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ERRATA

p 23 line 1: "collection" for collections"
p 23 line 2: "isofemale" for "isofemales"
p 33 line 18: at end of the sentence add "(Zu *et al.* 1998)"
p 39 line 1: "independence" for "independent"
p 40 line 3: should read " ($1^+/2^+$ representing chromosome 3) was crossed...."
p 41 line 5: "where" for "were"
p 47 line 2: should read "50-350 bp"
p 56 line 22: "in the linkage lines used in the present study" for "in these linkage lines"
p 111 line 4: order of reference "Gockel *et al.* 2001" before "Gockel *et al.* 2002"
p 111 line 28: "1991" for "1991a"
p 112 line 6: "1991" for "1991b"
p 112 line 6: order of reference "Hoffmann and Parsons 1991" before "Hoffmann *et al.* 2003"
p 112 line 22: "1995" for "1995a"
p 112 line 26: "1995" for "1995b"
p 113 line 20: order of references "Knibb 1982" before "Knibb *et al.* 1981"
p 113-114: order of Lakhotia references: Lakhotia 1987, Lakhotia 2001b, Lakhotia 2003, Lakhotia and Mukherjee 1980, Lakhotia and Mukherjee 1982, Lakhotia *et al.* 2001b, Lakhotia *et al.* 1999, Lakhotia and Sharma 1996, Lakhotia and Singh 1982b.
p 114 line 1: "2001" for "2001a"
p 114 line 4: "2001" for "2001b"
p 128 line 3: "obtained" for "obtain"
p 128 line 7: "sequences" for "sequence"

Adaptation of *Drosophila melanogaster* to altitudinal and latitudinal climatic gradients: the role of the heat-shock RNA gene *hsr-omega*

A thesis submitted for the degree of Doctor of Philosophy

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Abstract

The main focus of this work has been to investigate the hyper-variable tandem repeat-length polymorphism of the *heat-shock RNA omega* gene. *Hsr-omega* maps to the right arm of the third chromosome in *Drosophila melanogaster*, in a region that influences population variation for traits that vary across climatic regions.

Natural Australian populations of *D. melanogaster* were examined for latitudinal *hsr-omega* repeat-length variation using Southern analysis. Average repeat-length demonstrated a strong negative correlation with latitude, which was robust over three consecutive years. This association suggests the action of natural selection on that part of chromosome 3R marked by *hsr-omega* repeat-length variation.

Linkage associations were assessed between *hsr-omega* repeat-length and ten third chromosome genetic markers that also latitudinally cline. Using *D. melanogaster* collected from Coffs Harbour, a population central on the eastern Australian coast cline, a crossing scheme allowed the haplotype of seventy-eight independent third chromosomes to be scored. *Hsr-omega* repeat-length was not in strong linkage disequilibrium with any of the genetic markers, including a *hsr-omega*$^{1/5}$ (8 bp indel) polymorphism and the inversion *In(3R)P* in which the *hsr-omega gene* is situated. However *In(3R)P*, *hsr-omega*$^{1/5}$ and *DMU25686*, markers previously associated with interesting trait variation, showed strong linkage disequilibrium with each other.

Using this same central population, *hsr-omega* repeat-length variation was examined for possible contribution to variation in three traits among family lines (using trait data and lines described by Weeks *et al* 2002). Repeat-length did not associate with heat resistance or body size variation. However, the data suggest a mild association with a trait that varies latitudinally, cold tolerance variation, that should be investigated further.

Altitudinal variation was investigated in *D. melanogaster* for a number of phenotypic traits and genetic markers, including *hsr-omega* repeat-length, all of which are known to cline over latitude. For this analysis, traits and markers were scored from pairs of populations, one collected at high and one at low altitude, from five different latitudes along the eastern coast of Australia. Altitudinal differentiation was observed for cold tolerance, heat resistance, ovariole number, development time and a microsatellite marker, but not for *hsr-omega* repeat-length. Altitudinal differentiation observed here is likely to be the result of natural selection by temperature related factors.

The *hsr-omega* gene was partially sequenced in *D. simulans* and *D. serrata*, two close relatives of *D. melanogaster*. This sequence provides a strong foundation for future work on the role of *hsr-omega* in stress tolerance differences between species. *Hsr-omega* repeat-length variation was less polymorphic in *D. simulans* than in *D. melanogaster* and did not exhibit latitudinal variation.

A recently identified polymorphic marker of the *Tpi* gene in *D. melanogaster*, a TTA indel polymorphism that maps close to the end of the third chromosome, was examined for geographical variation. This exploratory study revealed both a latitudinal cline and altitudinal association in allele frequency, the directions of which were consistent if temperature is an important selective factor. The *Tpi* variation may prove useful in future studies to map and isolate genes that contribute to adaptive variation in thermal tolerance, body size and metabolic traits.

This thesis has revealed patterns of geographical variation and linkage associations that will contribute to a better understanding of climatic adaptation in nature. Most importantly, this study suggests new avenues of research for understanding the role of *hsr-omega* repeat-length variation and the traits it effects in natural populations of *Drosophila*.

Declaration

This thesis contains no material which has been accepted for the award of any degree or diploma in any university or other institution and to the best of my knowledge, contains no material previously published or written by another person, except where due reference is made in the text of this thesis.

Janelle Elyse Collinge, August 2004

Material not presented in this thesis has been published in (my contribution ~15%):

Anderson, A. R., Collinge, J. E., Hoffmann, A. A., Kellet, M. and McKechnie, S.W. (2003). Thermal tolerance trade-offs associated with the right arm of chromosome 3 and marked by the *hsr-omega* gene in *Drosophila melanogaster*. *Heredity* **90**, 195-202

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CHAPTER 1

General introduction

1.1 Geographical variation

Populations in nature are exposed to a wide range of environmental stresses. In order to survive these stresses organisms have responded by altering their phenotypic characteristics (morphology, life history, physiology or behaviour) to better suit the local environment (Hoffmann *et al* 2003). These phenotypic responses can be plastic (acclimation) or can result in changes in the genetic constitution of the population (adaptation). Plastic and genetic variation needs to be distinguished to be able to interpret the significance of the trait variation at the evolutionary level (Stearns 1980). Where genetic trait variation has been identified, rarely has this variation been directly linked to the underlying change in DNA sequence.

Variation along natural gradients provides some insight into how different traits and genes respond to stress. Gradual changes in measurable characteristics, often referred to as clines (Huxley 1938), occur over natural geographic gradients including gradients of latitude and altitude. Latitudinal clines have been observed in a wide variety of organisms from bryozoan to birds, for many different traits (Rhymer 1992; Kettle and Arthur 2000; Barnes and Arnold 2001; Storz *et al* 2001; Chirikova *et al* 2002; Smith *et al* 2002; Stenoien *et al* 2002). However, latitudinal clines have been most extensively studied in *Drosophila melanogaster*. Within this species, clinal variation in traits and genetic markers has been well described along the eastern coast of Australia (Knibb *et al* 1981;

James *et al* 1995a; Azevedo *et al* 1996; Gockel *et al* 2001; Hoffmann *et al* 2002a) and some of these clines are paralleled on other continents (Knibb 1982; Watada *et al* 1986; Capy *et al* 1993; Imasheva *et al* 1994; Van't land *et al* 1999). In general, the occurrence of latitudinal clines are evidence for natural selection and climatic adaptation, as they associate with a number of environmental parameters that also cline along latitudinal gradients such as regular increments in temperature, rainfall and daylength.

Altitudinal variation has been investigated less frequently, however, clines have been shown to exist in traits and genes in many organisms including snails, frogs and birds (Rand 1936; Berven 1982b; Tanaka and Brookes 1983; Baur and Raboud 1988; Bitner-Mathe *et al* 1995; Blanckenhorn 1997; Fleishman *et al* 1998; Gaston and Chown 1999). As with latitudinal clines, the occurrence of altitudinal clines are evidence of climatic adaptation as here too environmental parameters, such as temperature, relative humidity and rainfall change in a sequential way (Heath and Williams 1979; Beniston 1994). However, altitudinal gradients are particularly interesting as they usually occur over shorter geographical distances and continuous gene flow make local genetic adaptation probable only under strong selection pressures. Therefore, altitudinal differences in traits and genetic markers are less likely to be an affect of population history such as genetic drift and isolation, effects which confound a latitudinal study.

Latitude and altitude clines in traits and markers often provide evidence for natural selection (Levitan 1978) and can offer the first insight into which environmental factors are important. However, variation in traits and markers between populations can result from phenotypic plasticity, population history, or random genetic processes (Baur and Raboud 1988; Hoffmann and Parsons 1991b; Kirby and Stephan 1996). Parallel clines that show the same directional associations across different continents and different species are not easily explained by chance historic processes. However, they provide

strong evidence of natural selection and a genetic basis for clinal variation (Endler 1977; Hallas *et al* 2002).

Many climatic factors cline along latitude and altitude gradients, however, temperature is one of the shared common factors. Temperature has been implicated as the selective agent for many latitudinal clines in *D. melanogaster*, especially clines in heat resistance and cold tolerance (Hoffmann *et al* 2002). We anticipate altitude to reflect similar thermal adaptations in *D. melanogaster* and this is supported by initial investigations on altitudinal differentiation for body size and development time (Louis *et al* 1982).

