Genet. Res., Camb. (2006), **87**, *pp.* 13–21. © 2006 Cambridge University Press
doi:10.1017/S0016672306007919 Printed in the United Kingdom

An independent non-linear latitudinal cline for the sn-glycerol-3-phosphate (*α-Gpdh*) polymorphism of *Drosophila melanogaster* from eastern Australia

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(*Received 27 June 2005 and in revised form 7 October 2005*)

Summary

Latitudinal variation of the polymorphic sn-glycerol-3-phosphate (*α-Gpdh*) locus in *Drosophila melanogaster* has been characterized on several continents; however, apparent clinal patterns are potentially confounded by linkage with an inversion, close associations with other genetic markers that vary clinally, and a tandem *α-Gpdh* pseudogene. Here we compare clinal patterns in *α-Gpdh* with those of other linked markers by testing field flies from eastern Australian locations collected in two separate years. The *α-Gpdh* variation exhibited a consistent non-linear cline reflecting an increase in the α-*Gpdh*^{F} allele at extreme latitudes. This pattern was not influenced by the *In*(*2L*)*t* inversion wherein this locus is located, nor was it influenced by the presence of the *α-Gpdh* pseudogene, whose presence was ubiquitous and highly variable among populations. The *α-Gpdh* pattern was also independent of a cline in allozyme frequencies at the alcohol dehydrogenase (*Adh*) locus, and two length polymorphisms in the *Adh* gene. These results suggest clinal selection at the *α-Gpdh* locus that is partially or wholly unrelated to linear climatic gradients along the eastern coast of Australia.

1. Introduction

Natural populations of *Drosophila melanogaster* harbour extensive genetic variation at a number of allozyme and chromosomal inversion loci (Knibb *et al*., 1981; Oakeshott *et al*., 1982; Kreitman, 1983; Bubliy *et al*., 1999; van 't Land *et al*., 2000). Some loci show remarkable similarity in allele frequencies between populations, while others show extensive geographic variation (see Singh *et al*., 1982). Patterns of genetic variation across clines provide strong evidence for natural selection and indicate that the loci or traits exhibiting these patterns have adaptive significance (Oakeshott *et al*., 1982; Endler, 1986).

One polymorphic gene, sn-glycerol-3-phosphate dehydrogenase (*α-Gpdh*), is likely to provide adaptive versatility for *D. melanogaster*, since life-history and reproductive fitness effects that are likely to be of consequence in natural populations have been associated with this gene (Oakeshott *et al*., 1985; Barnes & Laurie-Ahlberg, 1986; Ochando & Ayala, 1999). Moreover, we now have extensive insight into the metabolic and physiological role of the enzyme product of *α-Gpdh* in lipid, carbohydrate, ethanol and flight metabolism (O'Brien & MacIntyre, 1972; Geer *et al*., 1983). An important aspect of fitness variation that is associated with the *α-Gpdh* polymorphism is evidence for epistatic interactions with a second linked polymorphic gene, alcohol dehydrogenase (*Adh*) (Cavener & Clegg, 1981; Izquierdo & Rubio, 1986; Oudman *et al*., 1991).

The *α-Gpdh* and *Adh* loci are among the most widely studied examples of latitudinal clines in *D. melanogaster*. It is generally assumed that these clines are influenced by temperature and/or temperature-related factors. The Adh^S allele tends to increase in frequency with decreasing latitude in both Northern and Southern Hemispheres (Oakeshott *et al*., 1982) and recent shifts in the clinal pattern of the Adh^S allele are likely to reflect conditions becoming warmer and drier over the last 20 years (Umina *et al*., 2005). Patterns for *α-Gpdh* have proven to be more difficult

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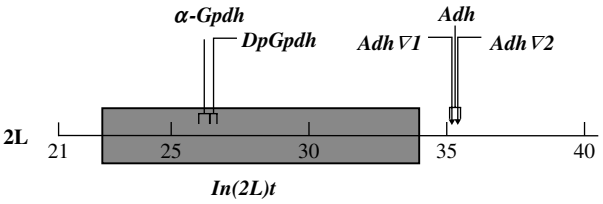

Fig. 1. Map of the left arm of chromosome 2 in *Drosophila melanogaster* showing the cytological positions of markers used in this study.

to interpret. The α-*Gpdh*^{F} allele is thought to increase with decreasing latitude, but worldwide patterns for α-*Gpdh* are far less consistent than for *Adh* and provide only equivocal evidence for selection (Oakeshott *et al*., 1982, 1984; Bubliy *et al*., 1999; van 't Land *et al*., 2000). There are a number of factors that may have confounded previous studies examining geographic variation of α-*Gpdh*. Many historical studies, from which latitudinal trends have been inferred, used flies that were collected several years apart, during different seasons and from diverse longitudes and altitudes (e.g. Berger, 1971; Oakeshott *et al*., 1982, 1984; van 't Land *et al*., 2000).

