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A WORLDWIDE POLYMORPHISM IN *ALDEHYDE DEHYDROGENASE* IN *DROSOPHILA MELANOGASTER*: EVIDENCE FOR SELECTION MEDIATED BY DIETARY ETHANOL

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Clinally varying traits in *Drosophila melanogaster* provide good opportunities for elucidating the genetic basis of adaptation. Resistance to ethanol, a natural component of *D. melanogaster*'s breeding sites, increases with latitude on multiple continents, indicating that the trait is under selection. Although the well-studied *Alcohol dehydrogenase* (*Adh*) polymorphism makes a contribution to the clines, it accounts for only a small proportion of the phenotypic variation. We describe an amino acid replacement polymorphism in *Aldehyde dehydrogenase* (*Aldh*), the gene encoding the second enzyme in the ethanol degradation pathway, that shows hallmarks of also contributing to the clines. The derived *Aldh* allele, like the *Adh-Fast* allele, increases in frequency in laboratory populations selected for ethanol resistance, and increases in frequency with latitude in wild populations. Moreover, strains with the derived allele have significantly higher ALDH enzyme activity with acetaldehyde (the breakdown product of ethanol) as a substrate than strains with the ancestral allele. As is the case with the *Adh-Fast* allele, chromosomes with the derived *Aldh* allele show markedly reduced molecular variation in the vicinity of the replacement polymorphism compared to those with the ancestral allele, suggesting a single, relatively recent origin. Nonetheless, the *Aldh* polymorphism differs from the *Adh* polymorphism in that the ethanol-associated allele remains in relatively low frequency in most populations. We present evidence that this is likely to be the result of a trade-off in catalytic activity, with the advantage of the derived allele in acetaldehyde detoxification being offset by a disadvantage in detoxification of other aldehydes.

KEY WORDS: **Adaptation, molecular evolution, physiology, selection—experimental, selection—natural, trade-offs.**

Elucidating the genetic basis of adaptation is one of the major goals of evolutionary biology. Recent methodological advances, such at QTL mapping and whole-genome expression profiling, have made it increasingly feasible to identify genes, or at least small chromosome regions, that contribute to adaptive phenotypic differences between populations or species. Nonetheless, identifying the precise molecular changes that contribute to adaptation remains challenging (cf. Hoekstra and Coyne 2007). One of the major difficulties is that in any identified gene or chromosome region, for every phenotypically significant molecular difference between a pair of populations or species, there are likely to be many with no phenotypic effects.

Clinally varying traits in the fruit fly *Drosophila melanogaster* provide good opportunities for characterizing the molecular basis of adaptations. In this cosmopolitan species, several physiological and morphological traits have been shown to

vary with latitude in a similar fashion on two or more continents (reviewed in Robinson et al. 2000; de Jong and Bochdanovits 2003; Hoffmann and Weeks 2007). Such parallel clines would be unlikely to result from genetic drift, and therefore give strong evidence that the traits in question are under natural selection. Moreover, outside of centromeric and telomeric regions, the population recombination rate in *D. melanogaster* appears to be sufficient to ensure that polymorphisms showing clinal variation are likely to be the targets of clinally varying selection, as opposed to merely being in linkage disequilibrium with the selected sites (Berry and Kreitman 1993). Therefore any clinal polymorphism in a gene known (e.g., through analysis of null mutations) to affect a clinal trait would be a good candidate for contributing to the phenotypic cline.

Ethanol resistance is one of the best-documented traits that shows consistent variation with latitude in *D. melanogaster*. Ethanol is a natural constituent of the decaying fruit in which many *Drosophila* species breed, and *D. melanogaster* appears to have a particular propensity for breeding in fruits high in ethanol, as well as in breweries and wineries (McKenzie and McKechnie 1979; Gibson et al. 1981; Merçot et al. 1994). At low concentrations, ethanol is a beneficial resource for *Drosophila*, but at high concentrations, it is toxic (Parsons et al. 1979). Statistically significant positive correlations of ethanol resistance with latitude have been reported among *D. melanogaster* strains collected from Northern Africa and Europe (David and Bocquet 1975; David et al. 1986), Southern Africa (David et al. 1986), India (Parkash et al. 1999), Australia (Anderson 1982; cf. Montooth et al. 2006), and North America (Cohan and Graf 1985). Although the reason that selection should favor higher ethanol resistance in temperate regions than in the tropics is not clear (see below), it seems unlikely that the clines are byproducts of climatic adaptation unrelated to ethanol consumption. Although *Drosophila* species with exclusively temperate ranges are considerably more cold tolerant than *D. melanogaster* (Gibert et al. 2001), the ethanol resistance of temperate *D. melanogaster* populations is exceptional within the genus (Merçot et al. 1994). The two main hypotheses for the maintenance of the clines are that ethanol levels in decaying fruits tend to be higher in temperate regions (Stanley and Parsons 1981), or that selection favors greater exploitation of ethanol-rich fruits in the temperate zone than in the tropics, independent of differences in availability (Eanes 1999).