Adaptation to stress, as discussed above, generally requires many generations of natural selection at the 'population' level. However, at the 'individual' level, the heat shock response provides short-term protection during exposure to thermal stress.

1.2 Heat shock response

The universal heat shock response is the activation or enhanced activity of heat shock genes in nearly all cell and tissue types exposed to elevated temperature (Feder and Hoftman 1999). This stress response was first discovered when *Drosophila* larvae placed at 37°C produced puffs along the polytene chromosomes (Ritossa 1962). Other stresses are known to induce heat shock genes such as cold stress, chemical stress and parasites (Goto and Kimura 1998; Feder and Hoftman 1999). Many genes in this family are also active during normal development (Feder and Hoftman 1999).

Temperature shock induces nine chromosome puffs in *D. melanogaster*, at cytological locations: 33B, 63BC, 64F, 67B, 70A, 87A, 87C, 93D, and 95D (Ashburner 1970). These puff sites represent regions of increased gene activity (transcription) and the

products of this activity are essential in protecting the 'cellular machinery' from damage during short periods of heat shock and other environmental stress (Lakhotia 2001b). Genes at these puff sites are therefore deemed to be good candidates for resistance to environmental stress.

Most heat-induced puffs in *D. melanogaster* can be attributed to the active transcription of genes that belong to the Heat shock protein (Hsp) family (Craig 1985; Lindquist 1986). However, one of the largest and most transcriptionally active heat shock gene puffs is not part of the Hsp family. The active gene at this puff site is the heat-shock RNA omega (*hsr-omega*) gene, located on chromosome 3R at locus 93D6-7 and genetic location 3-71 (Mohler and Pardue 1982; Mohler and Pardue 1984).

1.3 Hsr-omega and its RNA transcripts

The *hsr-omega* gene is expressed in almost all cell types during stress and at reduced level under normal developmental conditions (Bendena *et al* 1991; Mutsuddi and Lakhotia 1995; Lakhotia *et al* 2001a). *Hsr-omega* contains a translatable ORF but does not produce a detectable protein product (Lakhotia and Mukherjee 1982a; Garbe and Pardue 1986b; Fini *et al* 1989). Like many heat shock genes its activity is enhanced by heat and chemical stresses, such as carbon dioxide, 2-4 dinitrophenol, arsenic compounds and a variety of other chemicals (Ritossa 1963; Ashburner and Bonner 1979; Lakhotia 1987; Bendena *et al* 1989b; Bendena *et al* 1991; Lakhotia *et al* 2001a). However, *hsr-omega* appears to be more sensitive to environmental conditions than the other heat shock genes, as it is uniquely induced by benzamide, colchicine, colcemide, nicotinamide, formamide, acetamide and other amides (Lakhotia and Mukherjee 1980; Lakhotia and Sharma 1996; Tapadia and Lakhotia 1997). This unique puffing activity to amides has enabled the

identification of *hsr-omega* puff homologues in all *Drosophila* species examined (Peters *et al* 1980; Lakhotia and Singh 1982b; Burma and Lakhotia 1984; Chowdhuri and Lakhotia 1986; Scouras *et al* 1986; Garbe *et al* 1989; Drosopoulou *et al* 1996; Lakhotia 2003).

A series of publications over 25 years describe the unique feature, structure and more recently a model function of the *hsr-omega* gene in *Drosophila* species (see references in Pardue *et al* 1990; Lakhotia and Sharma 1996; Lakhotia 2001b). In *D. melanogaster hsr-omega* produces two primary RNA transcripts (Figure 1.1) by alternate termination of the gene (Lengyel *et al* 1980; Hovemann *et al* 1986). Generally, the RNA of heat shock genes are predominantly cytoplasmic, however, much of the *hsr-omega* RNA remains in the nucleus (Lengyel *et al* 1980). The larger *hsr-omega* RNA transcript is the nucleus limited, *omega-n*, which is greater that 10 kb in length (Lengyel *et al* 1980). More than 50% of this transcript consists of a large fragment of tandem repeats at the 3` end of the gene, for which laboratory stock of *D. melanogaster* are highly polymorphic in length (Waildorf *et al* 1984; Hogan *et al* 1995). The second RNA transcript is the 1.2 kb cytoplasmic limited, *omega-c*, which is spliced out from the nuclear 2 kb *omega-pre-c* transcript. These transcripts may play an important role in coordinating activity between and within the nucleus and cytoplasm, especially under stressful conditions (Bendena *et al* 1991). Similar nuclear and cytoplasmic limited RNA transcripts are found in *hsr-omega* homologues in other *Drosophila* species investigated (Lubsen *et al* 1978; Lengyel *et al* 1980; Lakhotia and Singh 1982b).

A unique and major feature of the *omega-n* RNA transcript is the tandem repeat fragment, which consists of more than 5 kb of 280 bp repeating monomers (Hogan *et al* 1995). These repeats show concerted evolution, a known feature of satellite DNAs (repeated DNA), generally considered to be non-coding junk (Doolittle and Sapienza 1980; Orgel and Crick 1980; Pavelitz *et al* 1999). In contrast to the *omega-n* specific

repeats, satellite DNAs generally occur across multiple chromosomes adjacent to other satellites and are not transcribed (Hogan *et al* 1995). The most intriguing feature of the *hsr-omega* tandem repeat fragment is that it is transcribed and appears to have a function in cellular stress response (Zu *et al* 1998).

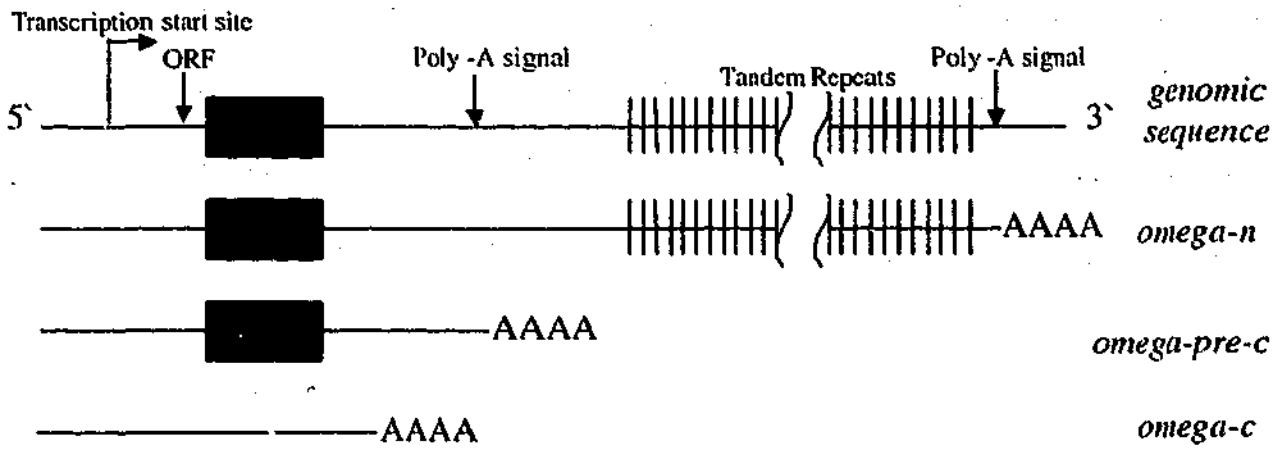


Figure 1.1 Schematic diagram of the *hsr-omega* gene and its RNA transcripts in *D. melanogaster*. The first and second poly-a signals are required for alternate termination of the RNA transcripts. The repeats at the 3` end are greatly truncated in this figure. The black box indicates the intron, which is sliced out in *omega-c*.

Previous studies provide strong evidence that *hsr-omega* is important in the thermotolerance phenotype in *D. melanogaster*. Both *hsr-omega*$^{a/b}$ and *hsr-omega*$^{L/S}$ polymorphisms cline along a latitudinal gradient on the eastern coast of Australia (McColl and McKechnie 1999; Anderson *et al* 2003). *Hsr-omega*$^{L/S}$ alleles associate with both heat and cold tolerance variation (Anderson *et al* 2003). The *hsr-omega*$^{a/b}$ polymorphism and *hsr-omega* RNA transcript levels have been strongly associated with heat stress through allele frequency changes in selection experiments for resistance to heat knockdown (McColl *et al* 1996) and changes to *hsr-omega* expression patterns in the selected lines (McKechnie *et al* 1998). The importance of this gene in heat tolerance was further highlighted in *hsr-omega* nullosomic *D. melanogaster*. These stocks, with greatly reduced

egg to adult viability, were unable to produce a 93D heat shock puff and those that did survive through to adults were less heat tolerant than wild type flies (Mohler and Pardue 1984; Pardue *et al* 1990; Lakhotia *et al* 1999). These studies provide evidence that this gene has vital functions in the cell especially for heat tolerance. However, is repeat-length variation also involved with the thermotolerance phenotype?

Although there is good evidence that the *hsr-omega* gene is important in thermotolerance variation in *D. melanogaster*, it is not known if variation in this gene is important in other species. *Hsr-omega* puffs have been identified in many *Drosophila* species, however, the structure of the gene and not the sequence is conserved across those species examined (Garbe *et al* 1986a; Garbe *et al* 1989). This creates a challenge to sequencing efforts and may be the reason why sequence information is not available in close relatives of *D. melanogaster*, ideal species for investigating thermotolerance association with the *hsr-omega* gene.