Patterns for α-*Gpdh* may also be variable because of interactions with other genetic markers located near this locus on the left arm of chromosome 2 (Fig. 1). The α-*Gpdh* locus is situated within the cosmopolitan inversion, *In*(*2L*)*t*, which is usually in positive linkage disequilibrium with the α-*Gpdh*^{F} and *Adh*^{S} alleles (Kamping & van Delden, 1999; van 't Land *et al*., 2000). Like other common inversions in *D. melanogaster*, *In*(*2L*)*t* is clinally distributed with latitude (Knibb *et al*., 1981; Knibb, 1982; Anderson *et al*., 1987) and therefore may confound latitudinal patterns of α-*Gpdh*. Latitudinal patterns may also be influenced by gene duplications in the α-*Gpdh* and *Adh* genes (Chia *et al*., 1985; Jiang *et al*., 1988; Takano *et al*., 1989; von Kalm *et al*., 1989; Kang *et al*., 1998). A presence/absence polymorphism occurs for a tandem duplication of the α-*Gpdh* gene in natural populations (Takano *et al*., 1989; Kusakabe *et al*., 1990). The α-*Gpdh* duplication (herein referred to as *DpGpdh*) is widespread and lacks the first and second exons suggesting a non-functional pseudogene (Symonds & Gibson, 1992; Kang *et al*., 1998). This pseudogene is likely to be selectively neutral (Kusakabe *et al*., 1990), although *D. melanogaster* containing the duplication have increased enzyme activity (Symonds & Gibson, 1992; Kang *et al*., 1998). Geographical patterns of *DpGpdh* and the extent of disequilibrium with the α-*Gpdh* polymorphism have not been thoroughly investigated.

Two sequence length indel polymorphisms of the *Adh* gene (*Adh*∇*1* and ∇*2*) may also interact with α-*Gpdh* and *Adh* (Kreitman, 1983). *Adh*∇*1* consists of a coupled insertion and deletion within the first intron of the *Adh* gene whose net product is a 5 bp insertion, while *Adh*∇*2* is a 37 bp point insertion (Kreitman, 1983; Berry & Kreitman, 1993). *Adh*∇*1* and ∇*2* are at low to intermediate frequencies in natural populations of *D. melanogaster*, show strong linkage disequilibrium with the *Adh*^{F} allele and have been implicated in differences in ADH protein levels (Kreitman & Aguadé, 1986; Aguadé, 1988; Mathew *et al*., 1992; Laurie & Stam, 1994).

In this paper, we re-examine latitudinal patterns in the α-*Gpdh* polymorphism by sampling multiple low-altitude sites along the east coast of Australia over two different time periods (2002 and 2004). The eastern coast is ideal for testing clinal patterns because of a recent history of colonization by *D. melanogaster*, high rates of gene flow and a lack of clinal variation at most microsatellite markers (Gockel *et al*., 2001; Kennington *et al*., 2003). To establish whether latitudinal clinal patterns are dependent on *DpGpdh*, *In*(*2L*)*t* and the *Adh* polymorphisms, these markers were scored along with α-*Gpdh*. We show that the α-*Gpdh* polymorphism exhibits a non-linear cline with latitude in contrast to linear patterns or a lack of clinal variation for the other markers.

2. Materials and methods

(i) *Fly collections*

D. melanogaster were collected from populations along the east coast of Australia between March and May in 2002 and again in 2004 (Table 1). Field males were stored in 100% ethanol at −20 °C for subsequent DNA extraction and genotyping. Field females were reared as individual isofemale lines at 25 °C under continuous light on a sugar (1·6% w/v), agar (3·2%), yeast (3·2%) and potato (1·6%) medium that was always treated with an antifungal agent (0·14% nipagin) and antibiotics (2% dihydrostreptomycin and 0·6% penicillin added to the medium surface). Once isofemale lines were established, field females were preserved in 100% ethanol and stored at −20 °C for genotyping.

(ii) *Markers*

DNA was extracted from field flies using a modified CTAB extraction protocol following Weeks *et al*. (2000). Each fly was then genotyped for *Adh*, α-*Gpdh*, *DpGpdh*, *Adh*∇*1*, *Adh*∇*2* and *In*(*2L*)*t*. *Adh* was scored using a Bi-PASA (bidirectional PCR amplification of specific alleles) method, utilizing four primers in a single PCR amplification (Liu *et al*., 1997). The outer primer sequences, which are not allele-specific, were: forward primer (P) -5′ AGCTCCCTGGCGGTAAG TTCATC; and reverse primer (Q) -5′ TGGGATTA

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Table 1. *Collection sites of adult* Drosophila melanogaster *along the east coast of Australia in 2002 and 2004*

