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Molecular similarity of *Drosophila melanogaster* alcohol dehydrogenase thermostable alleles from populations on different continents

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Allele specific oligonucleotide probes have been used to show that DNA, amplified by the polymerase chain reaction, from eleven thermostable *Adh* alleles extracted from populations on different continents contains the triplet TCC, which distinguishes Adh^{FChD} (Fast Chateau Douglas) from Adh^{F} (Fast). The molecular similarity of *Adh* thermostable alleles suggests that they had a common origin, and it is argued that the mutation probably occurred in China where high frequencies of Adh^{FChD} are found.

INTRODUCTION

Most natural populations of *Drosophila melanogaster* are polymorphic for two electrophoretically detectable alleles at the alcohol dehydrogenase (ADH; EC 1.1.1.1) locus (*Adh*) (Vigue and Johnson, 1973; Oakeshott *et al.*, 1982; Singh *et al.*, 1982). The proteins encoded by these two alleles, (Adh^{S} and Adh^{F}) differ by a single amino acid at residue 192, with lysine in ADH-S replaced by threonine in ADH-F (Thatcher, 1980). Within the ADH-F electrophoretic class a third category of variant has been identified because the ADH is very much more thermostable than either ADH-F or ADH-S. These thermostable variants were first found in a laboratory stock (Thörig *et al.*, 1975) and subsequently at low but polymorphic frequencies in natural populations in North America (Milkman, 1976; Sampsell, 1977), Australia (Lewis and Gibson, 1978; Wilks *et al.*, 1980), the Canary Islands (Hernandez *et al.*, 1986) and China (Jiang *et al.*, 1989). Comparisons of other biochemical properties of the *Adh* thermostability variants with those of ADH-S and ADH-F have shown that the thermostable variants tend to be intermediate with respect to the amounts of ADH protein and they have a secondary alcohol/primary alcohol activity ratio characteristic of ADH-S rather than ADH-F (Lewis and Gibson, 1978; Gibson *et al.*, 1980; Chambers *et al.*, 1981b; Gibson *et al.*, 1982*b*; Sampsell and Steward, 1983; Jiang *et al.*, 1989).

Chambers *et al.* (1981*a*) determined the amino acid sequence of 70 per cent of the protein produced by one of these thermostable variants, *Adh Fast Chateau Douglas* (Adh^{FChD}) extracted from an Australian population, and showed that it differed from ADH-F by the substitution of serine for proline at residue 214. Nucleotide sequence analysis of Adh^{FChD} has confirmed this structure (Collet, 1988) by showing that the relevant codon is TCC, compared to CCC in Adh^{F} alleles (Kreitman, 1983).

The similarities in biochemical properties of the Adh^{F} thermostable variants prompt the speculation that they all share the same mutation and are perhaps derived from a single mutational event. Although it seems very likely that the substitution of serine for proline in Adh^{FChD} is the cause of the increased heat stability of ADH-FChD protein it is possible that in other thermostable variants a different mutation has brought about increases in thermostability. Thus it is possible that the *Adh* thermostable alleles from geographically widely separated populations, or even within the same population, are molecularly heterogeneous.

To test whether *Adh* thermostable variants isolated from populations on different continents between 1971 and 1986 all have the nucleotide sequence TCC, which is found in Adh^{FChD} at posi-

tion 1555–1557 we have used allele specific oligonucleotides (ASO) to probe DNA from thermostable and normal Adh^F alleles. A similar experimental approach has been used to identify variants of β^s-globin alleles (Conner *et al.*, 1983) and α_1-antitrypsin deficiency alleles (Kidd *et al.*, 1983), and to investigate polymorphism at the HLA-DQα locus (Saiki *et al.*, 1986) in human material.

MATERIALS AND METHODS

The population origins of the *Adh* thermostable variants compared in this study are listed in table 1. In addition a number of single allele Adh^F lines and an Adh^S line, derived from North American, Australian and Chinese populations, were used as controls. All of the alleles used have been maintained as homozygotes in mass cultures since their isolation from natural populations.