At present, surprisingly little is known about the genetic basis of the ethanol resistance clines. Much work has been done on the role of the *Alcohol dehydrogenase* polymorphism in ethanol resistance variation; the ADH enzyme catalyzes the first step in ethanol metabolism, the oxidation of ethanol to acetaldehyde (reviewed in van Delden 1982; Chambers 1988; Geer et al. 1993; Heinstra 1993; Eanes 1999). The *Fast* electromorph of *Adh* confers greater ADH activity than the *Slow* electromorph, through both higher specific activity and higher level of ADH protein. The higher level of ADH protein of *Fast* strains is caused by polymorphisms in linkage disequilibrium with the mobility-altering replacement polymorphism, most notably an indel in the first intron (Laurie and Stam 1994; Stam and Laurie 1996). Consistent with its greater activity, the *F* allele usually (but not always) increases in frequency when laboratory populations are selected for ethanol resistance (Gibson 1970; van Delden et al. 1975; Cavener and Clegg 1978). The *F* allele also increases in frequency with latitude on multiple continents (Oakeshott et al. 1982), consistent with the ethanol resistance clines. Nonetheless, the *Adh* polymorphism apparently accounts for only a relatively small proportion of the latitudinal variation in ethanol resistance. Homozygous *F* and *S* strains from the same population generally show only modest differences in ethanol resistance (Oakeshott et al. 1980; Kerver and van Delden 1985; reviewed in Heinstra 1993). Moreover, Anderson (1982) found that adult ethanol resistance of isofemale lines collected from Australia showed a significant partial correlation with latitude controlling for *Adh* allele frequency, and our analysis of the combined data of David et al. (1986; their table 1) and Parkash et al. (1999; their tables 1 and 2) yields a similar conclusion (partial correlation of LC50 for ethanol with latitude, controlling for *Adh-F* allele frequency $= 0.63$, $P < 0.001$, $N = 43$ populations).

The second enzyme in the ethanol degradation pathway is aldehyde dehydrogenase (ALDH, E.C. 1.2.1.3), which catalyzes the NAD^+-dependent oxidation of the toxic intermediate acetaldehyde to the nontoxic acetate. ALDH is believed to be the main enzyme responsible for the oxidation of acetaldehyde in mammals (Weiner 1979). We have recently identified the main ALDH structural gene in *D. melanogaster*, *Aldh*, and shown that *Aldh* null mutants are killed by ethanol concentrations easily tolerated by wild-types (Fry and Saweikis 2006). Contrary to previous suggestions that adults and larvae metabolize acetaldehyde differently (Geer et al. 1985; Heinstra et al. 1989), with larvae relying on the acetaldehyde-oxidizing ability of ADH rather than ALDH (Heinstra et al. 1983; Eisses et al. 1985; Geer et al. 1985), *Aldh* null larvae were no less ethanol sensitive than adults.

Given the clear importance of *Aldh* in ethanol resistance, we have investigated whether this gene, like *Adh*, has amino acid replacement polymorphisms that contribute to the ethanol resistance cline. Here, we describe a replacement polymorphism in *Aldh* in which the derived allele, like the *Adh-F* allele, increases in frequency in laboratory populations selected for ethanol resistance, and increases in frequency with latitude in wild populations. Moreover, strains with the derived *Aldh* allele have significantly higher ALDH enzyme activity with acetaldehyde as a substrate than strains with the ancestral allele. As is the case with the *Adh-F* allele, chromosomes with the derived *Aldh* allele show markedly reduced molecular variation in the vicinity of the polymorphic site

compared with those with the ancestral allele, suggesting a single, relatively recent origin. Nonetheless, the *Aldh* polymorphism differs from the *Adh* polymorphism in that the ethanol-associated allele remains in relatively low frequency (20% or less) in most populations. We present evidence that this is likely to be the result of a trade-off in catalytic activity, with the advantage of the derived allele in acetaldehyde detoxification being offset by a disadvantage in detoxification of other aldehydes.

