

The latitudinal cline in the *In(3R)Payne* inversion polymorphism has shifted in the last 20 years in Australian *Drosophila melanogaster* populations

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Abstract

Clinal variation has been described in a number of inversions in *Drosophila* but these clines are often characterized by cytological techniques using small sample sizes, and associations with specific genes are rarely considered. Here we have developed a molecular assay for *In(3R)Payne* in *Drosophila melanogaster* from eastern Australia populations. It shows in repeated samples that the inversion cline is very tightly associated with latitude and is almost fixed in tropical populations while relatively rare in temperate populations. This steep cline has shifted in position in the last 20 years. The heat shock gene, *hsr-omega*, located centrally inside the inversion sequence, shows a different clinal pattern to *In(3R)Payne*. These results suggest strong ongoing selection on *In(3R)Payne* over the last 100 years since the colonization of Australia that is partly independent of *hsr-omega*.

Keywords: *Drosophila*, *hsr-omega*, *In(3R)Payne*, latitudinal variation, polymorphism, SNP

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Introduction

Inversion polymorphisms inhibit recombination in the inverted region of the chromosome, and help tie up blocks of genes, particularly around inversion breakpoints. A number of latitudinal clines in inversion frequencies in dipteran species have been identified, especially in *Drosophila* (Krimbas & Powell 1992; Bayoh *et al.* 2001). These clines are thought to be associated with natural selection arising from climatic variables, raising the issue of whether inversion polymorphisms are associated with climatic adaptation. Inversion clines can be established quickly, as seen in *Drosophila subobscura* in North and South America where they were established in less than 5 years (Prevosti *et al.* 1988). Inversion clines are often repeatable across different continents, as in the common cosmopolitan inversions of *Drosophila melanogaster* (Mettler *et al.* 1977; Inoue & Watanabe 1979; Knibb *et al.* 1981). While inversion clines are stable over time, they can also change, presumably because of shifts in climatic factors (Rodriguez-Trelles & Rodriguez 1998).

Perhaps the simplest hypothesis for inversion clines is that there are alleles tied up within the inversions that are under clinal selection and their strong disequilibrium with the inversion is what maintains the inversion cline. Clines for alleles associated with the inversion might then be as steep as those for the inversion itself, whereas alleles that act independent of the inversion may show geographical patterns that are independent of the inversion. For instance, in *D. melanogaster*, Frydenberg *et al.* (2003) studied the latitudinal variation of three small heat shock genes which are located within *In(3 L)Payne*. One of these genes, *hsp26*, showed a latitudinal cline that was present in inverted as well as in standard gene arrangements, indicating that evolutionary processes leading to the cline in *hsp26* were at least partly independent of the inversion.

More complicated hypotheses about inversion clines involve co-adapted gene complexes; inversions are thought to hold beneficial allele combinations which are selected as co-adapted gene complexes and may differ between populations (Dobzhansky 1970). If inversions hold together different allele combinations at different latitudes, clinal patterns in inversion frequencies might not lead to clinal patterns for genes inside the inversions. The absence of

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clinal patterns might also reflect the fact that crossing over within the inverted sequence breaks up any linkage disequilibrium between alleles and inversion breakpoints.

The cosmopolitan *In(3R)Payne* inversion polymorphism of *D. melanogaster* has become of increasing interest because of its potential association with quantitative traits that vary clinally in an adaptive manner. In particular, this inversion polymorphism has been implicated in body size clines in Australia and South America, based on associations between alleles within the inversion and size (Weeks *et al.* 2002) as well as quantitative trait loci (QTL) mapping of size diverged lines derived from cline ends (Calboli *et al.* 2003). Genes involved in the insulin-signalling pathway which are present in the inverted region may be responsible for this association (De Jong & Bochdanovits 2003). The *In(3R)Payne* inversion has also been associated with thermal resistance traits (Anderson *et al.* 2003), particularly via one of the heat shock genes, *hsr-omega*. This gene is located within the inversion but well away from the breakpoints, and shows a strong latitudinal cline in eastern Australia (McCull & McKechnie 1999) and has been implicated in heat resistance, a trait that also varies clinally in eastern Australia (Hoffmann *et al.* 2002).