Table 1 Geographical origins of alcohol dehydrogenase alleles encoding heat resistant ADH

Continent	Alleles
North America	*Sf*1—Cedar Rapids, Iowa, 1975 (Sampsell and Steward, 1983) *Si*44—Cedar Rapids, Iowa, 1975 (Sampsell and Steward, 1983) *AR*72—Tempe, Arizona, 1976. (Sampsell and Steward, 1983) *CS*47—Chicago, Illinois, 1980. (Sampsell and Steward, 1983) *BG*27—Bowling Green, Ohio, 1981. (Sampsell and Steward, 1983)
Europe*	71*k*—isolated in 1971 from a N^8/*delta*-49 laboratory stock held in Utrecht (Thörig *et al.*, 1975)
China	*H*15—Haikou 1986. (Jiang *et al.*, 1989) *H*20—Haikou 1986. (Jiang *et al.*, 1989) *G*15—Guangzhou 1986. (Jiang *et al.*, 1989)
Australia	*ChD*12—Chateau Douglas, Segenhoe, NSW, 1975. (Lewis and Gibson, 1978) *Cr*14—Craigmoor, NSW, 1980. (Gibson *et al.*, 1982*b*)

* The N^8/*delta*-49 stock probably originated in North America.

To reduce non-specific hybridisation to the allele specific oligonucleotide probes (*AF* and *AFD*, see table 2), which was found in preliminary experiments to occur with genomic DNA, and to obviate the need to clone each of the thermostable alleles, we have used synthetic oligonucleotide primers and the polymerase chain reaction (PCR) to amplify a 368 bp region of the *Adh* gene, which includes the nucleotides at position 1555–1557 specifying the proline or serine residue (fig. 1). Genomic DNA (approximately 1 μg), prepared as described by Chia *et al.* (1985), was amplified using 2 units of *Taq* DNA polymerase as described by Saiki *et al.* (1988). The amplification reaction mixture included the target DNA and 50 pmol of each of the primers

JG5 (5′-GTGGTCAACTTCACCAGCTC-3′)

and

JG6 (5′-CTTCTTAGATGCCGGAGTCC-3′)

which encompass a region of the *Adh* gene from nucleotide 1320–1687 (Kreitman, 1983) (fig. 1). The amplification reaction, carried out in 0·5 ml microcentrifuge tubes, was repeated for 35 cycles—

Table 2 DNA sequences of the synthetic oligonucleotide probes *AF* and *AFD*. The single nucleotide change distinguishing Adh^{FChD} from Adh^F is underlined

Gene	Probe	DNA sequence
Adh^F	*AF*	5′ CCC ACC CAG CCC TCG TTG G 3′
Adh^{FChD}	*AFD*	5′ CCC ACC CAG TCC TCG TTG G 3′

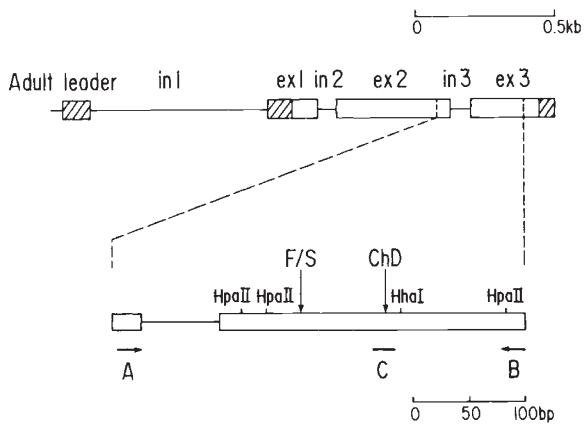

Figure 1 The structure of the *Adh* gene in *D. melanogaster* showing the relative sizes of the three exons and three introns. The non-translated regions are hatched. The 368 bp region amplified by the PCR is drawn in more detail. The sites of the Lys ← → Thr substitution distinguishing Adh^S and Adh^F and the Ser ← → Pro substitution in Adh^{FChD} are indicated by vertical arrows. The positions of some restriction endonuclease sites are shown. The positions of the two amplification primers used in the PCR reaction are indicated at A and B and the region to which the oligonucleotide probes *AF* and *AFD* hybridise is shown at C.

a cycle consisted of 1 min for denaturation of DNA at 94°C, 2 min for annealing of the DNA and primers at 50°C and 3 min for primer extension at 72°C—using a Perkin-Elmer Cetus Thermal Cycler.