1.4 Linkage and population level associations of polymorphic markers

Repeated and parallel clinal variation is usually attributed to natural selection, however linkage can have deep effects on adaptation and the course of natural selection (Eanes 1999). Linkage disequilibrium (LD) is distinguished by the tendency of certain allele combinations at linked loci to be more or less frequent than expected in a population (Charlesworth *et al* 1973). Generally LD occurs between closely positioned genes along a chromosome, however, the level of LD is not necessarily proportional to distance occurring between loci separated by hundreds of bases to tens of kilobases (Eanes 1999; Ayala *et al* 2002). Furthermore, gene polymorphisms that lie within or near chromosome

inversion breakpoints are more likely to be in linkage association with the inversion (Hasson and Eanes 1996; Andolfatto *et al* 1999; Rodriguez-Trelles 2003).

The *hsr-omega* gene is found within an inversion, *In(3R)Payne* (*In(3R)P*), which may be effecting our ability to investigate *hsr-omega* gene effects. Linkage associations with the *hsr-omega* repeat-length polymorphism need to be assessed, as non-random or hitchhiking associations with selected sites chromosomally close to *hsr-omega* including the inversion, may be influencing this polymorphism. Previous studies have observed significant linkage associations among 5` *hsr-omega* polymorphic markers (McKechnie *et al* 1998), however, it is unclear if these non-random associations extend to the 3` repeat-length polymorphism.

Gockel *et al* (2002) found that body size variation maps predominantly to the right arm of chromosome three. Additionally, *hsr-omega*^{US} and other nearby markers inside *In(3R)P*, were found to associate with body size variation (Weeks *et al* 2002). Therefore, repeat-length variation may also associate with this trait. However, these body size associations may be confounded by *In(3R)P*, in which the markers are situated. Further molecular markers that may be useful for finer scale mapping of body size variation need to be identified. The ubiquitously conserved Triosephosphate isomerase (*Tpi*) gene, which is important in glycolysis, is located 3` of the proximal breakpoint of *In(3R)P* within the region that has been shown to contribute to body size variation (Shaw-Lee *et al* 1991; Gockel *et al* 2002). Recently, molecular sequence variation was described in this gene, most involved singie nucleotides, however, a TTA indel polymorphism was observed (Hasson *et al* 1998). Characterisation of this indel molecular marker may prove useful as a mapping tool for body size variation as well as helping understand metabolic adaptation in future studies.

1.5 This Study

D. melanogaster is an attractive organism for this study. This species has been well characterised for clinal variation in traits and markers associated with climatic stress, especially latitudinal variation. Furthermore, it is easy to culture under laboratory conditions with a fast generation time and the entire genome has been sequenced (Adams *et al* 2000). Of all *Drosophila* species, the *hsr-omega* gene has been best characterised in *D. melanogaster*. Thermal tolerance variation is a major component of climatic adaptation and there is good reason to believe that natural heat tolerance variation in this species is strongly influenced by the *hsr-omega* gene.

This work will focus on the *hsr-omega* gene and its repeat-length polymorphism. The repeats are an intriguing component of a nucleus restricted RNA transcript for which the function is not well understood. While repeat-length is highly polymorphic, conspicuous natural repeat-length variation has not yet been examined in natural populations and such a study may help elucidate the role of repeat-length variation in climatic adaptation. In this report characterisation of the repeat-length polymorphism along gradients that occur in nature, both latitudinal and altitudinal gradients, and investigation of repeat-length linkage associations will be carried out. The altitudinal study will also include characterisation of phenotypic traits and other genetic markers. The *hsr-omega* gene and repeat-length polymorphism will be investigated in other *Drosophila* species closely related to *D. melanogaster*. Geographical variation and linkage associations will also be explored in the Triosephosphate isomerase (*Tpi*) indel polymorphism.

In brief ;

Chapter 2 examines natural populations of *D. melanogaster* for geographical *hsr-omega* repeat-length variation along a latitudinal gradient on the eastern coast of Australia.

In **Chapter 3**, repeat-length linkage associations with other candidate markers on chromosome three will be assessed. The *hsr-omega* repeat-length polymorphism will also be investigated for possible contribution to variation in quantitative traits.

In **Chapter 4** previously reported latitudinal variation along the eastern coast of Australia in traits and genetic markers in *D. melanogaster* will be investigated for altitudinal variation.

Chapter 5 explores geographical variation in an uncharacterised TTA indel polymorphism in the *Tpi* gene of *D. melanogaster*.

In **Chapter 6**, the *hsr-omega* gene will be sequenced in *D. serrata* and *D. simulans* and geographical repeat-length variation in *D. simulans* will also be investigated.

CHAPTER 2

Latitudinal variation in the *hsr-omega* repeat-length polymorphism of *D. melanogaster*

2.1 Introduction

Clines

The presence of genetic variation allows a population to adapt to new environments. In *Drosophila*, candidate genes for climatic stress resistance have been identified by correlating allele frequency with environmental gradients, especially temperature. Extensive research has found many traits and genetic markers show geographical variation, which may be important for adaptation to climatic extremes (see Chapter 1 for references). For example, in *D. melanogaster* several heat shock genes, including *hsr-omega*, have shown latitudinal variation in polymorphic alleles (McColl and McKechnie 1999; Bettencourt *et al* 2002; Frydenberg *et al* 2003). Genetic clines could be a consequence of random processors such as gene flow, genetic drift, founder effects or other historical events, however, repeated and parallel clinal variation is more likely to be due to natural selection by environmental stress (Levitan 1978).

Omega-n transcript and its unique tandem repeats

There is strong evidence that the *hsr-omega* gene is important in the adaptation to temperature stresses in *D. melanogaster*. Within the *hsr-omega* gene of *D. melanogaster* there are two good examples of genetic markers that cline over a latitudinal gradient and these markers strongly associate with temperature stress (McColl and McKechnie 1999; Anderson *et al* 2003). However, it is not known if the *omega-n* repeat polymorphism exhibits similar clinal variation and temperature associations.

The role of *omega-n* in the nucleus has been an ongoing interest for several years and significant progress has been made into understanding how this transcript functions. Both major *hsr-omega* RNA transcripts show increase in abundance after heat shock, however, chemicals that uniquely induce *hsr-omega* increase the abundance of *omega-n* only (Bendena *et al* 1989b). *Omega-n* RNA transcripts are found close to the site of transcription and are actively distributed within the nucleoplasm (Hogan *et al* 1994). The *omega-n* nucleoplasm speckles are generally found in close contact with heterogeneous nuclear RNA-binding proteins (hnRNPs), which are important for RNA processing and together form the omega speckles. Recent evidence suggests that *omega-n* transcripts have a role in adjusting the availability of hnRNPs for RNA processing activities in stressed and non-stressed cells (Prasanth *et al* 2000). During heat stress nuclear RNA processing is reduced, however, the level of *omega-n* increases to sequester away hnRNPs that have been concentrate at the 93D puff site and omega speckles form large and fewer cluster (Dangli *et al* 1983; Zu *et al* 1998; Prasanth *et al* 2000). Interestingly, in *hsr-omega* nullosomics, omega speckling distribution is completely disrupted (Prasanth *et al* 2000). However, mutants that over express *omega-n* transcripts form larger omega speckle clusters without heat shock and RNA processing is disrupted within the cell (Rajendra *et*

al 2001). These studies have highlighted the importance of *omega-n* in the cell, especially during stress.

The *hsr-omega* tandem repeat fragment at the 3` end of the gene is unique to the *omega-n* transcript and is highly polymorphic in repeat-length. The repeats constitute well over half of the *omega-* transcript length in *Drosophila* species (Garbe *et al* 1986a). This repeat fragment is known to vary in size from between 5 and 16 kb in laboratory populations of *D. melanogaster* and size has been demonstrated to be stable over many generations (Hogan *et al* 1995).

There is increasing evidence that it is the repeats that are associating with the hnRNPs in the nucleus. In *D. melanogaster* and *D. hydei* the repeats at the 3` end of the gene differ in repeat monomer size, at ~280bp and ~115bp in *D. melanogaster* and *D. hydei*, respectively (Peters *et al* 1984; Ryseck *et al* 1987), and alignments between the two species reveal that the sequence is highly diverged. However, there is a 9 nucleotide sequence motif (ATAGGTAGG) that is conserved in *D. melanogaster*, *D. hydei* and recent evidence suggests also in *D. pseudoobscura* (Garbe *et al* 1986a; Pardue *et al* 1990). This nanomer is arranged and concentrated uniformly throughout the repeats, occurring once in the short *D. hydei* repeat unit and twice in the larger *D. melanogaster* repeat unit (Garbe *et al* 1986a). This nanomer is within the size range to function as a binding site for proteins or RNA and the leftover sequence may act as spacers for this binding site (Garbe *et al* 1986a; Bendena *et al* 1989a; Garbe *et al* 1989; Pardue *et al* 1990). Indirect evidence suggests that the nanomer sequence motifs associate/bind with hnRNPs and coordinate their availability for RNA processing (Zu *et al* 1998). It is highly likely that the repeats have a major role to play in the function of the *omega-n* transcript.