2002 Populations	Latitude	Longitude	*n*	2004 Populations	Latitude	Longitude	*n*
Red Knight	42·8	147·2	30	Sorell	42·8	147·6	30
Bega	36·7	149·9	10	Millers	41·2	147·0	28
Wollongong	34·4	150·9	31	Yarra Valley	37·7	145·4	29
Belmont	33·0	151·7	20	Moruya	35·9	150·1	30
Coffs Harbour	30·3	153·1	30	Sydney	34·0	151·1	27
Alstonville	28·8	153·4	31	Tuncurry	32·2	152·5	24
Kingscliff	28·3	153·5	31	Red Rock	30·0	153·2	30
Redland Bay	27·6	153·3	55	Kingscliff	28·3	153·6	18
Rainbow Beach	25·9	153·1	30	Redland Bay	27·6	153·3	30
Maryborough	25·5	152·7	34	Maryborough	25·5	152·7	30
Mirriamvale	24·3	151·6	28	Rockhampton	23·3	150·5	30
Gladstone	23·9	153·3	30	Mackay	21·1	149·0	26
Rockhampton	23·3	150·5	29	Bowen	20·0	148·2	15
Sarina	21·4	149·3	29	Magnetic Island	19·2	146·8	19
Innisfail	17·5	146·0	31	Cardwell	18·3	146·0	29
Cape Tribulation	16·1	145·5	34	Kirrama	18·2	145·9	30
				Cairns	16·9	145·7	29
				Cooktown	15·5	145·3	29

TCACTTCTTAGATGCCG. The two inner primer sequences, which are allele-specific and have non-complementary 3′ ends to reduce megapriming and to switch from template-based amplification to self amplification of the smaller fragments, were: forward primer (A) -5′ ggggcggggcCACCCTGGTGCACAA; and reverse primer (B) -5′ ggggcgggcgCAACCAGG AGTTGAACG. This resulted in a non-specific 364 bp fragment (PQ), a 172 bp 'fast' fragment (PB) and a 223 bp 'slow' fragment (AQ).

For *α-Gpdh*, we first designed primers to amplify a 3610 bp fragment spanning exons 2–7 of the gene. This was done to eliminate the duplicated *α-Gpdh* sequence, which contains a copy of the single nucleotide polymorphism responsible for the α-*Gpdh*$^{S/F}$ enzyme polymorphism (see Bewley *et al*., 1989; Takano *et al*., 1989). The primer sequences were: forward -5′ TTATCAACGAGACGCACGAG; and reverse -5′ GACGGCATGATGGACGTATC. The resulting PCR product was then diluted 1 : 1000 and used as template DNA to score the α-*Gpdh*$^{S/F}$ polymorphism using a Bi-PASA method. The outer primer sequences for Bi-PASA were: forward primer (P) -5′ ATGCTGAAGAATAAGGGTCTGGAG; and reverse primer (Q) -5′ AAACAGAAGGTGCAT CAACTTAGC. The inner primers were: forward (A) -5′ ggcggcgcgAATCAGCTCAAGCCTAAA; and reverse (B) -5′ ggggcgggcgGTATGCAATCAATTAA ATCA, resulting in a non-specific 385 bp fragment (PQ), a 181 bp 'fast' fragment (PB) and a 260 bp 'slow' fragment (AQ).

Allozyme electrophoresis was used to determine the accuracy of the Bi-PASA markers for detecting the *Adh*$^{S/F}$ and α-*Gpdh*$^{S/F}$ polymorphisms. Female F_5 flies were taken from 90 isofemale lines (one per line), established using nine populations collected in 2004 spanning the east coast of Australia. Individual flies were ground with plastic pestles in 20 μl sterile water, of which 2·5 μl was used per well. Electrophoresis was carried out with the Titan III cellulose acetate system of Helena Laboratories, using a centre application and a novel staining recipe we developed (0·6 ml 1 M Tris-HCl, pH 8·0; 0·2 ml 3 mM NAD; 0·2 ml 100 % ethanol; 0·15 ml 120 mM α-glycerophosphate; 0·2 ml 3·5 mM PMS; 0·2 ml 5 mM MTT; 6 ml 1 % agar), allowing *Adh* and *α-Gpdh* to be stained on the same gel. DNA was extracted from the remaining 17·5 μl using the CTAB extraction protocol (Weeks *et al*., 2000) and genotyped by Bi-PASA. From a total of 90 individuals, allozyme and Bi-PASA genotypes matched in >94 % of cases for both *Adh* and *α-Gpdh*. This demonstrates that the nucleotide substitutions thought responsible for the fast/slow allozyme polymorphisms of *Adh* and in particular *α-Gpdh* (which was previously somewhat ambiguous; see Bewley *et al*., 1989; von Kalm *et al*., 1989) are accurate. There was a small bias in the directionality of the scoring discrepancy between the allozyme and Bi-PASA techniques; however, this bias was far too small to affect our findings.