The amplified DNA (5 μl) was denatured in 0·4 M NaOH for 10 min and then transferred to a Zeta-Probe membrane in the Bio-Rad slot blotting apparatus, using the manufacturer's instructions. The blotted membrane was rinsed in 2 × SSC, air-dried and then sandwiched between two sheets of Whatman 3MM filter paper soaked in distilled water. The membrane/filter paper sandwich was sealed in a plastic bag with the prehybridisation mixture which contained 5 × SSC, 20 mM NaH_2PO_4 *p*H 7·0, 4 per cent SDS, 10 × Denhardt's solution, 10 per cent dextran sulfate and denatured herring sperm DNA at 100 μg/ml, and incubated for 1 h at 59°C. One of the allele specific oligonucleotide probes (*AF* or *AFD*), labelled with [γ-^{32}P] dATP, was then added to the plastic bag and hybridised overnight at 59°C. The membrane was washed at 59°C for 30 mins in a solution containing 3 × SSC, 25 mM NaH_2PO_4 *p*H 7·5, 5 per cent SDS, 10 × Denhardt's solution, and then at the same temperature for 30 mins in 1 × SSC with 1 per cent SDS. The membranes were blotted with Whatman 3MM filter paper, wrapped in plastic film and exposed to Kodak film (XRP-1) for 3 hours at −20°C.

For Southern analyses the amplified DNA samples were digested with *Hha*I and electrophoresed on a 2 per cent agarose mini gel at 7 v/cm for 90 min at room temperature. The DNA fragments were transferred from the agarose gels to Zeta-Probe membranes by alkaline blotting (0·4 M NaOH). The membranes were rinsed in 2 × SSC, blotted dry and baked at 80°C for 45 min. The membranes were then wetted in distilled water, sandwiched between two sheets of filter paper and treated as described above.

The oligonucleotides were synthesised on an Applied Biosystems 380B DNA synthesiser.

RESULTS

The DNA sequences of the oligonucleotides used as probes are shown in table 2. Probe *AFD* was designed to be a perfect match to Adh^{FChD} and the mutation distinguishing this allele from Adh^{F} was placed close to the middle of the sequence of 19 nucleotides to maximise the thermal instability of mismatch hybridisation (Wallace *et al.*, 1981). Based on the length and G:C content of the two oligonucleotides, the T_ms for the probes were calculated to be 64°C for *AFD* and 66°C for *AF*. Theoretically a perfectly matched oligonucleotide-DNA duplex would be stable at 5°C below the T_m value, and a further 5°C reduction would be necessary to maintain hybrid stability for every mismatched base pair. (Wallace *et al.*, 1981). Preliminary experiments indicated that in hybridisations carried out at 54°C, probe *AF* hybridised with similar intensities to DNA from Adh^{F} and Adh^{FChD}. However, at 59°C both probes discriminated between the Adh^{FChD} and Adh^{F} control alleles (fig. 2).

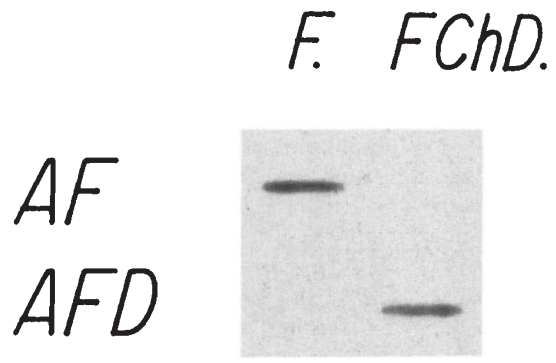

Figure 2 PCR amplified DNA from Adh^{F} and Adh^{FChD} control alleles blotted onto a Zeta-Probe membrane and probed with the oligonucleotides *AF* and *AFD* (see text); hybridisation was carried out at 59°C.

The fidelity of the polymerase chain reaction was checked by digesting samples of the amplified DNA with two restriction endonucleases: *Hha* I which was expected to generate fragments of 248 and 120 bp and *Hpa* II which cuts at three sites in the region giving fragments of 114, 27, 213 and 14 bp (fig. 1). In all cases investigated, the amplified DNA migrated as a single band of the size expected and the digestion products conformed to the sizes expected from the standard sequence (*Hha* I digestion shown in fig. 3 (a) and (d)), indicating that there was little non-specific amplification and that none of the alleles tested had any detectable insertions or deletions (>20 bp) in the amplified region.