This Project

While accumulated studies indicate that this gene has an important role in heat tolerance and viability in *Drosophila*, the exact cellular, molecular and physiological function is yet to be fully understood. Tandem repeat-length is highly polymorphic among laboratory stocks of *D. melanogaster*, however, repeat-length variation in natural populations has not been investigated. This study reports on latitudinal *hsr-omega* repeat-length variation along a climatic gradient, the eastern coast of Australia.

2.2 Materials and Methods

Collection sites

Adult *D. melanogaster* were collected by banana bait trapping at regular intervals down the eastern coast of Australia in February-March 2000 (Figure 2.1 and Table 2.1). This collection transect minimises other sources of variation such at longitude and altitude and spans more than 26° latitude (~3000 km). Isofemale lines were set up from field caught females and reared on potato medium (Appendix A) at 25°C. The progeny from these lines were used to genotype tandem repeat-length variation, which began six months after collection. This latitude collection and genotyping was repeated in 2001 and 2002 to observe any temporal variation in repeat-length.

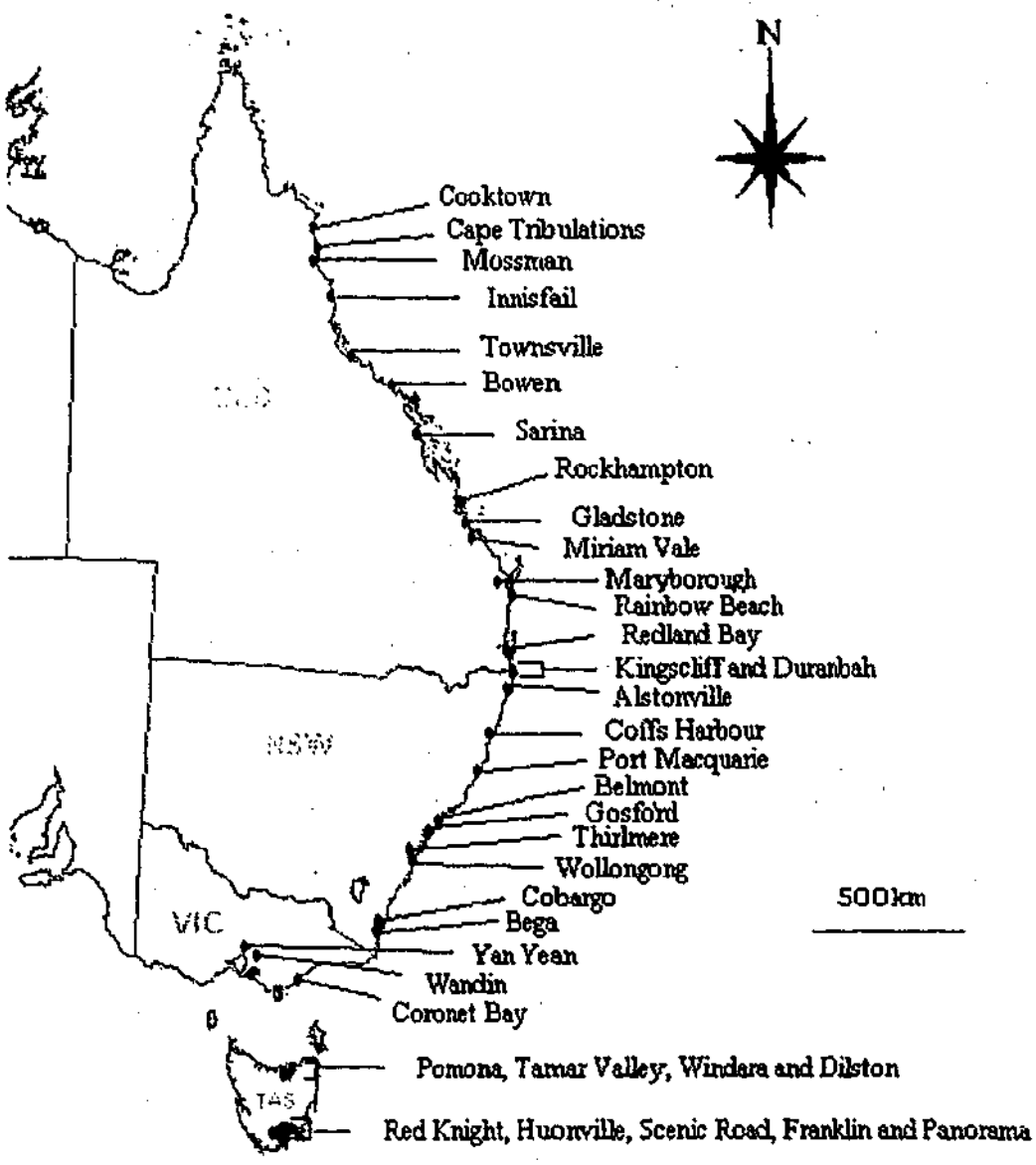


Figure 2.1 Map of locations for 2000-2002 collection sites

Table 2.1 The collections sites for 2000-2002, showing the number of isofemales lines scored for repeat-length variation.

		Number isofemale lines scored		
Location	**Latitude**	**2000**	**2001**	**2002**
Cooktown	15°22'		1	
Cape Tribulations	16°01'		5	5
Mossman	16°27'	8	2	
Innisfail	17°30'	11	3	5
Townsville	19°15'		14	5
Bowen	20°01'	7		
Sarina	21°25'	8	5	5
Rockhampton	23°22'	9	3	5
Gladstone	23°50'			5
Miriam Vale	24°19'	6		5
Maryborough	25°31'	11		5
Rainbow Beach	25°54'	2		5
Redland Bay	27°39'	7		5
Kingscliff	28°15'		9	5
Duranbah	28°19'	7		
Alstonville	28°50'	3		5
Coffs Harbour	30°19'	11	11	5
Port Macquarie	31°25'			5
Belmont	33°02'			5
Gosford	33°26'	7		
Thirlmere	34°11'		2	
Wollongong	34°25'			5
Cobargo	36°22'		14	
Bega	36°39'	13		5
Yan Yean	37°34'	6		
Wandin	37°47'	4	5	
Coronet Bay	38°26'			5
Pomona	41°06'		4	
Tamar Valley	41°11'	9	3	5
Windara	41°14'		6	
Dilston	41°19'			5
Red Knight	42°45'			5
Huonville	43°02'	8		
Scenic Road	43°02'	5		
Franklin	43°05'		9	
Panorama	43°07'		10	

Hsr-omega repeat-length genotyping

Tandem repeats were genotyped using the method described in Anderson *et al* (2003), which was based on a method referred to in Hogan *et al* (1995). DNA extractions were carried out on groups of twenty-five flies. In the 2000 and 2001 collection, extractions were performed on each isofemale line. In the 2002 collection, one extraction was performed per latitudinal collection site, where each extraction consisted of 5 isofemale lines pooled in equal proportions (5 flies per isofemale line).

High quality DNA was needed to genotype tandem repeat-length by Southern blots and so the following phenol/chloroform method was used. *D. melanogaster* were homogenised in 500 µl grind buffer (0.2M sucrose, 0.1M Tris pH 9.2, 50mM EDTA and 0.5%SDS) after which 2 µl proteinase K (at 20mg/ml) was added. The homogenate was then heated at 65°C for 10 min, removed from heat, then 150 µl 8M potassium acetate was added. The homogenate was vortexed and placed on ice for 15 min. Next 0.5 ml phenol/chloroform/isoamyl alcohol (25:24:1) was added to the homogenate and vortexed hard for 30 sec. The homogenate was then centrifuged for 5 min at 12 000rpm. The supernatant was transferred to 1 ml 100% ethanol, vortexed and the DNA was precipitated at room temperature for 4 h. Following this the precipitation was centrifuged for 10 min at 12 000rpm. The supernatant was removed and the DNA pellet was washed with 0.5 ml 70% ethanol. The pellet was air dried then dissolved overnight in milliQ water at 4°C.

The DNA (~3.5µg/µl) was double digested with *Pst I* and *Hind III* in Buffer B according to manufactures protocols (Promega) overnight at 37°C which cleaves each side of the repeat-length fragment (Hogan *et al* 1995). Samples along with Expand™ DNA molecular weight marker XV (Roche Diagnostics) and DNA of known repeat size (Southern standard see below), were loaded onto a 0.5% agarose gel and run with 1X Tris-acetate buffer (where 50X is 242g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5M

EDTA (pH 8) in 1 L). Electrophoresis was carried out at 2V/cm until Expand™ DNA molecular weight marker XV had separated out 3cm between the first and last molecular weight bands (for single isofemale extractions) or 6cm (for pooled extractions, which required greater resolution of the fragments). A polaroid image was captured of the ethidium stained agarose gel next to a ruler.