To date, association studies on *In(3R)Payne* have been hampered by the absence of a molecular marker for this inversion. Molecular markers enable inversions to be scored accurately and quickly on field-collected flies with large sample sizes. Here we develop a molecular marker for *In(3R)Payne* of *D. melanogaster* from eastern Australia and test its latitudinal association over 2 years. We then show how the latitudinal cline in *In(3R)Payne* has shifted in the last 20 years by comparing patterns with those described

by Knibb *et al.* (1981) based on cytological studies of flies collected in 1980. We also assess the association of *In(3R)Payne* with a previously characterized cline in *hsr-omega*. By comparing slopes of this cline with the *hsr-omega* cline and the *hsr-omega* molecular variation within the inversion, we show that these clines are partially independent. Evolutionary processes maintaining this inversion cline including Dobzhansky's co-adaptation hypothesis are discussed.

Materials and methods

Drosophila melanogaster field samples

Female and male *Drosophila melanogaster* were collected from the east coast of Australia between March and May in 2002 (16 sites) and in 2004 (18 sites) (Table 1). Field males were stored in 100% ethanol at -20°C for subsequent DNA extraction and genotyping of *In(3R)Payne* and *hsr-omega*. Field females were reared as individual isofemale lines at 25°C under continuous light and on a sugar (1.6% w/v), agar (3.2%), yeast (3.2%) and potato (1.6%) medium that was always treated with antibiotics (2% dihydrostreptomycin and 0.6% penicillin added to the medium surface) and an antifungal agent (Nipagin: 0.14% w/v). Once isofemale lines were established, field-collected females were preserved in 100% ethanol and stored at -20°C for genotyping.

Isolation of a single nucleotide polymorphism (SNP) in complete linkage disequilibrium with *In(3R)Payne*

Lines used for breakpoint analysis were characterized for *In(3R)Payne* by using basic cytological methods as described

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

2004 Populations	Latitude	<i>n</i>	2002 Populations	Latitude	<i>n</i>
Sorell	42°46'08" S	28	Red Knight	42°45'00" S	25
Millers	41°14'13" S	28	Bega	36°40'01" S	8
Yarra Valley	37°39'32" S	26	Wollongong	34°25'01" S	28
Moruya	35°54'53" S	27	Belmont	33°01'59" S	17
Sydney	33°57'18" S	25	Coffs Harbour	30°16'01" S	26
Tuncurry	32°10'30" S	18	Alstonville	28°49'01" S	28
Red Rock	29°59'02" S	30	Kingscliff	28°16'59" S	28
Kingscliff	28°15'54" S	17	Redland Bay	27°36'00" S	25
Redland Bay	27°36'40" S	25	Rainbow Beach	25°55'59" S	26
Maryborough	25°32'42" S	25	Maryborough	25°31'59" S	28
Rockhampton	23°19'44" S	25	Mirriamvale	24°19'59" S	24
Mackay	21°07'34" S	18	Gladstone	23°52'59" S	30
Bowen	20°00'58" S	15	Rockhampton	23°19'01" S	24
Magnetic Island	19°10'26" S	17	Sarina	21°22'59" S	24
Cardwell	18°16'01" S	28	Innisfail	17°30'00" S	24
Kirrama	18°13'05" S	28	Cape Tribulation	16°04'59" S	28
Cairns	16°54'25" S	27			
Cooktown	15°28'34" S	27			

The number of adults (*n*) genotyped for the *In(3R)Payne* marker and the *hsr-omega* indel are indicated.

by Yoon *et al.* (1973). Briefly, salivary glands were dissected from third instar larvae in Becker Ringers solution, placed in 45% acetic acid for 10 s and then 1 M HCl for 30 s prior to staining with lactoacetoorcein for 40 min. After staining, glands were squashed under a cover slip and visualized with a light microscope at $\times 1000$ magnification. Chromosome 3R was examined for presence/absence of a heterozygote loop and if absent, for the order of bands spanning the region where the inversion is found.

