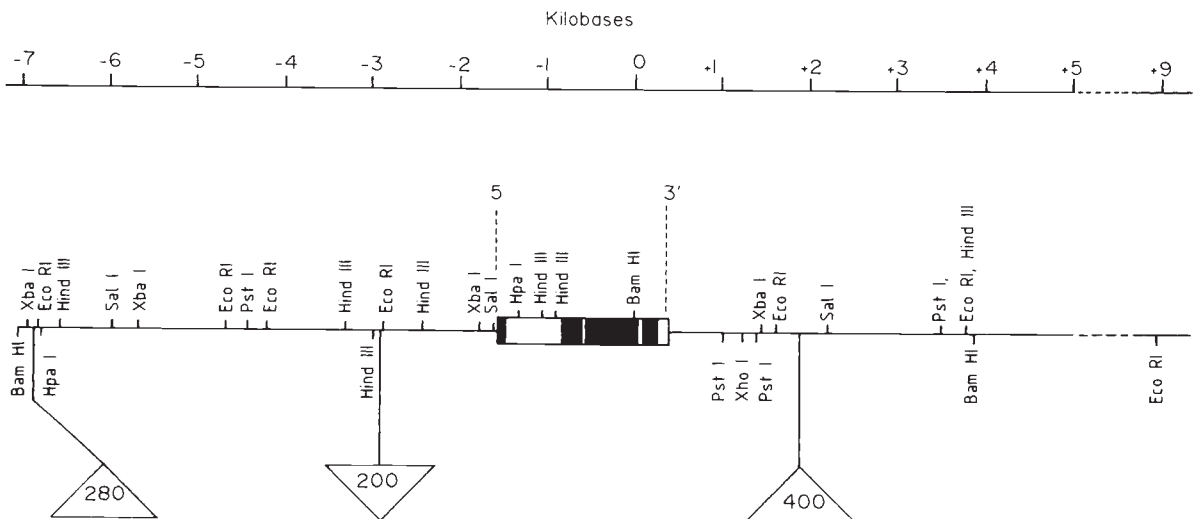
DNA fragments were transferred from agarose gels to nitrocellulose filters as described by Southern

(1975). Prehybridization of the filters was carried out as described by Miklos *et al.* (1984). The sAF-2 plasmid (Goldberg, 1980), which includes within an 11.5 kb insert the coding sequence of the *D. melanogaster Adh* gene together with 10 kb of flanking DNA, was labelled with [ $\alpha$ - $^{32}\text{P}$ ]-dCTP by nick translation (Rigby *et al.*, 1977). Autoradiography was carried out at  $-70^{\circ}\text{C}$  on Kodak film (XRP-1) using intensifying screens.

#### RESULTS

The total restriction endonuclease variation detected in the three Tasmanian populations is shown in fig. 1; the variation between *Adh* alleles from each population is listed in tables 1, 2 and 3. Following previous studies on restriction maps of the *Adh* region we have assigned the coordinate 0 to the invariant *Bam* HI site in the second exon of *Adh* (Chia *et al.*, 1985). The electrophoretic phenotypes, ADH-S and ADH-F, of the normal activity alleles are indicated.

Overall six of the eight enzymes (*Bam* HI, *Hind* III, *Hpa* I, *Pst* I, *Xho* I and *Eco* RI) exhibited restriction endonuclease site recognition variation due to the presence or absence of the recognition sites. Two insertions (of 280 bp and 400 bp) and one deletion (200 bp) were also detected. In total 11 different map variants were found although not all were present in each popu-



**Figure 1** Restriction endonuclease map variation detected in the *Adh* region of three Tasmanian populations. 3' is to the right of the figure. The boundaries of the *Adh* transcription unit are indicated and the adult 5' leader and three exons are shaded. Variable restriction endonuclease sites are indicated below the map; triangles represent insertions (points upward) or deletions (points downward) and the estimated sizes are shown. The *Eco* RI site (+9.0) was outside the probed region but could be scored unambiguously in all populations.

lation. For example, the *Hind* III (-3.0) site was not found in Tamar and the *Pst* I (+1.0) and *Bam* HI (+3.9) sites were absent from Cygnet and Huonville II. An analysis of the frequencies of the 11 restriction map variants in chromosomes bearing normal *Adh* alleles shows that the Cygnet and Huonville II samples do not differ from each other ( $\chi^2_{10} = 13.94$ ,  $p > 0.05$ ) but they do differ from Tamar ( $\chi^2_{10} = 18.69$ ,  $p < 0.05$ , and  $\chi^2_{10} = 36.4$ ,  $p < 0.001$ , respectively).