A Southern blot was performed as described in Sambrook *et al* (1989). The gel was soaked in 0.25M HCl for 10 min and rinsed in distilled water. The gel was gently agitated in denaturing solution (1.5M NaCl and 0.5M NaOH) for 45 min (changing the solution once) then rinsed with water. Following this the gel was gently agitated in neutralising solution (1M Tris (pH 7.4) and 1.5M NaCl) for 30 min, the solution was changed and the gel was soaked for a further 15 min. The DNA was transferred to a Zeta-probe membrane (Bio-rad) over 21 h using 10XSSC (1.5M sodium chloride and 0.15M sodium citrate) as the transfer buffer. The DNA was fixed to the membrane by exposure to UV for 3 min. The Zeta-probe membrane was then hybridised with a repeat monomer probe.

Probes were created by labelling purified *hsr-omega* repeat monomer DNA with radioactive ^{32}P. To amplify a repeat unit, primers were designed from the 280 bp tandem repeat monomer sequence published by Hogan *et al* (1995). The forward primer 5`- CGA AAA GGC TTA TCC TCT TGG - 3` and reverse primer 5`- ATA GTG ATT GGG GTA ATC GGG - 3` bind to each end of a 280 bp tandem repeat monomer. DNA for PCR was extracted from 25 flies as outlined in the phenol/chloroform method above, however, the homogenate was treated with 1 µl RNAase (at 4mg/ml from Roche Diagnostics) at 37°C for 30 min before the addition of 150 µl 8M potassium acetate. The reaction conditions were: 1.5mM $MgCl_2$, 0.2mM dNTPS, ~30ng of each primer, 1 units *Taq* DNA polymerase and ~10 µg DNA in a 50 µl reaction. To amplify this repeat unit, PCR was performed

under the following cycling conditions: denaturing at 94°C (5 min) and 36 cycles of 94°C (30 sec), 47°C (30 sec) and 72°C (10 sec). The PCR product was purified using the Wizard PCR Preps DNA Purification System (Promega). The purified PCR product was used in conjunction with a GIGAprime DNA Labelling Kit (Geneworks) to develop the ^{32}P labelled probe for hybridisation. To remove unincorporated radioactive nucleotides, orange G and dextran blue were added to the ^{32}P labelled probe and run through a sephadex G50 column. The blue fraction was collected and used for hybridisation.

The Zeta-probe membrane was placed DNA side inwards into a glass roller hybridisation tube. Prehybridisation solution (6XSSC, 5X Denhardts Reagent, 0.5% SDS, 1mg/ml Herring sperm) was added to the tube and incubated for 4 h at 68°C in a hybridisation oven. The prehybridisation solution was discarded and the hybridisation solution (6XSSC, 5X Denhardts Reagent, 0.5% SDS, 1mg/ml Herring sperm and radioactive probe ~20 counts/second) was added and incubated at 68°C overnight. The hybridisation solution was poured out and the membrane was briefly rinsed with wash solution 1 (2XSSC and 0.5% SDS). The membrane was then incubated with wash solution 1 at 37°C for 20 min and at 65°C for 30 min using fresh solution. A final wash was carried out using wash solution 2 (0.1XSSC and 0.5%SDS) at 70°C for up to 1 h or until the radioactivity on the membrane had dropped to approximately 5 counts/second. The membrane was exposed to x-ray film at –70°C, inside a x-ray cassette, containing an intensifying screen. The film was developed in an automated x-ray machine.

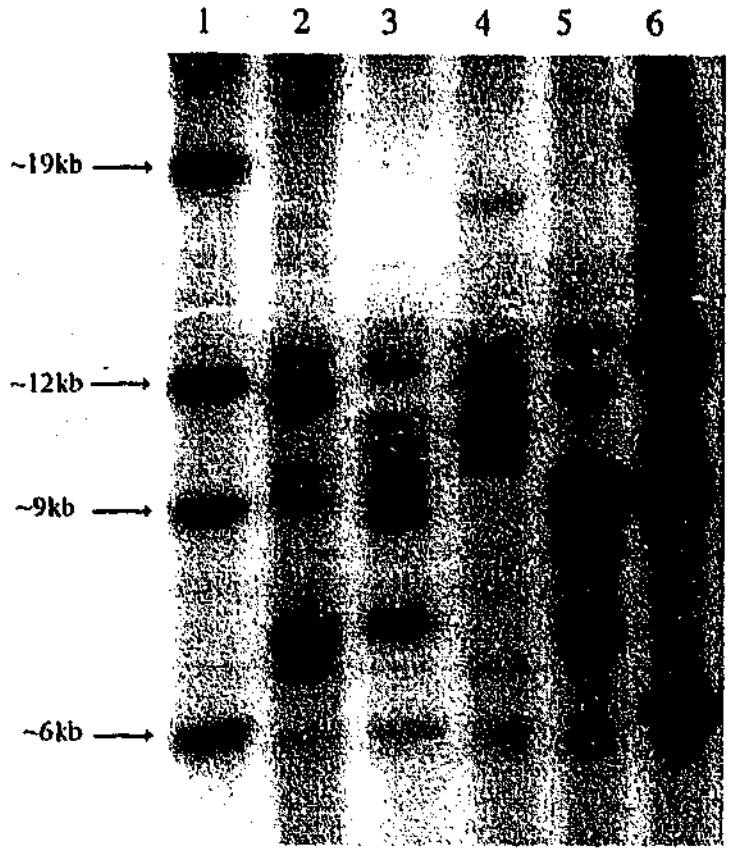
Repeat-length size on the x-ray film was determined by referring back to the original agarose gel. Size was estimated by measuring the distance travelled from the origin as indicated on the x-ray film, and this distance was used to determine how far this fragment had travelled on the agarose gel using the ruler on the polaroid image and hence the approximate size of repeat-length indicated by the Expand™ DNA molecular marker.

However, fragments had approximately 2.5 kb of sequence flanking the repeat region which was deducted from the size.

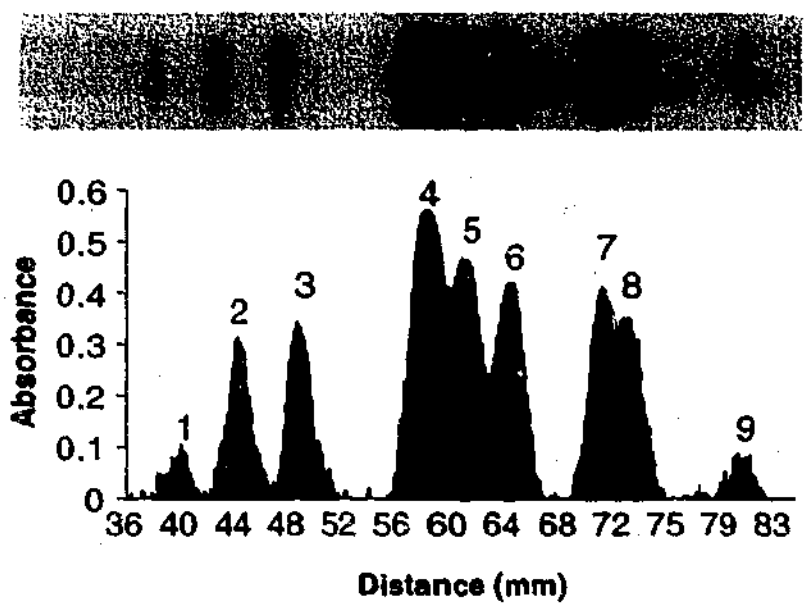
Analysis

Each year data were collected and analysed differently, as the technique became refined. In the 2000 collection, genotypes were analysed by binning alleles into five different categories based on size. Binning was aided by a Southern 'standard' which was run along side the samples on all gels at 3 well positions. The standard was created by pooling two isofemale lines, that between them, contained 4 different repeat-length alleles evenly spaced apart at 6, 9, 12 and 19 kb. The 'standard' enabled binning of alleles into five groups (Figure 2.2a): A (>19kb), B (12 to <19kb), C (9 to <12kb), D (6 to <9kb) and E (<6 kb). The frequency for each bin group was calculated for each latitudinal site and an average repeat number per group was determined (average repeat number of group A= 68, B= 43, C= 32, D= 22 and E= 18). In 2001, alleles were binned as before, however, allele groups were weighted based on the relative intensities of the bands within each lane. This was done by scanning the bands on the x-ray film using a Biorad imaging densitometer (Model GS-700) and weighted using Molecular Analyst Version 1.4 software (Figure 2.2b). The software produces a 2 dimensional profile to determine positions of bands in an image and the relative differences in intensities (area under peaks). In 2002, data was analysed as in 2001, however, each gel lane was equivalent to one latitudinal site and consisted of five equally pooled isofemale lines, rather than one isofemale line per lane. Therefore more allele bands were present and so gels were run a greater distance to separate and improve quantification of band intensities. This method provided a high throughput method of mass genotyping.

The average number of repeats for each latitudinal site was calculated using the average relative intensities of the bands in the allele bin groups. We estimated repeat unit



a)



b)

Figure 2.2 a) Southern blot image. Lanes 1 and 6 are standard size markers while lanes 2 to 5 each show typical repeat-length variation from five pooled isofemale lines. b) Absorbance graph of lane 5 from part a), used to weight allele groups.

contribution to intensity by dividing the intensity in a bin group by the average repeat number for that group. As allele bin groups were not weighted in 2000, repeat monomer intensity was determined in 2000 by the proportion of alleles in each allele group (as a percentage of total alleles) divided by the average repeat number for that group. The frequency of an allele group was calculated by dividing repeat monomer contribution for an allele group by the total repeat monomer contribution across all allele groups for a latitudinal site. A single value, average number of repeats, was then determined for each latitudinal site by multiplying the allele group frequency by average repeat number for that allele bin group summed across all allele bin groups. To estimate the proportion of variation in the average number of repeats explained by latitude, data were analysed by linear regression (using SPSS 11.5 for Windows).