Bacterial artificial chromosomes (BAC) known to hybridize to cytological positions around the proximal inversion breakpoint of *In(3R)Payne* were used as initial probes in *in situ* hybridizations (Philips *et al.* 1994). BACR01H23 (Berkeley Drosophila Genome Project website, <http://www.fruitfly.org>) hybridized to both the proximal and distal cytological breakpoints in inverted homozygote polytene chromosomes, suggesting the molecular breakpoint was within this fragment. Polymerase chain reaction (PCR) was used to produce DNA fragments of various sections of the BAC to locate the breakpoint. All probes hybridized to the 89 region in standard arrangements. In inverted arrangements, they could hybridize to the 89 region, 96 region or both (breakpoint within the probe), depending on their location in respect of the breakpoint (see Fig. 1 and Appendix 1). Results showed there was some degree of duplication around the breakpoint region as more than one fragment hybridized to both cytological breakpoints.

To develop a marker to score *In(3R)Payne*, a single nucleotide polymorphism (SNP) was found by sequencing a 550-bp area directly before the proximal breakpoint duplicated region (primers marked by asterisk in Appendix 1). Three independent lines fixed for *In(3R)Payne* from Innisfail in 2002 and three lines fixed for the standard arrangement from Red Knight in 2002 were sequenced using Applied Biosystems PRISM BigDye Terminator chemistry (version 3.1) at Monash University Micromon sequencing facility. Sequences (GenBank Accession nos AY676332–AY676346) were aligned using CLUSTAL W (Thompson *et al.* 1994) and a single nucleotide transversion was found that differed consistently between standard (A) and inverted (C) arrangements, respectively, at position 12253813 bp of the *D. melanogaster* genome sequence (Berkeley Drosophila Genome Project database).

The Bi-PASA (bidirectional PCR amplification of specific alleles) method of Liu *et al.* (1997) was used to genotype the SNP. This method uses four primers in a PCR amplification; two outer primers which are not allele specific and two inner primers which are allele specific. This results in the amplification of up to three possible fragments; the two outer primers amplify a nonspecific 570 bp fragment, one outer and inner pair amplify the C allele specific 326-bp fragment, and the other primer pair amplify the A allele specific 298-bp fragment. The inner primers have non-

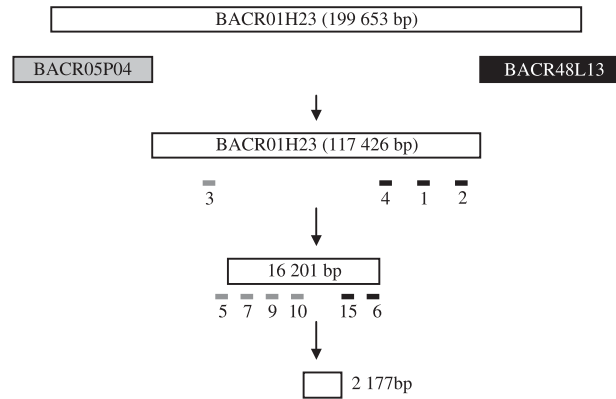


Fig. 1 Schematic representation showing the process to determine the site of the *In(3R)Payne* proximal breakpoint in *Drosophila melanogaster*. Grey blocks represent hybridization of BACs at 89 C and black blocks represent hybridization of BACs at 96 A of third instar polytene chromosomes of *D. melanogaster*. Probe numbers correspond to probe sequences in Appendix 1. BACR05P04, BACR48L13, and BACR01H23 were *in situ* hybridized to an inverted homozygote; BACR05P04 hybridized at 89 C, BACR48L13 hybridized to 96 A and BACR01H23 hybridized to both. Because of the overlap of BACs the breakpoint was within 117 426 bp of BACR01H23. Hybridization of large (up to 10 kb) PCR probes (1–4) decreased this area to 16 201 bp. Further hybridization of smaller probes (5–14) reduced the area that contained the breakpoint to 2177 bp. This area was not reduced further as probes covering this area hybridized to both breakpoints in inverted homozygotes (suggesting sequence repetition in this region).

complementary 3' ends to reduce megaprimering and to switch from template-based amplification to self amplification of the smaller fragments.