Materials and Methods

STRAINS AND REARING CONDITIONS

Flies were reared on standard cornmeal-molasses-Brewer's yeast-agar medium at 25°C. The following fly strains were used: four ethanol-selected and two control populations from Fry et al. (2004); 76 iso-second chromosome lines collected in central Pennsylvania in 1998 and 1999 (Lazzaro et al. 2004), kindly provided by B. Lazzaro; 13 isofemale lines collected in 1994 at Lake Kariba, Zimbabwe, kindly provided by C. Aquadro; 36 isofemale lines collected in 2004 from six locations in Cameroon (Pool and Aquadro 2006), kindly provided by J. Pool; preserved single flies from 7 to 10 isofemale lines from each of five locations in Europe, kindly provided by C. Schlötterer (collecting locations and dates: Weil am Rhein, Germany, 2000; Harjavalta, Finland, 1996; Copenhagen, Denmark, 1999; Katowice, Poland, 2000; and Naples, Italy, 2001); 104 isofemale lines collected along a latitudinal transect in eastern Australia in 2004 (Umina et al. 2005), kindly provided by A. Hoffmann; 294 iso-second chromosome lines collected along a latitudinal transect in the eastern United States in 1997 (Verrelli and Eanes 2001), kindly provided by W. Eanes; two isofemale lines collected near Vienna, Austria in 2004, kindly provided by C. Schlötterer.

SEQUENCING AND GENOTYPING

DNA was extracted using the Puregene DNA extraction kit (Gentra Systems, Minneapolis, MN), and the entire *Aldh*-coding region amplified in five segments using the primers shown in online Supplementary Table S1. PCR products were directly sequenced in both directions on an Applied Biosystems (Foster City, CA) Automated 3730 DNA Analyzer, using Big Dye Terminator chemistry and AmpliTaq-FS DNA Polymerase. To avoid heterozygosity, flies from the experimental populations and the isofemale lines were usually either crossed to a strain with a deficiency for the *Aldh* region, in which case DNA was extracted from a single hemizygous offspring, or used to generate iso-second chromosome lines by crossing to a balancer stock, following Fry and Saweikis (2006). Single individuals from five of the Cameroon lines were sequenced without these precautions; one showed evidence for heterozygosity and was excluded from the analyses. Chromatograms were visually inspected and sequences aligned using Sequencher software (Gene Codes Corporation, Ann Arbor, MI). All sequences were deposited in GenBank (accession numbers EU154355-EU154402).

Sequencing the entire coding region in six lines (see Results) revealed a single replacement polymorphism in which the derived allele ("*Phe*") creates a BbsI restriction site. Digestion with BbsI (New England Biolabs, Ipswich, MA) was therefore used to genotype the polymorphism in all the lines described above. This method would be misleading if some *Phe* alleles had additional substitutions near the replacement substitution that disrupted the BbsI site. Sequencing the region surrounding the replacement polymorphism in an additional 42 lines from four continents (see Results), however, revealed complete correspondence between genotype inferred by BbsI digestion and that revealed by sequencing. The additional sequences also provide information on levels of molecular variation in phenylalanine (*Phe)* and leucine (*Leu*) (ancestral) alleles, and allow inference of whether the *Phe* replacement substitution arose once or multiple times.

ALDH ACTIVITY ASSAYS

To determine whether the replacement polymorphism is associated with variation in catalytic activity, ALDH activity of whole-fly homogenates was measured in 24 of the Pennsylvania lines, 8 *Phe* and 16 *Leu* (see Fry et al. 2004 and Fry and Saweikis 2006 for methods). Substrates were acetaldehyde (3 mM final concentration), butyraldehyde (3 mM), and benzaldehyde (5 mM), with two, one, and two replicate assays per line, respectively. (Chemicals were purchased from Sigma-Aldrich, St. Louis, MO.) Replicates were performed at different times (blocks), with the order of samples randomized within blocks. Although only acetaldehyde is relevant to ethanol metabolism, aldehyde dehydrogenases are thought to be important in detoxification of a wide range of endogenous and exogenous aldehydes (Klyosov 1996; Vasiliou et al. 2000). Butyraldehyde and benzaldehyde, although not necessarily biologically relevant for *Drosophila*, were chosen to give a wide range of molecular weights and hydrophobicities of the assayed substrates. Only males (2- to 4-day old) were assayed; preliminary assays on 15 lines showed no line by sex interaction, and males tended to have lower coefficients of variation within lines than females (P. Jones and J. Fry, unpubl. data). Protein concentration of the homogenates was measured as described in Fry et al. (2004), and the results expressed as nM NAD^+ reduced per milligram total protein per minute, using the extinction coefficient of NADH at 340 nm of 6200 L mol^{-1} cm^{-1}.