Climatic data were obtained from the Australian Bureau of Meteorology website (www.bom.gov.au) to look for association between average number of repeats and climatic variables. Climatic data (based on averages typically over 100 years) were acquired for the nearest neighbouring weather station for each latitudinal collection site. Six climatic variables were chosen; average daily maximum temperature for the hottest month (Tmax), average daily maximum temperature for the coldest month (Tmin), average annual number of days above 30°C (T>30), average annual number of days below 2°C (T<2), mean monthly rainfall for the wettest month (Rmax), mean monthly rainfall for the driest month (Rmin). These climatic variables were initially tested for associations with latitude by Pearson correlation (r). Spearman's coefficients of rank correlation (r_s) was used to test for associations between the average number of repeats and climatic variables. Partial correlations were performed to look for associations between the average number of repeats and climatic variables while controlling for latitude. These correlations were carried out using SPSS 11.5 for Windows.

2.3 Results

Within Australian *D. melanogaster* a large number of allele sizes were detected for repeat-length in the *hsr-omega* gene. It was not unusual to observe more than 10 different band positions, 10 alleles, from a single population on high-resolution gels. Repeat-length in natural populations along the eastern coast of Australia, varied from ~5 kb to ~19 kb (corresponding to about 18 to 68 tandem repeat monomers) with an average of ~12 kb (corresponding to about 43 repeats).

Linear regression revealed that a significant proportion of variation in *hsr-omega* repeat-length was explained by latitude in 2000 (42%), 2001 (48%) and 2002 (32%). Data were consistent in direction as the average number of repeats showed a negative association with latitude for each year (Figure 2.3). The slope of the regression between the average number of repeats and latitude was analysed for homogeneity between years using a univariate general linear model (SPSS 11.5 for Windows). The linear association between the average number of repeats and latitude was homogeneous among years (MS = 267.62, df 1, $P<0.001$).

Hsr-omega repeat-length variation was investigated for associations with six climatic variables using Spearman's coefficients of rank correlation (Table 2.2). The three sets of clinal data for the average number of repeats were pooled and a weighted average taken for correlations with climatic variables. The frequency of the average number of repeats from the pooled three-year data set was negatively associated with latitude (r_s=-0.646, $P<0.001$). All clinal climatic variables showed significant associations with the average number of repeats, however, all climatic variables (except Rmin) were also associated with latitude (Table 2.2). As latitude and climatic variables were associated with average number of repeats, partial correlation controlling for latitude was performed. Partial correlations did not result in any significant associations (Table 2.2).

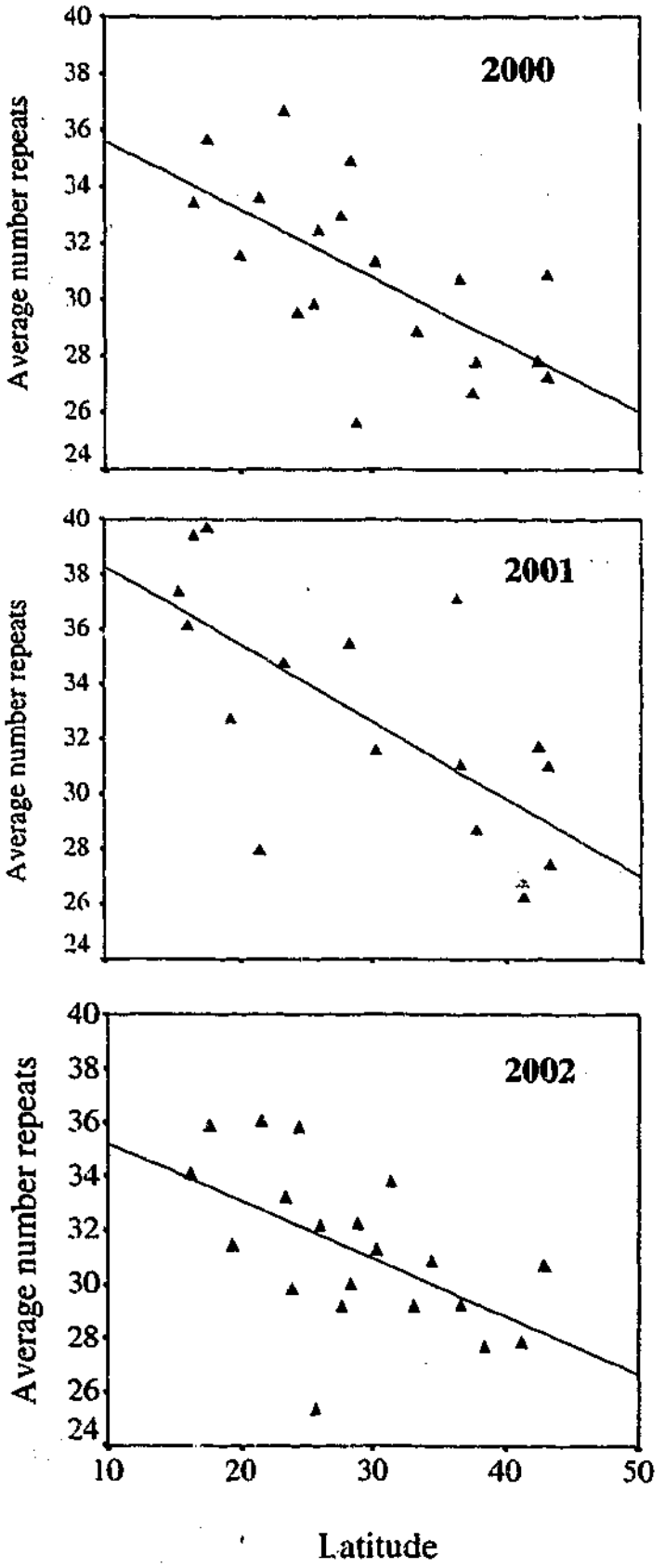


Figure 2.3 Association of average number of repeats with latitude in 2000 (R^2= 0.42, P<0.01), 2001 (R^2=0.48, P<0.01) and 2002 (R^2= 0.32, P<0.01). Raw allele group frequency data in Appendix B.

Table 2.2 Associations between climatic variables, latitude and average number of repeats, and partial correlations with latitude.

	Correlation		
Climatic Variable	**Latitude (r)**	**Average No. repeats (r_s)**	**Partial correlation~**
Tmax	-0.915***	0.568***	-0.064
Tmin	-0.877***	0.510***	-0.116
T>30	-0.808***	0.471***	-0.064
T<2	0.647***	-0.472***	0.152
Rmax	-0.551***	0.351**	-0.078
Rmin	0.182	-0.274*	-0.147
Average No. repeats	-0.624***	-	-

*** P<0.001, ** P<0.01, *P<0.05
~ between average number of repeats and climatic variables that controls for latitude
(Tmax) = average daily maximum temperature for the hottest month
(Tmin) = average daily maximum temperature for the coldest month
(T>30) = average annual number of days above 30°C
(T<2) = average annual number of days below 2°C
(Rmax) = mean monthly rainfall for the wettest month
(Rmin) = mean monthly rainfall for the driest month

2.4 Discussion

Repeat-length variation of the *Drosophila hsr-omega* gene, in natural populations has not previously been investigated. Hogan *et al* (1995) reported that repeat-lengths vary within laboratory stocks of *D. melanogaster* from between 5 and 16 kb with only two to three alleles occurring per stock. Similar to Hogan *et al* (1995) repeat-lengths in Australian samples showed a lower limit of ~5 kb, however, we observed an upper limit of at least 19 kb of repeats which is likely to be close to the upper limit since over 300 isofemale lines were scored. Hogan *et al* (1995) reported 7 alleles from 6 laboratory populations, however, in this study I have detected a larger number of alleles segregating in Australian natural populations. In this study, natural populations of *D. melanogaster* were highly polymorphic and frequently had 10 or more alleles per population when fragments were

run for longer periods of time on agarose gel ie. as in the 2002 collection. No doubt, this marker is highly polymorphic. If higher resolution techniques were developed it is conceivable that more repeat-length alleles could be resolved and internal sequence variation among different alleles of the same repeat number are likely.

This study found that repeat-length variation, measured as the average number of repeats, showed a robust latitudinal cline along the eastern coast of Australia, with the number of repeats being negatively associated with latitude ie. cool temperate populations had fewer repeats. Temporal analysis of this cline shows that this relationship was consistent over three years suggesting that repeat-length was relatively stable in Australian natural populations. The stable cline suggests that natural selection was acting on repeat-length variation although the selective agent remains elusive. The inability to detect an association with climatic factors does not exclude these or related physical variables as being directly or indirectly an underlying cause of the repeat-length cline. The role of natural selection on geographical variation in repeat-length needs to be further investigated.