The two sequence length polymorphisms of *Adh*, *Adh∇1* and *∇2*, were scored in the same PCR reaction using one reverse and two forward primers based on nucleotide polymorphisms described in Kreitman (1983). Primer sequences were: forward primer (A) -5′ GGCATATAATATACTAATAC; forward primer (B) -5′ TTATAAACATACAAACCGA; and reverse primer -5′ AGAGCTTTTCATTATCTACC. *DpGpdh* was scored using a forward primer (-5′ GGCAAA ATTTGTAAATCCCCTA) complementary to a region within exon 8 and a reverse primer (-5′ TCAGGATGTCAGCGTTCTTG) complementary

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Table 2. *Sequence locations of* Drosophila melanogaster *markers on the left arm of chromosome 2 used in this study*

Marker	Sequence location
In(2L)t	2204115..2298835–13172747..13212655
α-Gpdh	5943682..5949092
DpGpdh	5949748..5960458
Adh	14615555..14618902
Adh∇1	14615704..14615733
Adh∇2	14616108[a]

[a] *Adh∇2* is a single point insertion.

to a region within exon 3. *In(2L)t* was scored using the protocol of Andolfatto *et al*. (1999). The cytological positions and sequence locations of all markers used in this study are shown in Fig. 1 and Table 2, respectively.

(iii) *Statistical analysis*

All loci were tested for deviations from Hardy–Weinberg equilibrium using the web-based program GENEPOP (http://www.wbiomed.curtin.edu.au/genepop/). For statistical comparisons, allele frequencies were angular-transformed prior to analysis. Associations between the frequency of *α-Gpdh*, *Adh∇1*, *Adh∇2* and *DpGpdh* with latitude for populations collected in 2002 and 2004 were determined using regression analyses. We have previously published the regression data for *Adh* and *In(2L)t* (Umina *et al*., 2005). For *α-Gpdh* and *DpGpdh*, we also tested the significance of quadratic terms in the regression analyses. Slopes of linear regression lines were compared using *t*-tests following Zar (1996), to determine whether the slopes of the regression lines differed between years. If slopes did not differ significantly, then the elevations were compared using *t*-tests (Zar, 1996). For *α-Gpdh*, a significance test for additional independent variables in multiple regression was performed following Sokal & Rohlf (1995). The *F* probability distribution was used to test for the degree of diversity across entire curves between years based on Motulsky (1999). We also conducted bivariate and partial correlations using the statistical program SPSS for Windows (v. 11.5), to examine the relationships and interdependence of the different markers in collections from both years.

To determine linkage disequilibrium (LD) between *α-Gpdh*, *Adh* and *In(2L)t*, we increased sample sizes for genotyping in four populations in 2002 (Red Knight, Wollongong, Maryborough and Innisfail) and four populations in 2004 (Sorell, Sydney, Maryborough and Cardwell) and only considered these populations in analyses. Estimates of the absolute D and Hedrick's D' (D/D_{max}) were made using the computer program 2LD (Zhao, 2004). In several populations, the D/D_{max} ratio resulted in unrealistically high estimates of D'. This is due to low allele frequencies and the presence of only two or three of the four possible haplotypes (van 't Land *et al*., 2000). These D' values are therefore not shown. To test for LD between markers in these populations, we performed Fisher's exact tests using the GDA computer program (http://lewis.eeb.uconn.edu/lewishome/software.html).

All multiple comparisons were corrected at the table-wide $\alpha' = 0{\cdot}05$ level using the Dunn–Sidák method (Sokal & Rohlf, 1995).

3. Results

(i) *Latitudinal clines*

α-Gpdh variation showed a curvilinear association with latitude across the entire east coast of Australia for the 2002 and 2004 collections (Fig. 2), with significant quadratic regressions found in both years (Table 3). The frequency of the $\alpha\text{-}Gpdh^F$ allele was highest in populations collected from the highest and lowest latitudes (either end of the cline). However, no latitudinal association was evident if we only consider data from 22° S and further south. The frequency of the $\alpha\text{-}Gpdh^F$ allele therefore probably does not change much in southern locations. A significant linear relationship with latitude was also found for the 2004 collection, but the addition of a quadratic term to the regression model significantly increased the proportion of variation explained by the model ($Fs = 6{\cdot}985$; df$V1 = 1$; df$V2 = 15$; $P < 0{\cdot}05$), indicating a quadratic relationship as in 2002. To determine whether the association with latitude had changed between collection dates, the F probability distribution was used to test for the degree of diversity across entire curves (Motulsky, 1999). The F ratio (4·161) was not significant ($P = 0{\cdot}335$) based on a combined SS of 0·247 (df = 31) and separate SS of 0·219 (df = 28), indicating no shift in clinal patterns for *α-Gpdh* between 2002 and 2004.

The $Gpdh^F$ allele was positively correlated with *In(2L)t* and the Adh^S allele in 2002 ($r = 0{\cdot}129$, $n = 479$, $P < 0{\cdot}01$; $r = 0{\cdot}099$, $n = 479$, $P < 0{\cdot}05$, respectively) and 2004 ($r = 0{\cdot}132$, $n = 484$, $P < 0{\cdot}01$; $r = 0{\cdot}117$, $n = 483$, $P < 0{\cdot}05$, respectively); however, there was no significant LD detected between *α-Gpdh* and these markers (see below). If the *α-Gpdh* cline is independent of *In(2L)t*, there should still be a significant relationship between *α-Gpdh* and latitude when only chromosomes with the standard arrangement are considered (and ignoring populations with few (<10) standard arrangements). In this case, significant relationships between latitude and *α-Gpdh* are evident in both