Southern blots prepared from *Hha* I digests of amplified DNA from thermostable Adh^{F} alleles and from control Adh^{F} and Adh^{S} alleles were probed with *AFD* and *AF*. These results show that *AF* hybridised to the 248 bp *Hha* I fragment of the Adh^{F} alleles but not to DNA from the

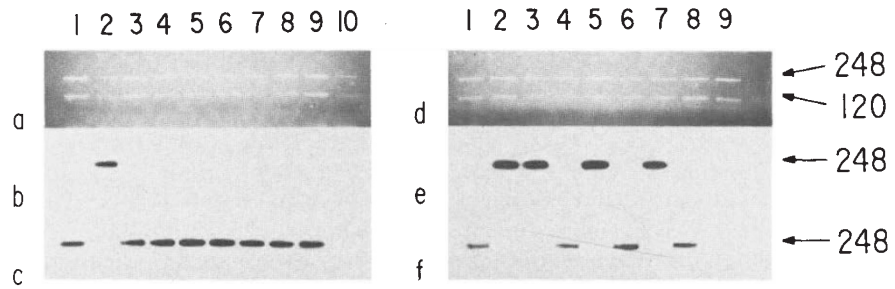

Figure 3 PCR amplified DNA digested with *Hha* I: (a) and (d) after electrophoresis on agarose gel, (b), (c), (e) and (f) similar gels blotted on a Zeta-Probe membrane and probed at 59°C; for (b) and (e) the probe was *AF*, for (c) and (f) the probe was *AFD*. *Adh* variants in lanes 1-10, in (a), (b) and (c): *FChD*12, *F*, *CS*47, *Si*44, *BG*27, *AR*72, *Sf*1, *71K*, *FCr*14, *S*.; in lanes 1-9 in (d), (e) and (f): *FChD*12, *F*, *G*1, *G*15, *H*6, *H*15, *H*36, *H*20, *S*.

The thermostable alleles are underlined, F is a control Adh^F and S is a control Adh^S, the other variants are all electrophoretically "fast". The origins of the thermostable *Adh* alleles are given in table 1; *G*1 was extracted from Guangzhou and *H*6 and *H*36 from Haikou.

thermostable *Adh* alleles or the Adh^S allele (fig. 3(b) and (e)). The probe *AFD* hybridised to the same *Hha* I fragment, but only to DNA from *Adh* alleles encoding heat resistant ADH; it did not hybridise to DNA from the Adh^F or the Adh^S control alleles (fig. 3(c) and (f)). The *AFD* probe hybridised to heat resistant *Adh* alleles from each continent; in no case did the *AFD* probe fail to hybridise to a heat resistant *Adh* allele.

DISCUSSION

The results show that under stringent hybridisation conditions the two synthetic oligonucleotides discriminate between Adh^F and Adh^{FChD} and identify similar thermostable alleles. Neither probe hybridised to Adh^S DNA under these stringency conditions (although both do when the stringency is relaxed). The collated data on the nucleotide sequences of six Adh^S alleles and six Adh^F alleles (table 3) indicate that the *AF* probe is likely to be a perfect match to Adh^F alleles but not to all Adh^S alleles. These data suggest that the Adh^S allele we used as a control (which was isolated from the same Tasmanian population as one of the Adh^F controls) has the sequence CCA at position 1555-1557 and thus has at least one mismatch with both probes.

The Southern blots show that thermostable *Adh* alleles isolated over a fifteen year period from eight different populations on three continents are indistinguishable in these experiments from Adh^{FChD}. It therefore is highly likely that all of these thermostable variants have the DNA sequence TCC and have serine at residue 214 as does ADH-FChD.

The highly consistent relationship between the relatively high thermostability of the ADH protein and the substitution of serine for proline at residue 214 in a series of alleles with a variety of genetic backgrounds indicates that protein structural changes accompanying this substitution cause the change in thermostability (Chambers *et al.*, 1981*a*).