Data for acetaldehyde and benzaldehyde were analyzed using the MIXED procedure in SAS (Littell et al. 1996), with genotype (*Phe* or *Leu*) as a fixed effect, and line within genotype, block, and the block by genotype interaction as random effects. For the unreplicated butyraldehyde data, ALDH activity of the two genotypes was compared by a *t*-test.

Results

IDENTIFICATION OF POLYMORPHISM

We initially sequenced the *Aldh*-coding region from six strains: one from each of two laboratory populations derived from North Carolina and selected for ethanol resistance for approximately 200 generations (the "HE" populations of Fry et al. 2004); one from each of two corresponding control populations ("R" populations); and two strains from Zimbabwe. The "HE" and "R" populations were chosen because the former had evolved significantly higher ALDH activity than the latter (Fry et al. 2004), suggesting the possibility that the two treatments had diverged in frequency in one or more replacement polymorphisms in *Aldh*. Comparing the six sequences and the genome database sequence revealed a single replacement polymorphism, a transition that changes codon 479 from CTC (*Leu*) to TTC (*Phe*).

In a tBLASTn (protein-translated nucleotide) search of the 12 other sequenced *Drosophila* species using *Aldh* amino acids 421–520 as a query (the carboxy-terminal 100 amino acids, in which the polymorphic residue is near the center), the best hit recovered in every species (88–100% amino acid sequence identity, no gaps) had *Leu* at the homologous position. Thus, not only is the *Leu* allele ancestral, but the polymorphic residue is strongly conserved in other species. The conservation of the residue extends even to the two sequenced mosquito species, *Anopheles gambiae* and *Aedes aegypti* (82–83% identity, no gaps). This conservation over more than 200 million years of evolution suggests that the *Leu* −> *Phe* replacement is likely to have significant functional consequences.

ALLELE FREQUENCIES IN EXPERIMENTAL POPULATIONS

To determine whether changes in the frequency of the *Phe* allele might have occurred in response to selection for ethanol resistance, eight individuals from each R and HE population, plus three to six independent isochromosomal lines per population, were genotyped by digestion with BbsI, which cuts only the *Phe* allele. Eight flies from each of two additional populations that had been selected for ethanol resistance at a lower intensity than the HE populations ("M" populations of Fry 2001) were also genotyped, as were 20 flies preserved from the base population ancestral to all six experimental populations. The *Phe* allele rose to high frequency in all four ethanol-selected populations, while remaining close to the estimated ancestral frequency of 0.15 in the two control (R) populations (Table 1). *Phe* allele frequencies were slightly higher in the two HE populations than in the two M populations, possibly reflecting the greater selection intensity in the former lines (constant exposure to medium with 16% ethanol in the HE lines, compared to intermittent exposure to medium with 12% ethanol in the M lines; see Fry et al. 2004 for details). The probability of the allele frequency rankings paralleling the rankings in selection intensity (i.e., both HE populations > both M populations > both R populations, or vice versa) by chance alone is $(2\ 2!\ 2!\ 2!)/(6!) = 1/45$.

Table 1. Frequencies of the *Aldh-Phe* allele in the ethanol-selected and control populations of Fry et al. (2004).