The outer primer sequences were; outer primer 1'-TTTGCCGCAAATTATTGTGAG and outer primer 2'-ATCGCGTGACAGGTTGGC. Inner primer 1 specific for the A allele was cgggcggcgggTTATATTTTATAGTGTGCTA and the inner primer 2 specific for the C allele was cgggcggcAATACAATTGCATGCAC. The outer and inner primer pair 1 produce a 298-bp fragment that marks the standard arrangement, whilst the outer and inner pair 2 produce a 326-bp fragment that marks *In(3R)Payne*.

To determine if the SNP marker was in complete linkage disequilibrium with the standard and inverted arrangements, we characterized larvae from isofemale lines collected in 2004 from several populations along the cline using both cytological methods and by scoring the SNP marker on the same individuals. Briefly, salivary glands were dissected from a third instar larva and cytologically genotyped as above. DNA was then extracted using a CTAB (cetyltrimethyl ammonium bromide) method (Weeks *et al.* 2002) from the remaining portion of the larva and scored for the SNP marker. A single larva from 46 isofemale lines from three northern populations (21 from Cardwell, 14 from Bowen and 11 from Sarina), 34 isofemale lines from

three middle populations (10 from Red Rock, 18 from Sydney and 6 from Moruya) and 28 isofemale lines from two southern populations (19 from Sorrell and 9 from Yarra Valley) were characterized cytologically and with the SNP marker. From a total of 14 inverted homozygotes, 47 heterozygotes and 47 standard homozygotes, the SNP was in complete linkage disequilibrium with gene arrangement *In(3R)Payne*. Therefore, the SNP was used as a marker for *In(3R)Payne*.

Genotyping clinal populations for the presence of *In(3R)Payne*

Field females and males collected from populations along the east coast of Australia in 2002 and 2004 (Table 1) were genotyped for *In(3R)Payne* using the SNP marker and an 8 bp indel in the heat shock gene, *hsr-omega*, that has previously been shown to form a cline (Anderson *et al.* 2003). DNA was extracted using CTAB (Weeks *et al.* 2002), with 2 µL of DNA used in the Bi-PASA to score the SNP as above, and 1 µL was used to genotype the 8 bp indel in *hsr-omega* as in Anderson *et al.* (2003). This polymorphism is in complete linkage disequilibrium with the A/T polymorphism investigated in other research on *hsr-omega* and linked to knock-down resistance for heat stress (McCull *et al.* 1996; McKechnie *et al.* 1998; McCull *et al.* 1999; Anderson *et al.* 2003).

Data analysis

Associations between the frequency of the SNP marking *In(3R)Payne* and *hsr-omega*^S with latitude for populations collected in 2004 and 2002 were determined using linear regression in SPSS (version 11.5). For statistical comparisons, frequencies were angular transformed. However regressions presented in figures are not transformed to maintain biological meaning and because this is not necessary for descriptions of associations (Zar 1996). Zero frequency data points were omitted from slope analyses because of the effect on residuals after angular transformation. Slopes of regression lines were compared using analysis of covariance (ANCOVA) and *t*-tests following Zar (1996) to determine whether the slopes of the regression lines differed between years [including data from Knibb *et al.* (1981) for *In(3R)Payne* and Anderson *et al.* (2003) for *hsr-omega*] and between markers for 2004 and 2002. If slopes did not differ significantly, then the elevation of the population regressions were compared following Zar (1996). Upper and lower confidence limits (95%) for coefficients of determination (R^2) were determined by permutation (10 000) by drawing the residuals of a regression model at random and recalculating R^2 [using the Excel add-in POPTOOLS (Hood 2004)]. Previous research has shown that *In(3R)Payne* is not in linkage disequilibrium with other cosmopolitan

inversions (Weeks *et al.* 2002; P. Umina, unpublished) and endemic inversions on the right arm of chromosome 3 are extremely rare (<1%) and therefore neither were considered here.