The role of the repeats in the functioning of this gene is not clear. However, recent evidence suggests that the conserved nanomer motif (ATAGGTAGG) of the repeats associates with the hnRNPs in the nucleus. In relation to the latitudinal cline in repeat-length, populations in warmer tropical regions, at lower latitudes, on average have more repeating monomers and therefore have more nanomer motif as binding sites for hnRNP sequestering. Repeat-length variation may be an adaptation to an environmental condition that clines along a latitudinal gradient, which provide an adaptive advantage related to the *omega-n* speckles in the nucleus. However, further research is needed to investigate this motif as a binding site for hnRNPs, the cellular processes involved and the traits affected.

It is likely that this tandem repeat-length cline is not independent of other polymorphic variation under selection, which may be confounding the latitudinal association. The repeat-length polymorphism could be in linkage disequilibrium with the *hsr-omega*$^{I/S}$ polymorphism at the other end of the gene. This polymorphism, like the repeats, is also known to latitudinally cline along the eastern coast of Australia and is associated with environmental temperature. Furthermore, the association between repeat-length and latitude may be confounded by the inversion polymorphism, *In(3R)P*, in which the *hsr-omega* gene is cytologically located. A feature of inversions is suppression of recombination so that groups of genes inside the inversion can be locked up in blocks that persists in the population (Strobeck 1983). Previous studies have provided evidence that some inversion polymorphisms are subject to natural selection in *Drosophila* (Dobzhansky 1948; Stalker and Carson 1948; Knibb 1982). There is strong evidence to suggest that natural selection is acting on *In(3R)P*, as parallel latitudinal clines do exist on different continents (Knibb *et al* 1981). The *hsr-omega* repeat-length variation may be under epistatic selection or genetic hitchhiking with the inversion, and/or some other closely linked polymorphism, such as *hsr-omega*$^{I/S}$, under natural selection, rather than selection acting on repeats independently. Estimates of repeat-length linkage disequilibrium with other polymorphic markers of *hsr-omega* and the inversion, need to be determined before further progress can be made in understanding why the cline exists and whether or not repeat-length variation has any effect on fitness traits.

2.5 Summary

Natural populations were found to be highly polymorphic and contained many alleles with between 5 and 19 kb of tandem repeat sequence. The presence of a strong negative association with latitude in repeat-length was demonstrated, which was robust as the slope of the regression did not differ significantly between years. This cline in itself is not direct evidence of natural selection but rather a correlation that strongly indicates the action of selection. In the following chapters the repeat-length polymorphism will be further investigated to try to understand what selection pressures, if any, are involved with shaping this clinal pattern.

CHAPTER 3

Hsr-omega repeat-length variation: associations with chromosomal markers and phenotypes in *D. melanogaster*

3.1 Introduction

Many markers and traits in *D. melanogaster* have been associated with latitude along the eastern coast of Australia and a number of these clines are replicated on other continents and in other species (Chapter 1 references; Loeschcke *et al* 2000; Starmer and Wolf 1997; Hoffmann and Shirriffs 2002). Replicated clinal variation is usually attributed to natural selection, however, linkage associations can have major effects on clinal patterns (Eanes 1999). Linkage disequilibrium is the association of alleles across loci in the gamete phase at the population level (Charlesworth *et al* 1973). When attempting to understand the causes of association between specific allelic variants and environmental factors or phenotypic traits, linkage disequilibrium needs to be taken into account.

There are two primary causes for linkage disequilibrium between loci of a chromosome, firstly epistatic natural selection and secondly random historical factors such as genetic drift or founder effects. Linkage by epistatic natural selection favours fitter combinations of alleles in a population (supergenes), whereas historical factors generally results in random oscillations of gamete frequencies (Ohta 1982). It is often difficult to

determine the cause of linkage, however, when chromosomes consistently carry the same set of alleles in many populations, genetic drift can be eliminated as a causal factor, in favour of selection (Rodriguez-Trelles 2003).

Hsr-omega is linked in a block of chromosome three genes

Many genes located on chromosome three of *D. melanogaster* are thought to be important for thermotolerance and so this chromosome is particularly interesting when investigating genes involved with climatic adaptation (Lindquist 1986; Goto 2000; Goto 2001; Lakhotia 2001b; Anderson *et al* 2003). In a recent study from our laboratory, Weeks *et al* (2002) investigated a number of clinal chromosome three markers, in a large set of single pair mating lines, for associations with each other. Allelic variation of three chromosome 3R markers, *hsr-omega*^{LS}, *AC008193* and *DMU25686*, were strongly correlated at the population level. Since all three are found within the third chromosome right arm inversion, *In(3R)P* (not scored in Weeks *et al* 2002 study), which also shows a latitudinal cline (Knibb *et al* 1981), this inversion is a possible reason for the association of these markers. Inversions are known to lock up blocks of genes maintaining certain allele combinations by suppressed recombination (Langley 1974).

Clinal variation in the *hsr-omega* repeat-length polymorphism may be neutral and hitchhiking with *In(3R)P*, or other closely linked polymorphisms (such as *hsr-omega*^{LS}), that are under natural selection. In order to determine if *hsr-omega* repeat-length variation has any effects on fitness, it is important to first tease apart associations of *hsr-omega* repeat-length with other nearby clinal genetic markers.

Hsr-omega trait associations

To understand if *hsr-omega* repeat-length variation has any effects on fitness, it is important to investigate possible associations of *hsr-omega* repeat-with environmental factors and phenotypic traits. Trait association with *hsr-omega* repeat-length has not been investigated previously, however, clinal variation in heat and cold tolerance has been associated with polymorphic markers in the *hsr-omega* gene (McKechnie *et al* 1998; Anderson *et al* 2003). Body size is also a trait of interest as positive latitudinal clines in body size have been observed in *D. melanogaster* (James *et al* 1995) and quantitative trait locus mapping indicates that a large portion of variation in *D. melanogaster* body size has been attributed to the right arm of chromosome three (Gockel *et al* 2002). Recently, Week *et al* (2002) found direct associations between body size and markers, *hsr-omega*$^{L/S}$, *DMU25686* and *AC008193*, which are chromosomally near to the *hsr-omega* repeat length polymorphism. Therefore the *hsr-omega* repeat-length polymorphism needs to be assessed for possible contribution to variation in traits, particularly cold tolerance, heat resistance and body size.

This Project

The *hsr-omega* repeat-length polymorphism latitudinally clines along the eastern coast of Australia. Over this same geographical gradient, clinal variation on chromosome three has also been demonstrated in several gene polymorphism (including *hsr-omega*$^{L/S}$ (Anderson *et al* 2003), *TPI* (Chapter 5), *hsp 70* (Bettencourt *et al* 2002) and *Clk* (Saleem *et al* 2001; S. W. McKechnie Unpublished)), four microsatellites (*DMU25686*, *DMU14395*, *DMTRXIII*, *AC008193* (Gockel *et al* 2001)), and two inversions (*In(3R)Payne* and *In(3L)Payne* (Knibb *et al* 1981)). In order to obtain further clues to selective processes that might underlie the latitudinal association in *hsr-omega* repeat-

length, this polymorphism is investigated for independent of these above mentioned clinal markers, most importantly *In(3R)P*. *Hsr-omega* repeat-length variation is also explored for association with candidate quantitative clinal traits, cold tolerance, heat resistance and body size, that have been associated with this region of the chromosome.

3.2 Materials and Methods

Derivation of lines for the linkage disequilibrium study

Adult *D. melanogaster* were collected from Coffs Harbour, Australia (30°19'00 S 153°08'00 E) in March 2001 using banana bait traps (Tidon and Sene 1988). Coffs Harbour, centrally located along the eastern Australia coast cline, was chosen as the *D. melanogaster* population of origin for this study as it was expected to contain many of the segregating alleles for the genetic markers under investigation. Fifty isofemale lines were established from this population and reared at 18°C on *Drosophila* medium (Appendix A).

In order to score the haplotype of individual third chromosomes from this population, a crossing scheme was established that allowed independent chromosome lines to be derived (Figure 3.1). *Hsr-omega*$^{L/S}$ (8 bp indel polymorphism) scoring during the derivation of these lines allowed 2 independent chromosomes to be obtained from each isofemale line. Seventy-eight independent chromosomes were obtained, as for some lines only one chromosome was isolated ie. lines homozygous for the *hsr-omega*$^{L/S}$ polymorphism. These lines were scored for the haplotype of eleven genetic markers along different third chromosomes from the Coffs Harbour population.