Selection regime	Population	Frequency of *Phe* allele (No. of chromosomes)
High Ethanol	HE1	1.000 (19)
	HE2	0.905 (21)
Mixed	M1	0.750 (16)
	M2	0.813 (16)
Control	R1	0.316 (19)
	R2	0.000 (22)
–	Base	0.150 (40)

ASSOCIATION OF POLYMORPHISM WITH ALDH ENZYME ACTIVITY

To determine whether the *Phe* allele is associated with faster turnover of acetaldehyde than the *Leu* allele, we measured ALDH enzyme activity in a set of homozygous second chromosome lines derived from a Pennsylvania population and sharing the same genetic background for the other chromosomes (Lazzaro et al. 2004). Of 76 lines, only eight had the *Phe* allele; the enzyme activity of these eight was compared to that of a random sample of 16 of the *Leu* lines. As might be expected based on the selection experiment results, the *Phe* lines had significantly higher ALDH activity than the *Leu* lines, by an average of 52%, when acetaldehyde was used as a substrate (Fig. 1; $P < 0.001$). In contrast, when butyraldehyde, a four-carbon aldehyde, was used as a substrate, the two groups did not differ in ALDH activity (Fig. 1; $P > 0.5$). Surprisingly, with the seven-carbon benzaldehyde as a substrate, *Phe* lines had 60% *lower* ALDH activity than *Leu* lines (Fig. 1; $P < 0.001$), with no overlap between the groups.

These results give evidence that the *Leu* −> *Phe* substitution, or possibly another amino acid substitution in linkage disequilibrium with it, causes a shift in substrate specificity of the ALDH enzyme. (The results cannot be explained by expression variation, which would affect enzyme activities with different substrates similarly). To determine whether another replacement substitution could be responsible, we sequenced the complete *Aldh*-coding region in four of the lines for which enzyme activity was measured, two *Leu* and two *Phe*; no other replacement polymorphisms were found.

With acetaldehyde and benzaldehyde as substrates, significant variation in ALDH activity among lines within genotypes was also observed ($P < 0.001$ in each case; no similar analysis could be done for butyraldehyde because observations were unreplicated). Because other replacement polymorphisms are apparently rare, all or most of this variation can be attributed to variation in level of ALDH protein.

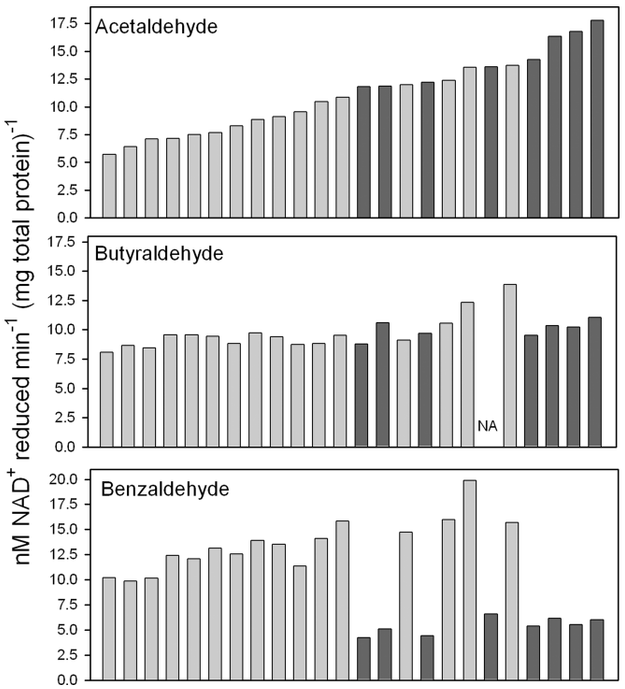

Figure 1. ALDH enzyme activity with three aldehyde substrates in 24 iso-second chromosome lines from Pennsylvania. Lines are ordered by activity with acetaldehyde. Dark and light bars represent *Phe* and *Leu* lines, respectively.

ALLELE FREQUENCIES IN WILD POPULATIONS

To determine whether the *Phe* allele increases in frequency with latitude, as expected of an allele associated with ethanol resistance, we used BbsI digestion to genotype lines from Europe and tropical Africa, and from latitudinal transects from eastern Australia and the eastern United States. The average *Phe* allele frequency found by genotyping a single fly from each of 44 isofemale lines from five locations in Europe (41–61°N) was 0.18 (range: 0–0.40). In contrast, genotyping single flies from African isofemale lines revealed no *Phe* alleles in 36 lines from six locations in Cameroon (4–11°N), and only one homozygote in 13 lines from a single location in Zimbabwe (17°S, allele frequency = 0.08).