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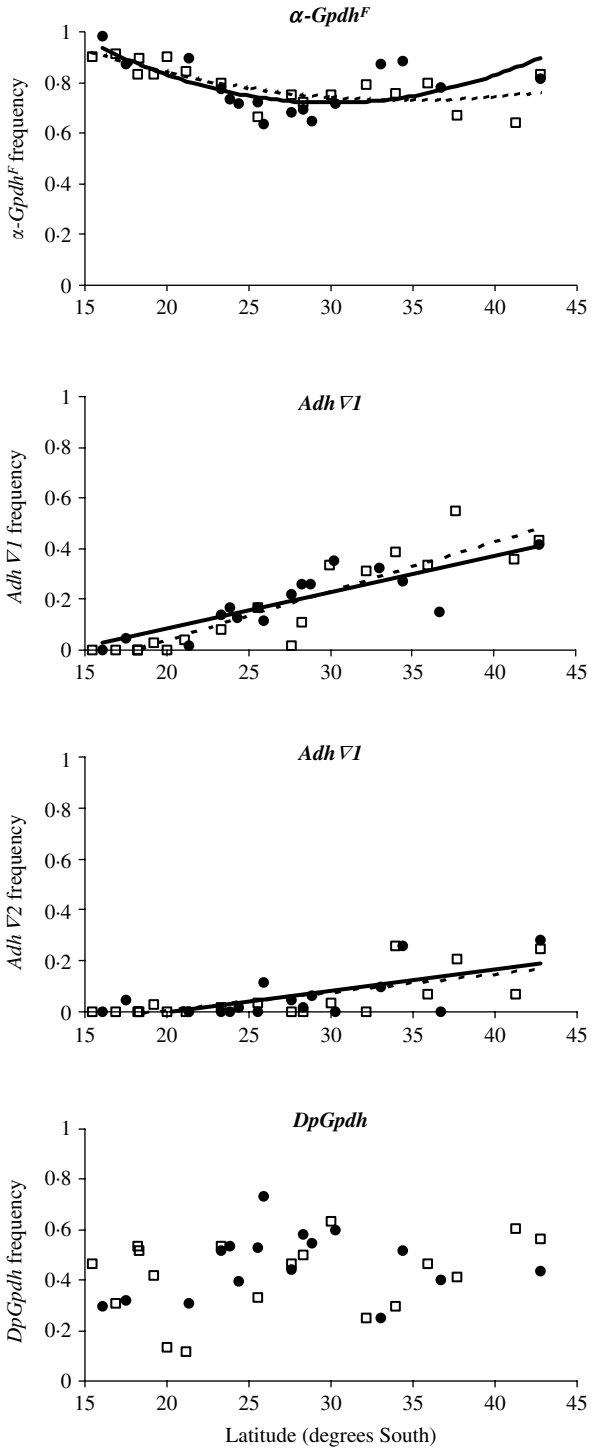

Fig. 2. Relationship between latitude and frequency of *α-Gpdh*, *Adh∇1*, *Adh∇2* and *DpGpdh* from *Drosophila melanogaster* populations collected along eastern coastal Australia in 2002 (●, data points; —, trendline) and 2004 (□, data points; ----, trendline).

the 2002 ($R^2=0{\cdot}472$; $t=5{\cdot}358$, $P<0{\cdot}05$) and 2004 collections ($R^2=0{\cdot}547$; $t=7{\cdot}860$, $P<0{\cdot}01$). This suggests that the curvilinear relationship between latitude and the frequency of *α-Gpdh* is largely independent of *In(2L)t*.

Table 3. *Regression analysis examining associations between markers and latitude in 2002 and 2004 collections of* Drosophila melanogaster *from eastern coastal Australia*

Marker	Year	Regression equation	t	P	R^2
α-Gpdh^F	2002	$y=0{\cdot}0015x^2-0{\cdot}0943x+2{\cdot}447$	6·63	<0·05	0·51
	2004	$y=0{\cdot}0007x^2-0{\cdot}0479x+1{\cdot}848$	12·58	<0·001	0·63
Adh∇1	2002	$y=0{\cdot}022x-0{\cdot}184$	29·32	<0·001	0·68
	2004	$y=0{\cdot}0315x-0{\cdot}507$	91·78	<0·001	0·85
Adh∇2	2002	$y=0{\cdot}0157x-0{\cdot}262$	6·58	<0·05	0·32
	2004	$y=0{\cdot}0168x-0{\cdot}304$	21·96	<0·001	0·58

There was a strong linear relationship between the frequency of the *Adh∇1* allele and latitude, with highly significant regressions and high coefficients of determination (R^2) for both the 2002 and 2004 collections (Table 3). In both years, the *Adh∇1* allele increased in frequency as latitude increased (Fig. 2). Slopes for the regression of *Adh∇1* frequency on latitude did not differ significantly among the two years ($F_{1,30}=3{\cdot}209$, $P=0{\cdot}083$), while the elevations of the population regressions also did not differ significantly among years ($F_{1,31}=2{\cdot}444$, $P=0{\cdot}128$). To separate possible selection on *Adh∇1* from $Adh^{S/F}$, we performed partial correlation analysis controlling for $Adh^{S/F}$. The relationship between the *Adh∇1* allele and latitude did not remain significant in the 2002 ($r=0{\cdot}033$, $n=476$, $P=0{\cdot}466$) or 2004 collections ($r=0{\cdot}065$, $n=480$, $P=0{\cdot}158$), indicating that the clinal pattern of *Adh∇1* is partly or wholly due to LD with $Adh^{S/F}$ (see below).