Of the 58 chromosomes investigated all except one had the expected single copy of the *Adh* gene and flanking DNA. Tamar 40 was exceptional in that the region which hybridised to the sAF2 clone was 16 kb. This chromosome is being analysed in detail but it appears that a duplicate of the region between -6.2 and -3.2 is inserted at +5.2 and a duplicate of the region from -1.2 to 0, which includes part of the *Adh* coding region, is inserted at +4.0.

The tabulations show that although 14 different haplotypes occurred, each of the twelve *Adh* null bearing chromosomes had the same haplotype, regardless of the population from which they were derived. It is noteworthy that the null allele haplotype does not include either of the two insertions or the deletion detected in the Tasmania populations.

Inspection of the data reveals that in the Tamar sample the three null alleles share the haplotype of one normal *Adh* allele, Tamar 33. In the Cygnet samples two chromosomes, Cygnet 19 and 25, and in Huonville II one chromosome, Huonville 16, have the haplotype of the null allele. Each of these four chromosomes carries the *Adh*<sup>S</sup> allele.

Amongst the chromosomes carrying normal *Adh* alleles there was also some significant gametic disequilibria in each population (table 4). The

same three disequilibria were present in Cygnet and Huonville II, but none of these were significant in Tamar.

## DISCUSSION

The overall level of restriction endonuclease variation in the *Adh* region of the Tasmanian populations is similar to that previously described for this gene region in other populations of *D. melanogaster* (Cross and Birley, 1986; Aquadro *et al.*, 1986). In particular the estimates of overall heterozygosity (Nei and Tajima, 1981) (table 5) are very similar to those reported by Cross and Birley (1986) for the Australian Chateau Tahbilk population which is located on the mainland 525 km north of Tamar. Amongst chromosomes bearing normal *Adh* alleles there was significant heterogeneity between the Tasmanian populations with Cygnet and Huonville II differing from Tamar in the frequencies of the eleven restriction map variants and in the patterns of gametic disequilibria. However, the proportion of polymorphic nucleotide sites,  $\hat{p}$  (Hudson, 1982), and the estimated heterozygosity per nucleotide pair,  $\hat{\theta}$  (Ewens, Spielman and Harris, 1981), were each significantly lower ( $p < 0.05$ ) in Huonville II than in Cygnet or Tamar (table 5). Elsewhere we will compare the *Adh* restriction map variation in the Tasmanian populations with similar data we have collected for a number of mainland populations spanning 30 degrees of latitude along the *Adh*<sup>S</sup> cline described by Wilks *et al.* (1980).

The main aim, however, of the experiments described here was to investigate the molecular landscape surrounding the *Adh* locus in a sample of separately isolated null activity alleles so that they could be compared with one another and with

**Table 4** Significant gametic disequilibria in the Tasmanian populations

Population	Number of comparisons	Significant gametic disequilibria
Tamar	55	<i>Bam</i> HI (-7.2): <i>Adh</i> , $p < 0.01$ <i>Hpa</i> I (-6.9): <i>Bam</i> HI (+3.9), $p < 0.01$ <i>Adh</i> (+0.2): <i>Bam</i> HI (+3.9), $p < 0.01$ <i>Pst</i> I (+1.0): $\Delta$ 400, $p < 0.01$ <i>Xho</i> I (+1.2): <i>Eco</i> RI (+9.0), $p < 0.05$
Cygnet	45	<i>Hind</i> III (-3.0): <i>Adh</i> , $p < 0.05$ <i>Hind</i> III (-3.0): $\nabla$ 200, $p < 0.001$ $\nabla$ 200: <i>Adh</i> (+0.2), $p < 0.05$
Huonville II	10	<i>Hind</i> III (-3.0): <i>Adh</i> , $p < 0.05$ <i>Hind</i> III (-3.0): $\nabla$ 200, $p < 0.001$ $\nabla$ 200: <i>Adh</i> (+0.2), $p < 0.05$

**Table 5** Estimates of the proportion of polymorphic nucleotides,  $\hat{p}$ , overall heterozygosity,  $h$  (Nei and Tajima, 1981) and estimated heterozygosity per nucleotide pair,  $\hat{\theta}$  (Ewens, Spielman and Harris, 1981). Standard errors are given in parentheses and that for  $\hat{\theta}$  assumes free recombination