Genotyping single flies from isofemale lines from temperate southern (36–43°S, $N = 16$) and tropical northern (15–19°S, $N = 20$) Australia revealed *Phe* allele frequencies of 0.22 and 0.025, respectively. To determine whether significant clinal variation was present among the entire set of 104 Australian lines, DNA was mass extracted from 15 males per line, amplified, and digested with BbsI; the proportion of lines with a detectable band corresponding to the *Phe* allele showed a significant positive correlation with latitude (Fig. 2A; Spearman rank correlation $r_s = 0.56$, $P = 0.008$ one-tailed). Only three lines appeared to be fixed for the *Phe* allele, however, consistent with the low *Phe* allele frequencies found by genotyping individual flies.

Phe allele frequency in isochromosomal (homozygous) lines from nine locations in the eastern United States showed a nearly significant positive correlation with latitude (Fig. 2B; $r_s = 0.53$, $P = 0.072$ one-tailed). *Phe* allele frequencies were relatively low (range: 0–0.13) in all populations.

MOLECULAR VARIATION IN THE VICINITY OF THE REPLACEMENT POLYMORPHISM

To determine whether the *Phe* allele arose once or multiple times, we surveyed molecular variation in a 229-bp window centered on the replacement polymorphism (Table 2). Among the 31 *Leu* alleles sequenced, there were four relatively common haplotypes (termed *Leu1–Leu4*) defined by synonymous polymorphisms at positions 1434, 1440, and 1452; each of these was found in North America and on at least one other continent (Africa, Europe, or Australia). Three additional *Leu* haplotypes, differing from the

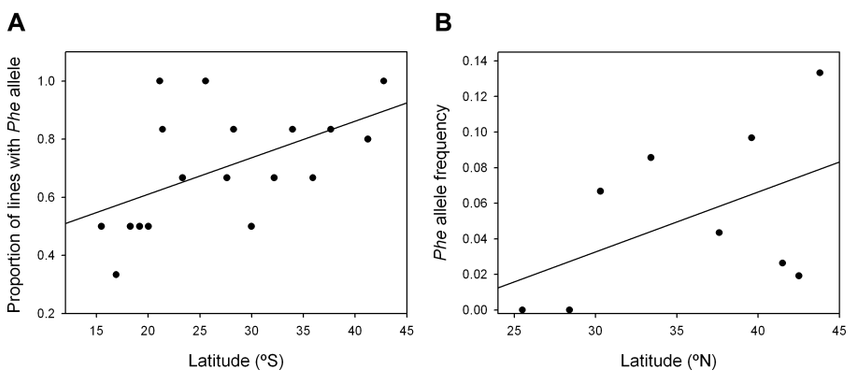

Figure 2. Relationship between *Aldh-Phe* allele frequency and latitude. (A) Proportion of isofemale lines from 18 locations in eastern Australia fixed or segregating for the *Phe* allele. Points are based on six lines each, except for the samples from 21.1° ($N = 3$) and 41.2° ($N = 5$). (B) *Phe* allele frequencies in the eastern United States; number of isochromosomal lines per site ranged from 14 to 52 (mean 32.7).

Table 2. **A molecular variation in a 229-bp region of *Aldh* centered on the *Phe/Leu* replacement polymorphism (sites 1320–1548 in cDNA, with first base of start codon = 1). (A) Observed haplotypes; differences from the reference sequence (Flybase Release 4.3) are shown. Asterisks denote replacement polymorphisms; all others are synonymous. (B) Observed frequencies of haplotypes. Because *Phe* and *Leu* alleles were usually identified before sequencing, the frequencies of the two alleles in a given sample do not necessarily reflect their frequencies in the respective population.**

(A)

Haplotype	Nucleotide position (cDNA)							
	1332	1350	1430	1434	1435	1437	1440	1452
Leu1/reference	G	C	A	C	C	C	T	G
Leu2								A
Leu3				T				A
Leu4				T			C	A
Leu1+			G*					
Leu2++		T				A		A
Leu4+	A			T			C	A
Phe					T*			

B.

Continent	Description	Haplotype							
		Phe	*Leu1*	*Leu2*	*Leu3*	*Leu4*	*Leu1+*	*Leu2++*	*Leu4+*
North America	HE (selected) populations	5		1					
	R (control) populations	2	1	1	2	2			
	Pennsylvania, 1998–1999	8		4		6	1	1	1
Europe	Austria, 2004		2						
Africa	Cameroon, 2004			2	1	3			
	Zimbabwe, 1994	1				2			
Australia	2004	1	1						
Total		17	4	8	3	13	1	1	1

common haplotypes by either one or two singleton substitutions, were each observed only once. Only the singleton at site 1430 was nonsynonymous, replacing an asparagine (AAT) with a serine (AGT). Nucleotide diversity (π, calculated for the 229-bp window using equation 9.2 in Li 1997) within the entire worldwide sample of *Leu* lines was 0.0034 ($N = 31$), about the same as within the Pennsylvania population alone ($\pi = 0.0040$, $N = 13$). Although the number of bases sequenced is small, these are fairly typical values for *D. melanogaster* autosomal genes (cf. Andolfatto 2001).