The molecular similarity of the *Adh* thermostable alleles lends support to the hypothesis (Jiang

Table 3 Amino acid and DNA sequences of alcohol dehydrogenase alleles in the region covered by the synthetic oligonucleotide probes *AF* and *AFD* (variation has not been reported in the flanking sixteen nucleotides)

	Allele sequenced		
Origin of allele	Adh^S	Adh^F	Adh^{FChD}
U.S.A. (Seattle)*	Pro CCA	Pro CCC	—
U.S.A. (Palm Beach)*	Pro CCA	Pro CCC	—
U.S.A. (Palm Beach)*	Pro CCA	—	—
France (Bully)*	Pro CCA	Pro CCC	—
Africa (Burindi)*	Pro CCA	Pro CCC	—
Japan (Ishigaki)*	Pro CCC	Pro CCC	—
Australia (Huonville)†	—	Pro CCC	—
Australia (Segenhoe)‡	—	—	Ser TCC

Data from * Kreitmann (1983); † Gibson and Wilks (unpublished); ‡ Collet (1988).

et al., 1989) that they are derived from the same mutation. The amino acid and nucleotide sequences of Adh^{FChD}, Adh^{F} and Adh^{S} alleles show that Adh^{FChD} was derived from Adh^{F}. There is evidence that Adh^{S} is ancestral to Adh^{F} as the ADH's of *D. simulans* and *D. mauritiana* both have lysine at residue 192 rather than threonine (Ashburner *et al.*, 1984). Estimates of the age of the Adh^{F}/Adh^{S} polymorphism in *D. melanogaster* range from 610,000 years to 3·5 Myr (Stephens and Nei, 1985) with those authors preferring 1 Myr as the best estimate available for the time of the Adh^{F} mutation. Based on the relatively low level of sequence variation between a single Adh^{FChD} and five Adh^{F} haplotypes Collet (1988) estimated the time for the divergence of Adh^{FChD} from Adh^{F} as 260,000 to 473,000 years. The proline/serine change does not occur in other members of the species group (Bodmer and Ashburner, 1984), so the region is not a "hot spot" for mutational change.

The population distribution of Adh^{FChD} is intriguing and relevant to hypotheses about its origin. Far fewer natural populations have been scored for *Adh* thermostable alleles than for Adh^{F}, but in the data that are available the highest frequency of Adh^{FChD} is in southern China where it reaches 30 per cent (Jiang *et al.*, 1989). Although the allele occurs at low frequency in the Canary Islands (Hernández *et al.*, 1986) it has not so far been reported from the African continent where the *melanogaster* subgroup originated (Lemeunier *et al.*, 1985; Lachaise *et al.*, 1988).

In populations sampled in North America and Australia, where most data are available, the frequencies of Adh^{F} thermostable alleles tend to be less than 5 per cent (Sampsell, 1977; Wilks *et al.*, 1980). The highest frequencies occur in populations with intermediate levels of Adh^{F}, and this pattern of distribution does not appear to track any climatic variable. The exceptionally high frequencies in southern China are not matched at sites elsewhere with similar climates. This leads us to suggest that the present geographical distribution of Adh^{FChD} could have arisen if the mutation first occurred in southern China and from there dispersed. However, as the mutation is estimated to have occurred 260,000 to 473,000 years ago, this would imply that *D. melanogaster* colonised the Eurasian continent very much earlier than the minimum of 6500 to 9500 years ago suggested by Lachaise *et al.* (1988). It is believed that *D. melanogaster* probably became a colonist of North America (Sturtevant, 1920) and Australia in the past two hundred years (Jiang *et al.*, 1989).

Further speculation on the origin and geographical distribution of Adh^{FChD} will benefit from more extensive data on population frequencies in Africa, in other parts of Asia, and particularly in South America where no data are presently available. It will then be possible to compare the complete nucleotide sequences of a number of Adh^{FChD} thermostable alleles with those of Adh^{F} to discern their lineages and obtain further evidence on the time of occurrence of the Adh^{FChD} mutation. Whether Adh^{FChD} alleles turn out to have had a single or multiple origin, the evidence that heat resistant alleles are molecularly similar will facilitate population comparisons of *Adh* frequencies (Gibson *et al.*, 1982*a*).

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