Results

In(3R)Payne marker cline

The frequency of the *In(3R)Payne* marker forms a steep cline with latitude for both 2004 and 2002 collections (Fig. 2a) with highly significant linear regressions for both years (Table 2). Coefficients of determination (R^2) were 0.92 and 0.57 for 2004 and 2002, respectively (Table 2), showing a strong negative relationship between *In(3R)Payne* and latitude. Confidence intervals for R^2 suggested that the association with latitude was tighter for 2004 than either 2002 or 1979 (Table 2) although more populations were sampled in 2004 than in 2002 (Table 1) and 1979 (data from Knibb *et al.* 1981).

Slopes of the three regression lines did not differ significantly ($F_{2,42} = 2.791, P = 0.073$), however, there was a significant

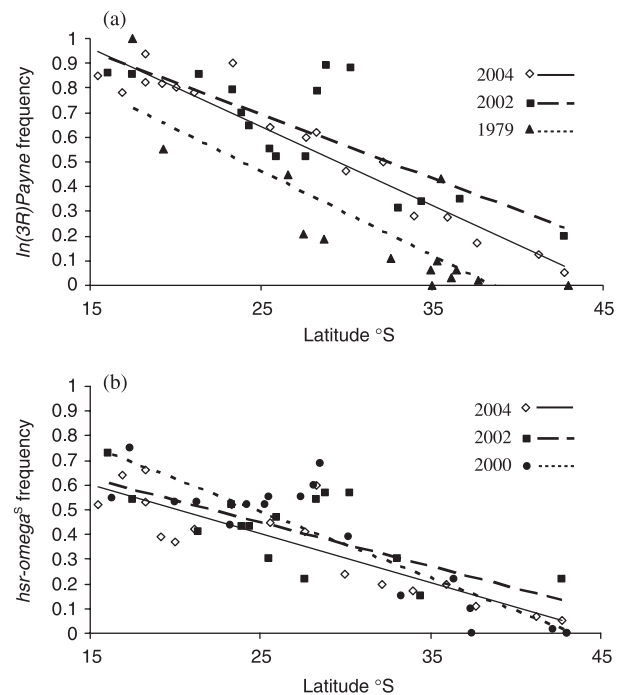


Fig. 2 (a) Frequency of *In(3R)Payne* from populations of *Drosophila melanogaster* collected along the east coast of Australia in 2004 (◇ data points, — trendline), 2002 (■ data points, - - trendline) and 1979 [▲ - - data points, trendline; data taken from Knibb *et al.* (1981)]. (b) Frequency of *hsr-omega*^S from populations of *D. melanogaster* collected along the east coast of Australia in 2004 (◇ data points, — trendline), 2002 (■ data points, - - trendline) and 2000 [● data points, trendline; data taken from Anderson *et al.* (2003)].