In contrast, no variation was observed among the 17 *Phe* alleles sequenced, including one each from Australia and Africa. These results indicate that the mutation giving rise to the *Phe* allele arose only once, apparently on the *Leu1* haplotype (Table 2). Although a larger and more systematic survey of variation would be needed to estimate the age of the *Phe* allele, the complete absence of variation on *Phe* alleles suggests that it arose relatively recently.

Discussion

We have identified a world-wide replacement polymorphism in *Aldehyde dehydrogenase*, a gene essential for ethanol resistance in *D. melanogaster* (Fry and Saweikis 2006), that appears to be under selection mediated by dietary ethanol. The derived *Phe* allele causes faster turnover of acetaldehyde than the ancestral *Leu* allele, increases in frequency in laboratory populations selected for ethanol resistance, and occurs at higher frequency in ethanol-resistant temperate populations than in ethanol-susceptible tropical populations. Sequence comparisons show that the *Leu* −> *Phe* substitution occurred only once, relatively recently, at a residue that is conserved in other Diptera. Taken together, the results suggest that the substitution contributes to the latitudinal clines in ethanol resistance in *D. melanogaster* (although the contribution could be small; see below).

Drosophila Aldh belongs to the ALDH1/2 group of the aldehyde dehydrogenase superfamily (Sophos and Vasiliou 2003).

Although members of this group are best known for their role in detoxification of ethanol-derived acetaldehyde, they typically have broad substrate specificity, and appear to be optimized for detoxification of larger, more hydrophobic aldehydes than acetaldehyde (Klyosov 1996; Perozich et al. 1999). For this reason, we tested how the *Leu −> Phe* substitution affected enzyme activity with two other aldehyde substrates, butyraldhyde and benzaldehyde. Surprisingly, with the latter substrate, ALDH activity of strains with the *Phe* allele was markedly lower than that of strains with the *Leu* allele, the opposite of the pattern observed with acetaldehyde. Although it is not clear if benzaldehyde is biologically relevant for *Drosophila*, there are potential natural substrates of *Drosophila* ALDH that resemble benzaldehyde more than acetaldehyde in molecular weight and hydrophobicity. For example, members of the ALDH1/2 group in mammals have been shown to play an important role in detoxification of 4-hydroxynonenal (4-HNE), a highly toxic aldehyde generated as a byproduct of oxidative damage to lipids (Chen and Yu 1996; Srivastava et al. 1998; Murphy et al. 2003), and known to be present in *Drosophila* (Singh et al. 2001). Although Singh et al. (2001) identified a *Drosophila* glutathione-S-transferase that can detoxify 4-HNE, this does not preclude a role for ALDH; both enzymes are important for 4-HNE detoxification in mammals (Chen and Yu 1996; Srivastava et al. 1998).

The possibility that the *Leu −> Phe* substitution may reduce catalytic activity of *Drosophila* ALDH with important natural substrates other than acetaldehyde could explain two of our observations. First, although the *Phe* allele appears to be advantageous on diets high in ethanol, it remains the minority allele even in ethanol-resistant temperate populations. Second, all other sequenced Diptera, including 12 *Drosophila* species and two mosquito species, have *Leu* at the corresponding residue. Neither of these observations would make sense if acetaldehyde were the only important natural substrate of *Drosophila* ALDH. Instead, they are consistent with the hypothesis that the *Phe* allele is disadvantageous *except* in flies feeding on high amounts of ethanol, when its higher catalytic activity with acetaldehyde may be sufficient to offset its (presumed) lower activity with other substrates. Because the other substrates may well be endogenous compounds like 4-HNE, there is no need to assume that the disadvantage of the *Phe* allele is specific to certain diets. In fact, the disadvantage could be relatively constant across species and populations, with the appearance of the *Phe* allele only in *D. melanogaster*, and then only in significant frequencies in temperate populations, resulting from stronger selection for ethanol resistance in these populations than in other *Drosophila*. Indeed, temperate *D. melanogaster* populations have a particular propensity for breeding in breweries and wineries (e.g., McKechnie and Geer 1993), and are the most ethanol-resistant *Drosophila* known (Merçot et al. 1994).