There was a shallow and significant linear relationship between the frequency of the *Adh∇2* allele and latitude for both the 2002 and 2004 collections (Table 3). As with *∇1*, the *Adh∇2* allele increased in frequency as latitude increased (Fig. 2). Slopes for the regression of *Adh∇2* frequency on latitude did not differ significantly between 2002 and 2004 ($F_{1,30}=0{\cdot}134$, $P=0{\cdot}717$). Elevation of the population regressions also did not differ significantly among years ($F_{1,31}=0{\cdot}015$, $P=0{\cdot}905$). No relationship between *Adh∇2* and latitude was evident in the 2004 collection following partial correlation controlling for $Adh^{S/F}$ ($r=0{\cdot}068$, $n=480$, $P=0{\cdot}138$). However, the association between *Adh∇2* and latitude in the 2002 collection remained significant following partial correlation ($r=0{\cdot}161$, $n=476$, $P<0{\cdot}001$), suggesting the clinal pattern observed in 2002 is independent of $Adh^{S/F}$. However, the correlation to test for independence is not very powerful due to the extremely low frequency of *Adh∇2* and the relatively high frequency of the Adh^S allele along the cline (see Fig. 2; Umina *et al.*, 2005).

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We examined the presence of the partial tandem duplication of *α-Gpdh* (*DpGpdh*) described by Takano *et al.* (1989) and found it is widespread along coastal eastern Australia (Fig. 2). *DpGpdh* showed no significant linear or quadratic association with latitude in either the 2002 (linear: $F=0{\cdot}373$; df = 15; $P=0{\cdot}551$; quadratic: $F=2{\cdot}509$; df = 15; $P=0{\cdot}120$) or 2004 (linear: $F=1{\cdot}537$; df = 17; $P=0{\cdot}233$; quadratic: $F=1{\cdot}137$; df = 17; $P=0{\cdot}347$) collections. Despite the lack of any association with latitude, *DpGpdh* was highly correlated with the α-$Gpdh^S$ allele across all populations in 2002 ($r=0{\cdot}303$, $n=480$, $P<0{\cdot}001$) and 2004 ($r=0{\cdot}293$, $n=484$, $P<0{\cdot}001$). *DpGpdh* was highly polymorphic in natural populations, ranging in frequency from about 10% at some locations to greater than 70% at others. Overall the average frequency of *DpGpdh* was relatively high in both 2002 (46·3%) and 2004 (42·0%), supporting the suggestion that the duplication is probably not deleterious (Kusakabe *et al.*, 1990).

(ii) *Hardy–Weinberg and linkage disequilibrium*

For the *α-Gpdh* and *Adh* loci, populations collected in 2002 and 2004 conformed to Hardy–Weinberg expectations. For *In(2L)t*, the 2002 (but not 2004) populations were also in Hardy–Weinberg equilibrium. In 2004 there were significant deviations detected in four populations, caused by a shortage of heterozygotes. For *Adh∇1* and *∇2*, neither the 2002 or 2004 collections were in Hardy–Weinberg equilibrium. For both loci there was a deficiency of heterozygotes across several populations. A sign test was performed for all loci not conforming to Hardy–Weinberg equilibrium to test whether the number of populations in heterozygote deficiency and heterozygote excess deviated significantly from 50%. For *Adh∇1* in 2004, there were significantly more populations with a heterozygote deficiency ($P=0{\cdot}016$); however, for all other cases there was no significant difference. Franklin (1981) has previously analysed deviations from Hardy–Weinberg equilibrium in *Adh* and other loci, and suggested that different deviation patterns across loci can be used as evidence of selection.

As expected, we found strong LD across the entire cline between all markers in the *Adh* gene. Chi-square values were highly significant ($P<0{\cdot}001$ in all cases) for LD between *Adh∇1*/*Adh∇2*, Adh^F/*Adh∇1* and Adh^F/*Adh∇2* in the 2002 and 2004 collections. To examine the gametic associations between the different markers in more detail, we genotyped additional flies from four populations collected at different latitudes in each of 2002 and 2004, and determined the degree of LD between different markers at each population. Exact tests of LD in the populations from varying latitudes revealed significant levels of disequilibria between *In(2L)t* and *Adh* in southern latitude populations (Table 4). The two most southern populations in 2002 (Red Knight and Wollongong) and 2004 (Sorell and Sydney) had relatively high standardized D' values which were statistically significant at the 5% level after corrections for multiple comparisons. The more northern populations did not show any significant LD between *In(2L)t* and *Adh* in both 2002 and 2004 (Table 4). Interestingly, there was also no significant LD detected between *In(2L)t*/*α-Gpdh* or *Adh*/*α-Gpdh* in any of the 2002 or 2004 populations examined.