Table 2 Linear regressions of *In(3R)Payne* and *hsr-omega^S* frequency onto latitude for 3 years in *Drosophila melanogaster* collected along the east coast of Australia

Marker	Year	Regression equation	<i>t</i>	<i>P</i>	<i>R</i> ² (95% CI)
<i>In(3R)Payne</i>	2004	$y = -0.036x + 1.847$	13.397	< 0.001	0.918 (0.763–0.928)
	2002	$y = -0.028x + 1.694$	4.314	< 0.001	0.571 (0.173–0.649)
	1979*	$y = -0.050x + 2.057$	5.080	< 0.001	0.721 (0.334–0.814)
<i>hsr-omega</i>	2004	$y = -0.023x + 1.258$	7.784	< 0.001	0.791 (0.487–0.819)
	2002	$y = -0.020x + 1.220$	3.750	< 0.01	0.501 (0.118–0.627)
	2000†	$y = -0.029x + 1.496$	5.753	< 0.001	0.703 (0.341–0.766)

*Data from Knibb *et al.* (1981); †data from Anderson *et al.* (2003).

difference in elevation ($F_{2,44} = 12.692$, $P < 0.001$). We used *t*-tests (Zar 1996) to determine which differences among elevations were significant. There was no significant difference in the elevation of the population regressions between the 2004 and 2002 samples ($t = 1.49$, d.f. = 31, $P = 0.147$). However, elevations in the 2004 and 2002 population regressions differ significantly from the 1979 population regression ($t = 3.660$, d.f. = 29, $P < 0.001$; $t = 3.902$, d.f. = 27, $P < 0.001$, respectively, for 2004 and 2002), suggesting a significant shift in elevation over the last 20 years.

hsr-omega cline

There was a significant linear relationship between the frequency of the *hsr-omega^S* allele and latitude for both the 2004 and 2002 collections (Table 2), and this allele increased in frequency as latitude decreased (Fig. 2b). *R*² values for the latitude association were 0.79 for 2004 and 0.50 for 2002, but confidence limits for the *R*² values overlapped (Table 2). *R*² values were similar to those previously published based on collections in 2000 (Anderson *et al.* 2003).

Slopes for the regression of *hsr-omega^S* frequency onto latitude did not differ significantly among years ($F_{2,44} = 0.985$, $P = 0.381$). Elevation of the population regressions also did not differ significantly among years ($F_{2,49} = 0.921$, $P = 0.405$).

In(3R)Payne and *hsr-omega* cline comparison

The significant relationship between latitude and the *hsr-omega^S* cline could largely be because of the relationship of *In(3R)Payne* with latitude, given that the *hsr-omega* gene is found within this inversion. The cline in *hsr-omega^S* has a smaller slope than the cline in *In(3R)Payne* (Fig. 2a,b), suggesting that the gene and inversion are not completely associated. When we combine the data from the 2004 and 2002 collections [as slopes do not differ between collections within *In(3R)Payne* or *hsr-omega^S*] and compare regressions for *In(3R)Payne* and *hsr-omega^S*, there was no significant difference in slope ($t = 1.01$, d.f. = 64, $P = 0.114$), but there was a significant difference in elevation ($t = 4.470$, d.f. = 65, $P < 0.001$).

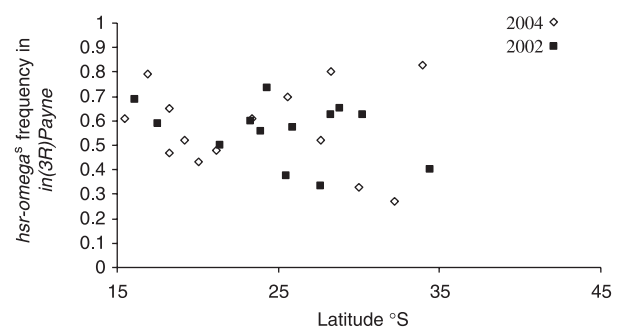


Fig. 3 Frequency of *hsr-omega^S* in *In(3R)Payne* chromosomes carrying the inverted arrangement from populations of *Drosophila melanogaster* collected along the east coast of Australia in 2004 (◇) and 2002 (■). Only populations where more than 10 inverted *In(3R)Payne* chromosomes were sampled are shown.