Population	$\hat{p}$	$\hat{h}$	$\hat{\theta}$
Tamar	0.019 (0.007)	0.86 (0.057)	0.007 (0.0026)
Cygnnet	0.017 (0.007)	0.95 (0.014)	0.006 (0.0024)
Huonville II	0.009 (0.004)	0.85 (0.06)	0.003 (0.0015)

normal alleles from the same populations. The results show that, regardless of the population or year of origin, each of the 12 *Adh* null alleles has the same haplotype as revealed by the 8 hexanucleotide restriction endonucleases we have used. The data for the Huonville II and Tamar populations are particularly revealing as 8 and 3 null alleles respectively were available for comparison with normal alleles. Amongst the normal *Adh* alleles the null allele haplotype occurred at a frequency of 0.07 in each population yet all three Tamar nulls were identical ( $p < 0.001$ ), as were the eight null alleles from Huonville II ( $p < 0.001$ ).

It is significant that the null alleles do not contain any insertions or deletions in the size range which could be resolved with the technique used (greater than 50 bp). This observation eliminates the possibility that the loss of ADH activity and the relatively high frequency of null alleles were brought about by the insertion of a mobile element of this size in, or close to, the *Adh* gene. In this context it is also worth mentioning that attempts to use *P* element insertion to induce mutations at the *Adh* locus have not been successful (Kidwell, 1986).

The data described here strongly suggest that the *Adh* regions of the separately extracted null alleles from the Tasmanian populations share a common ancestry, which implies that the null alleles may well be multiple copies of the same mutation. A possible, but in our view unlikely, alternative hypothesis which cannot be discounted with the present data, is that the null alleles derive from multiple mutations at an *Adh* allele in a specific molecular landscape. If this were so it might be expected that the separate mutations would possibly differ in structure and in biochemical properties. The observation that all the nulls investigated here have been shown to produce mRNA's that are larger than normal, and all have similar biochemical properties, mitigates against

this hypothesis (Freeth, 1986). Clearly, DNA sequence data from null alleles from the Tasmanian populations is required to fully test this possibility and to elucidate the molecular lesion leading to loss of ADH activity.

The observation that the four chromosomes which share the null allele haplotype all carry the *Adh<sup>S</sup>* allele suggests the mutation which resulted in the loss of ADH activity occurred in that allele. Again, the DNA sequence of a null allele will be required to confirm this suggestion. It is interesting that the frequency of *Adh<sup>S</sup>* is higher in the south of Tasmania than in the north (see data in Anderson and Gibson, 1985; in the samples used in the present study the *Adh<sup>S</sup>* frequencies were 0.45 in Cygnnet, 0.42 in Huonville II and 0.15 in Tamar) whereas in Australasia as a whole the frequency of *Adh<sup>S</sup>* is highest in populations from the northern latitudes (Wilks *et al.*, 1980).

If, as seems likely, the null alleles turn out to be multiple copies of the same mutant, then the null has increased in frequency and spread to populations (at least 300 km apart) which to some extent are genetically differentiated at the *Adh* locus. Tests of the Huonville II population show that the null has persisted over at least 3 years and this population, in common with the other Tasmanian populations, experiences severe bottlenecks in the winter months. An *Adh* null allele has also been detected at the All Saints population in northern Victoria (350 km north of the Bass Strait which is approximately 275 km wide and separates Tasmania from the mainland) but as this null was detected in a sample of frozen flies it could not be isolated (Freeth, 1986). It will be interesting to analyse null alleles from mainland populations to see whether they too share the same haplotype.

We have little evidence, as yet, to suggest why the null allele increased in frequency. Our estimates of the depression of heterozygote fitness, based on the average frequency of *Adh* null alleles in Tasmania, is about 0.0003 (Freeth and Gibson, 1985). ADH activity is completely absent from the null homozygotes and the level in heterozygotes between the null allele and *Adh<sup>F</sup>* or *Adh<sup>S</sup>* is about half that in normal homozygotes (Freeth, Gibson and de Couet, 1986). This reduction is not trivial but in view of the data of Middleton and Kacser (1983) the difference may not be large enough to affect ethanol metabolism *per se*, although other reactions on which the enzyme impinges may be changed. There is evidence that heterozygotes between the nulls and normal alleles have a higher viability on ethanol media than expected from their ADH levels (Freeth, Gibson and de Couet, 1986).

Future studies on the fitness and population distribution of the *Adh* null alleles will be aided by the information that they are probably all copies of the same allele. By using tetra nucleotide endonucleases, detailed restriction maps of the cloned *Adh* null alleles can be constructed and should provide evidence on the origin of the alleles. The nucleotide divergence between alleles will give an indication of when the mutation occurred.

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