4. Discussion

By examining the eastern coastal cline of Australia, we have demonstrated a curvilinear association between the frequency of the α-$Gpdh^F$ allele and latitude in the cosmopolitan species, *D. melanogaster*. This relationship was demonstrated over two independent collections made 2 years apart, and provides evidence that *α-Gpdh* is under selection from a number of environmental variables, most likely climatic conditions. This contrasts with previous studies, which have suggested a linear relationship with latitude where the frequency of the α-$Gpdh^F$ allele decreases with increasing distance from the equator (Oakeshott *et al.*, 1982, 1984; van 't Land *et al.*, 2000).

Why have only linear relationships been previously reported for *α-Gpdh*? This may be due to sampling issues, or possibly to an evolutionary change over time. In the classic study first characterizing the *α-Gpdh* cline in Australasia, Oakeshott *et al.* (1982) used populations from different longitudes as well as collections made over different years and seasons. The study of van 't Land *et al.* (2000) involved samples from only six populations from Central America and another nine populations from South America, with these two regions separated by a gap of over 16° latitude. Although van 't Land *et al.* (2000) observed a cline for *α-Gpdh* when data from both South America and Central America were combined there was no clinal pattern for each region considered separately.

Moreover, when the entire cline of Oakeshott *et al.* (1982) is re-analysed following angular transformation of allozyme frequencies, the quadratic component is marginally significant ($F=3{\cdot}673$; df = 32; $P=0{\cdot}037$) while the linear regression is not significant ($F=2{\cdot}306$; df = 32; $P=0{\cdot}139$), suggesting a curvilinear association with latitude in the same direction to our findings here. However, it should be noted that when only the east coast populations are analysed neither a linear or curvilinear association between *α-Gpdh* and latitude is present. Therefore, it appears we have witnessed a clinal relationship develop or at least become tighter over the last 20 years along eastern Australia, although further examination of

https://doi.org/10.1017/S0016672306007919 Published online by Cambridge University Press

Table 4. *Linkage disequilibrium between Adh, α-Gpdh and In(2L)t in populations of* Drosophila melanogaster *collected from four latitudes along eastern coastal Australia in 2002 and 2004. Sample size* (*n*), *absolute D* (*D*), *Hedrick's multiallelic version of D* (*D′*) *and the significance of LD* (*P*) *are indicated*

Markers	Year	Population	Latitude	*n*	*D*	*D′*	*P*
Adh × *α-Gpdh*	2002	Red Knight	42·8	104	−0·042	0·39	0·06
		Wollongong	34·4	66	−0·051	0·55	0·62
		Maryborough	25·5	112	0·035	0·67	0·17
		Innisfail	17·5	88	−0·019	0·27	0·36
	2004	Sorell	42·8	91	−0·011	0·08	0·10
		Sydney	34·0	111	−0·029	0·27	0·17
		Maryborough	25·5	79	0·004	0·07	0·29
		Cardwell	18·3	105	−0·010	0·18	0·03
Adh × *In(2L)t*	2002	Red Knight	42·8	104	−0·039	0·87	<0·001[a]
		Wollongong	34·4	66	−0·127	0·93	<0·001[a]
		Maryborough	25·5	112	0·003	0·04	0·33
		Innisfail	17·5	88	−0·039	–	0·13
	2004	Sorell	42·8	91	−0·020	0·77	<0·01[a]
		Sydney	34·0	111	−0·056	0·42	<0·01[a]
		Maryborough	25·5	79	−0·008	0·28	0·05
		Cardwell	18·3	105	−0·009	0·52	0·09
α-Gpdh × *In(2L)t*	2002	Red Knight	42·8	104	0·020	–	0·17
		Wollongong	34·4	66	0·051	–	0·20
		Maryborough	25·5	112	0·027	0·76	0·05
		Innisfail	17·5	88	0·056	0·83	0·14
	2004	Sorell	42·8	91	0·004	0·28	0·20
		Sydney	34·0	111	0·032	0·54	0·26
		Maryborough	25·5	79	0·059	–	0·04
		Cardwell	18·3	105	0·009	0·34	0·91

[a] Remained significant after correction for multiple comparisons.

these patterns in high-latitude regions would be worthwhile. Additional samples from the northern end of the cline (<22° S latitude) are also needed as the association between latitude and *α-Gpdh* alleles appears to be the strongest in this region.