If the *hsr-omega^S* cline is independent of the *In(3R)Payne* inversion cline, there should be a significant relationship between *hsr-omega^S* and latitude when only chromosomes with the inverted *In(3R)Payne* arrangement are considered [and ignoring populations with few (< 10) inverted arrangements]. In this case, no relationship between latitude and *hsr-omega^S* is evident in either the 2004 or 2002 collections (Fig. 3; $F_{1,12} = 0.045$, $P = 0.836$; $F_{1,11} = 1.539$, $P = 0.241$, respectively). This suggests that the relationship between latitude and the frequency of *hsr-omega^S* is partly or wholly because of the disequilibrium with *In(3R)Payne*.

Discussion

The development of a molecular marker for *In(3R)Payne* has enabled genotyping of field caught females faster than would otherwise be possible with cytological analysis. With this marker, it should be possible to separate effects of the inversion polymorphism on traits such as body size (Gockel *et al.* 2002) and thermal tolerance (Anderson *et al.* 2003) from genes located within the inversion.

As has previously been reported, *In(3R)Payne* shows a strong negative relationship with latitude and forms a steep cline in Australian east coast populations, suggesting

the inversion is under strong selection by climatic variables. The steepness of the cline has not significantly altered between the two years sampled in this study, nor has it changed in the last 20 years since it was originally sampled in 1979. However, the elevation of the population regression has shifted from 1979 to 2002/2004. When regression lines in Fig. 2a are compared, it is evident that this shift is equivalent to more than 5° of latitude, or a distance of more than 500 km. This shift is likely to be an evolutionary response to changing conditions rather than ongoing changes in inversion frequencies towards an equilibrium point because inversion clines tend to be established quickly (Prevosti *et al.* 1988). Climatic factors that might be responsible for such a shift are currently unknown. Other studies particularly in *Drosophila robusta* (Etges & Levitan 2004) and *Drosophila subobscura* (Solé *et al.* 2002) indicate changing patterns of inversion polymorphisms, possibly in response to climate change.

A potential confounding effect in this comparison is that the inversion was sampled in 1979 in isofemale lines that had been maintained in the laboratory for up to nine generations, whereas in the current study field-caught females were genotyped. Laboratory culture might result in a decrease in inversion frequency in mass-bred populations but this is likely to be small (Singh & Das 1992) and we have not found any systematic shift in the frequency of *In(3R)Payne* arrangements in any of our laboratory populations.

The *hsr-omega* L/S indel polymorphism formed a cline with latitude in 2000 (Anderson *et al.* 2003) and this cline is also evident in the 2002 and 2004 collections. The cline in this gene appears to be stable over 4 years (as does the inversion cline over 2 years), with no difference in elevation or slope over the three collections. It is not known if the cline has shifted in the last 20 years because it was not scored at the time when the original inversion cline was established. The *hsr-omega*^S allele is in strong linkage disequilibrium with the inversion and only occurs rarely with the standard arrangement. This is despite the fact that *hsr-omega* is at genetic location 3–71, whereas the breakpoints of the inversion are approximately at 3–59 (proximal) and 3–86 (distal), well away from *hsr-omega*. Other studies have suggested that disequilibrium between markers is only strong near inversion breakpoints (Andolfatto *et al.* 2001; Laayouni *et al.* 2003), although in *Drosophila pseudoobscura* markers away from breakpoints can also be in disequilibrium and are likely maintained by selection (Schaeffer *et al.* 2003).

When only variation within the inverted arrangement of *In(3R)Payne* is considered, a cline in *hsr-omega*^S is no longer evident. Although sample sizes were reduced when we considered only the *hsr-omega* polymorphism within this chromosome arrangement, the *hsr-omega* cline is steep and should nevertheless have been detected even with these sample sizes. The absence of a cline suggests that *hsr-omega*

may not be under direct latitudinal selection that is independent of selection on the inversion. In contrast, the *hsp26* gene did show latitudinal variation within the *In(3L)Payne* inversion (Frydenberg *et al.* 2003).