The *Aldh* polymorphism described here bears interesting similarities and differences to the well-studied *Adh* polymorphism. Both loci exhibit clines on multiple continents in which the derived allele increases in frequency with latitude. For both loci the derived allele confers faster ethanol detoxification, and increases in frequency in laboratory populations selected for ethanol resistance. In addition, both genes appear to have functions in addition to ethanol detoxification. (In the case of *Adh*, the existence of alternative functions is suggested by the apparently low fitness of null mutants even in the absence of ethanol; van Delden and Kamping 1988). In a related vein, for both genes it is not certain why the ancestral allele is favored in the tropics. The *Adh-Slow* allozyme is more thermostable than the *Fast* allozyme *in vitro* (reviewed in van Delden 1982), but this effect is of questionable importance *in vivo* at temperatures encountered by wild flies (van Delden 1982; Vigue et al. 1982).

In contrast to these similarities, the two enzymes differ markedly in their evolutionary histories. The common ancestor of the ALDH1/2 family, an NAD^+-dependent tetrameric aldehyde dehydrogenase, was apparently present in the common ancestor of eubacteria and eukaryotes over two billion years ago (Rzhetsky et al. 1997; Perozich et al. 1999). Conservation of these enzymes within the metazoa is high (e.g., 70% amino acid identity between *Drosophila Aldh* and human *ALDH2*, the liver enzyme chiefly responsible for detoxification of ethanol-derived acetaldehyde). In contrast, *Drosophila* ADH is unrelated to mammalian ADH, and appears to have evolved from another short-chain dehydrogenase in the common ancestor of a relatively small number of Dipteran families, possibly of just the Drosophilidae itself (Ashburner 1998).

An important feature of the *Adh* polymorphism that has not been explored in detail for *Aldh* is the role of expression variation. The amino acid substitution responsible for the *Fast–Slow* difference alters the specific activity of ADH protein but does not affect ADH protein level (Choudhary and Laurie 1991). The replacement substitution, however, is in linkage disequilibrium with polymorphisms that affect expression; as a result, *Adh-Fast* homozygotes produce about 60% more ADH protein than *Slow* homozygotes (Laurie and Stam 1988, 1994; Stam and Laurie 1996). Substantial variation in ADH activity within electromorphs is also present (Aquadro et al. 1986). This parallels our observation of significant variation in ALDH activity among lines within genotypes (*Phe* or *Leu*) in the Pennsylvania population. We are currently investigating whether some of this variation results from noncoding polymorphisms in the *Aldh* gene region. Although we have not yet determined whether the *Leu*/*Phe* polymorphism is in linkage disequilibrium with polymorphisms that affect expression, among isofemale lines fixed for the *Leu* allele, lines from Vienna had significantly higher ALDH activity than lines from Cameroon, with no overlap between the groups (J. Fry and K. Donlon, unpubl.

data). Because sequencing two lines from each region revealed no additional replacement polymorphisms that could account for the activity difference, the difference must reflect higher levels of ALDH protein in the more ethanol-resistant European lines. This offers a possible contrast to the situation with *Adh*, in which there appears to be little or no geographic variation in expression independent of the Fast/Slow polymorphism (Anderson 1982).

Another difference between the *Adh* polymorphism and the *Aldh* polymorphism is that the *Aldh-Phe* allele remains in relatively low frequency even in the most ethanol-resistant temperate populations. Thus, whatever the effect of the *Leu −> Phe* substitution on ethanol resistance, it cannot account for more than a small fraction of the phenotypic difference between temperate and tropical populations. The results reported here, together with previous work, give evidence that the ethanol resistance cline has a complex genetic basis, with contributions from the (itself complex) *Adh* polymorphism, a contribution (possibly small) from the *Aldh* replacement polymorphism, a possible contribution of *Aldh* expression differences independent of the replacement polymorphism, and a contribution of one or more genes on the third chromosome (Chakir et al. 1996). We are currently working on identifying the latter genes, and on quantifying the contribution of variation at *Aldh* (including expression variation) to the resistance clines.

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