The non-linear nature of the *α-Gpdh* cline reflects a higher frequency of α-$Gpdh^F$ in populations derived from the highest and lowest latitudes. This suggests complex selection patterns acting on this gene. *α-Gpdh* is important in the biosynthesis of phospholipids and triglycerides (O'Brien & MacIntyre, 1972; Geer *et al*., 1983) and plays an essential part of the α-glycerophosphate cycle that facilitates rapid ATP production for flight in adults (Zera *et al*., 1985; Barnes & Laurie-Ahlberg, 1986) and the oxidative degradation of ethanol and carbohydrates in larvae (Geer *et al*., 1983). The non-linear cline could be the result of two separate functions that independently select for α-$Gpdh^F$ in southern and northern locations. For example, α-$Gpdh^{FF}$ genotypes have been shown to have better survival (Oakeshott *et al*., 1985) and a higher flight output at high temperatures (Serra & Oller, 1984; Barnes & Laurie-Ahlberg, 1986), which are presumably more important fitness components in the tropics than cooler temperate regions. On the other hand, α-$Gpdh^{FF}$ genotypes produce more productive females under cool crowded conditions (Ochando & Ayala, 1999 – possibly through more efficient synthesis of lipids), which would be more important in temperate regions where food supply is scarce. While speculative, such fitness changes could lead to more intense selection for the α-$Gpdh^F$ allele at the two extremes of the cline than at intermediate latitudes, resulting in a quadratic clinal pattern. A non-linear clinal pattern has been described for some traits in *D. melanogaster* from eastern Australia (Mitrovski & Hoffmann, 2001) and may be associated with similar selection pressures affecting *α-Gpdh*.

The partial tandem duplication of the *α-Gpdh* gene has been associated with higher GPDH protein levels and activity in natural populations of *D. melanogaster* (Symonds & Gibson, 1992; Kang *et al*., 1998), which may explain the high duplication frequencies in natural populations along the entire east coast of Australia. *DpGpdh* was tightly associated with the α-$Gpdh^S$ allele, supporting previous findings that α-$Gpdh^{SS}$ flies carry more duplications than flies with a α-$Gpdh^{FF}$ genotype (Kang *et al*., 1998). The absence of a clinal pattern with latitude suggests that the duplication is influenced by different evolutionary pressures to those acting on the α-$Gpdh^{S/F}$ polymorphism. There are different explanations for the observed association between α-$Gpdh^S$ and the presence of *DpGpdh* at the *α-Gpdh* locus. The duplication

https://doi.org/10.1017/S0016672306007919 Published online by Cambridge University Press

may have arisen in a chromosome carrying this allele, and the association may be breaking down over time. An alternative explanation involves co-selection between the *α-Gpdh* polymorphism or closely linked polymorphisms and *DpGpdh*. It is not possible to distinguish between these alternatives based on current data.

The two *Adh* indel polymorphisms, *Adh∇1* and *∇2*, showed a linear relationship with latitude, increasing with distance from the equator. These polymorphisms have been positively linked with levels of ADH protein (Laurie & Stam, 1994) and perhaps a higher amount of *Adh* enzyme is required in the cooler temperate regions than in the tropics. The clinal patterns observed for *Adh∇1* and *∇2* could be a result of neutral hitchhiking. Both *Adh∇1* and *∇2* were in strong linkage disequilibrium with the Adh^F allele in the 2002 and 2004 collections. Additionally, when we controlled for the $Adh^{S/F}$ polymorphism using partial correlations, only the relationship between the low-frequency *Adh∇2* and latitude in 2004 remained significant. This suggests that *Adh∇1* (and more than likely *Adh∇2*) are not involved in climatic adaptation along the cline. These markers (and *DpGpdh*, which also showed no clinal pattern with latitude) are more than likely influenced by demographic factors such as migration and colonization history (Gockel *et al*., 2001).

Another important factor to be considered in relation to the maintenance of clinal patterns for *α-Gpdh* and the other polymorphisms on the left arm of chromosome 2 is the influence of *In(2L)t*, which shows a linear clinal pattern with latitude in eastern Australia (Umina *et al*., 2005). We found strong LD between *In(2L)t* and the Adh^S allele in southern populations, but not in the north. The higher level of linkage at temperate latitudes is consistent with the findings of van 't Land *et al*. (2000) who found a strong association between Adh^S and $\alpha\text{-}Gpdh^F$ at temperate latitudes in Central and South America that was most likely caused by *In(2L)t*. Strong gametic disequilibrium has also been observed for *Adh* and *α-Gpdh* in the laboratory and a semi-natural population (van Delden & Kamping, 1989; Kamping & van Delden, 1999) although the degree of linkage varied considerably over seasons and under different environmental conditions. We did not detect significant LD between *Adh* and *α-Gpdh* in any field populations, despite the relatively high D' values in several instances. There was also no evidence of significant LD between *α-Gpdh* and *In(2L)t*, despite the *α-Gpdh* locus being located inside the inversion. Unless gametic phases are known and/or large samples sizes are used to characterize LD, however, estimates of D' must be met with some caution.

We are grateful to Jenny Griffiths and Vanessa Kellerman for fly collecting, the Australian Research Council for financial support via their Special Research Centre Program and two anonymous reviewers for valuable comments on this manuscript.

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