The *hsr-omega*^S allele does not reach the same high frequency reached by *In(3R)Payne* in low latitude populations. Additional markers will need to be considered to determine if *hsr-omega* is a target of any direct selection independently of *In(3R)Payne*, or if selection on *In(3R)Payne* depends more directly on other genes or gene combinations. *hsr-omega* might yet be under clinal selection in combination with other loci rather than only through disequilibrium with the inversion, particularly as it is located central to the inversion breakpoints where recombination rates are expected to be highest because of double crossovers or gene conversion (Navarro *et al.* 1997). This would represent a component of Dobzhansky's co-adaptation hypothesis, where combinations of genes within inversions are co-adapted to have a high fitness within populations. Under this hypothesis, alleles are adapted to act together within inversions as well as between different gene arrangements, leading to high fitness for inversion heterozygotes.

However, when heterozygote advantage is present, steep clines in inversions such as those found here for *In(3R)Payne* are not expected to exist; the fixation or near fixation of an inverted arrangement at one end of the cline and a standard arrangement at the other end is not consistent with strong overall heterozygote advantage. Instead, the steepness of the cline suggests that it may be associated with adaptive clinal variation in traits; perhaps genes in complete disequilibrium with the inversion, some or most of which form co-adapted blocks locked within the inversion, help contribute to the strong clinal patterns observed for several quantitative traits in *Drosophila melanogaster*. This is in contrast to other species such as *Drosophila simulans* that do not show strong clinal differentiation (Gibert *et al.* 2004) and lack latitudinally varying inversion polymorphisms.

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Appendix 1

Primer pair	Position in BAC01H23	Hybridization result	Probe number
AAACTCACGAAGTCACTCACTGCTTG CGAAACGGGTGCTATCAGTG	115020–115045 125017–124998	1 signal (96 A18)	1
ACATTGCCAGTGGGGCTGAG CGCACGAGTTGCCAATGAGTATG	157627–157646 167163–167141	1 signal (96 A18)	2
TTGGTATCGCTATTGCTTCTCCCC CTTGTGTGCCCTCACGATGA	69656–69679 77648–77627	1 signal (89C2-3)	3
GGAGTACATCCTGCTCTGCTGCC GGCGTGACCGTGAGCAGTAGTG	93849–93871 101222–101201	1 signal (96 A18)	4
AGAAGATGGAGAACTGTGGGGCAT TGTGATAGGCCCGCGCAG	85684–85661 84661–84679	1 signal (89C2-3)	5
GCTTTTGGCAGCAGAGCAGGAT ATCCCCGAATGTCGCCACG	93877–93856 92869–92886	1 signal (96 A18)	6
ACAACATCGGTCAAACACGGAGAA CGCTAAATGGGCAGGTGCAAT	85147–85176 86189–86169	1 signal (89C2-3)	7
GCCGCACACCCTCACAGTTG CGACTGGCTAACTGACTGGCTG	87699–87719 88739–88718	2 signals (faint 96)	8
GCCGCACACCCTCACAGTT* CGCGTGTGGACTCACTGC*	87699–87717 88270–88253	1 signal (89 C2-3)	9
GCAGTGAGTCCACACGCG CCGGGTGTATTTCAGTAGCC	88253–88272 88716–88697	1 signal (89C2-3)	10
CAGCCAGTCAGTTAGCCAGTCG CCTCTTGACCGTTCCTTC	88718–88739 89239–89214	2 signals	11
GGATTGGAACCGATTTCACAC ACCCTTTGAGTCCCCTTCTCC	89233–89254 90229–90209	2 signals	12
CTGCATTGGGTAATGAAAGCG CGCTTTCATTACCCAATGCAG	89737–89757 90229–90209	2 signals	13
CTTATCCAGGGTCTTGCCACA CGGTTGACGGAGCCAA	90266–90286 90832–90816	2 signals	14
GGCAAGAGTGAAACTGTCTCCC ATTTACCGATGGTGCCCC	90833–90854 91393–91375	1 signal (96 A18)	15