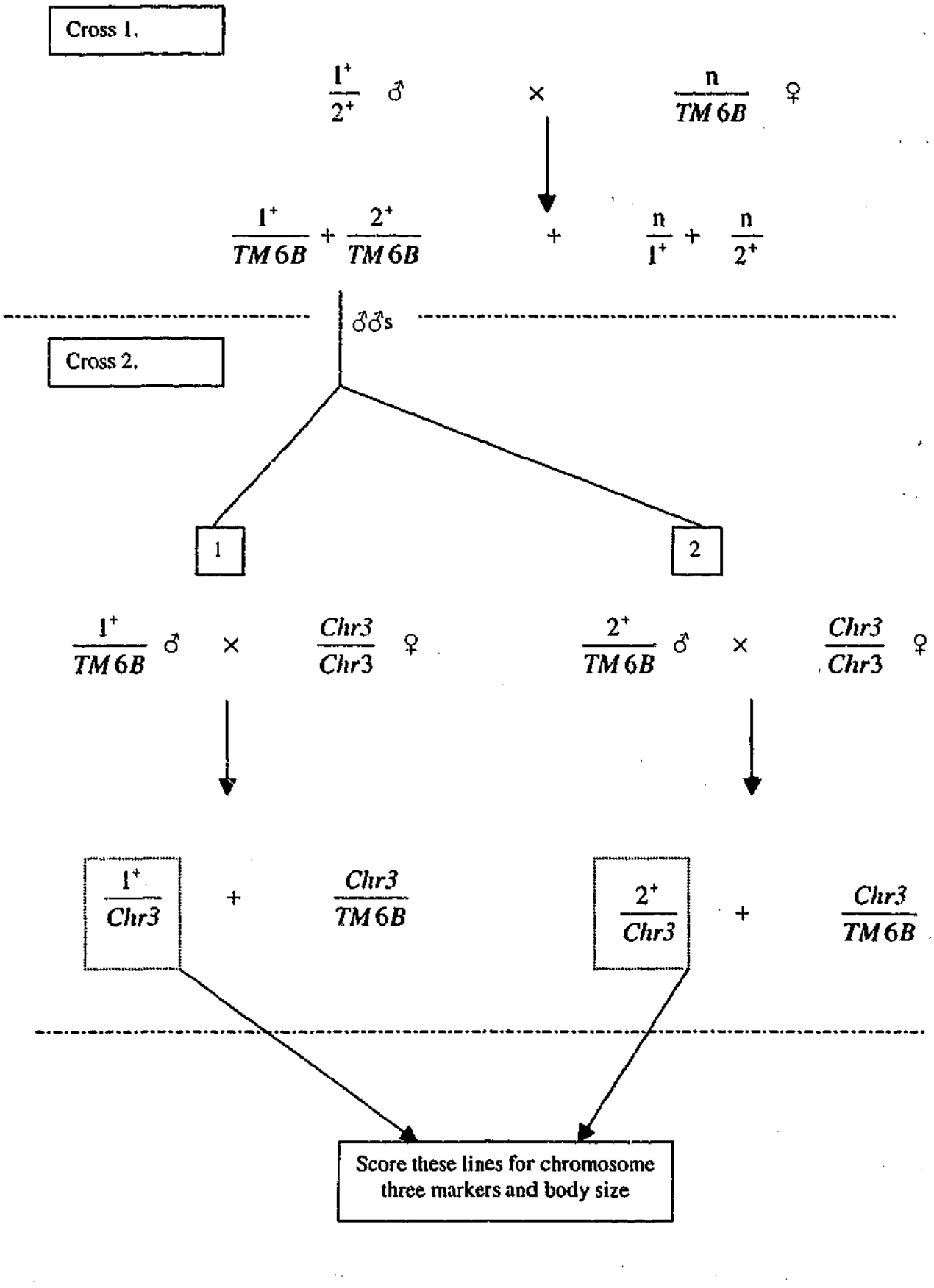


Figure 3.1 Crossing scheme used to isolate two independent chromosomes from each single Coffs Harbour isofemale line for scoring chromosome three haplotypes. An isofemale line ($1^+/2^+$) was crossed to a chromosome three balancer (*TM6B*) to separate independent chromosomes and then to a line homozygous for all chromosome three markers of interest (*Chr3*) to isolate independent chromosomes in separate lines.

To obtain these lines, for each of the fifty Coffs Harbour isofemale lines, several wild males were mated to several virgin chromosome three balancer (TM6B) females in single pair matings. These parental males were genotyped for *hsr-omega*$^{L/S}$ to determine which lines to use for the second cross that would maximise the number of independent chromosomes obtained. One line was derived from each isofemale line were the parental male used was heterozygous for *hsr-omega*$^{L/S}$ (or two lines retained where two parental males were each homozygous for the alternate allele). These lines were used in the second cross.

For the second cross, several single pair matings were set up between the male progeny from the first cross containing the balancer phenotype (tubby and humeral) and virgin females from a laboratory stock previously found to be homozygous for all genetic markers of interest. After mating, parental males were genotyped for *hsr-omega*$^{L/S}$ to determine which lines would be used in haplotype scoring that would maximise the number of independent chromosomes isolated in separate *D. melanogaster* lines. Progeny that did not contain the balancer phenotype were used for scoring a series of genetic markers on chromosome three and for scoring wing size (refer to Chapter 4 for method of wing size measurements) from seventy-eight independent chromosome lines.

Marker scoring for the linkage disequilibrium study

Each independent third chromosome was scored for eleven genetic markers. The location of these markers on chromosome three and other genetic marker information is given in Table 3.1 and in Figure 3.5. All marker were genotyped from adult flies, except *In(3R)P*, which was scored by polytene chromosome squashes of larvae from the second cross.

Table 3.1 Chromosome three genetic markers

Locus	Cytological location	Genetic location	Polymorphism type	No. alleles segregating	MCA	MCA Frequency	Expected Heterozygosity (H_E)
In(3L)P	63B8..9-72E1..2	3L	Chromosome inversion	2	Standard arrangement	0.74	0.387
DMU14395	65D1..3 (3L)	3-18	(TC) repeat	9	11 repeats	0.71	0.475
Clk	66A5-66A12(3L)	3L	(CAG) repeat	6	30 repeats	0.63	0.583
hsp 70	87A5..7(3R)	3-51	139Lp Indel	2	56H8	0.75	0.375
DMTRXIII	88B3 (3R)	3-54.2	(CT) repeat	11	18 repeats	0.45	0.753
In(3R)P	89C2..3-96A18..19	3R	Chromosome inversion	2	Standard arrangement	0.57	0.490
hsr-omega$^{L/S}$	93D6-7 (3R)	3-71	8bp Indel	2	Long allele	0.62	0.473
hsr-omega^{TR}	93D6-7 (3R)	3-71	280bp repeat	8	Group 4	0.48	0.709
DMU25686	93F (3R)	3-73	(AT) repeat	9	5 repeats	0.31	0.785
AC008193	94D (3R)	3-77	(TG) repeat	9	14 repeats	0.23	0.845
Tpi	99E(3R)	3-101.3	(TTA) Indel	2	Deletion	0.53	0.499

MCA= most common allele, H_E = expected heterozygosity, *hsr-omega*^{TR} = *hsr-omega* repeat-length

Polytene chromosome squashes to score *In(3R)P*, were performed as described in (Yoon *et al* 1973). The salivary glands of larvae were dissected in Ringers solution (6.5g NaCl, 0.14g KCl, 0.2g $NaHCO_3$, 0.12g $CaCl_2$ 0.01g NaH_2PO_4 in 1 L) and placed in 45% acetic acid for 10 sec. The glands were removed from the acetic acid and placed into 1M HCl for 30 sec and then stained in lactoacetoorcein for 40 min. The glands were then rinsed in lactoacetic acid and placed on to a drop of lactoacetic acid on a glass microscope slide. A glass coverslip was placed over the preparation and the glands were squashed using hand pressure until the arms of the chromosomes had spread clear of each other when viewed under a compound microscope. The squash was scored for the inversion as detailed in Figure 3.2.

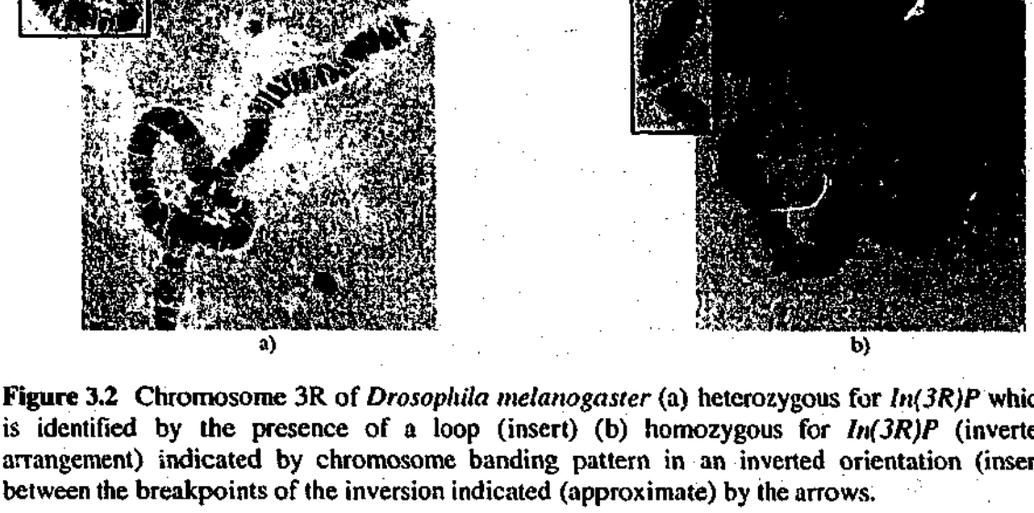


Figure 3.2 Chromosome 3R of *Drosophila melanogaster* (a) heterozygous for *In(3R)P* which is identified by the presence of a loop (insert) (b) homozygous for *In(3R)P* (inverted arrangement) indicated by chromosome banding pattern in an inverted orientation (insert) between the breakpoints of the inversion indicated (approximate) by the arrows.

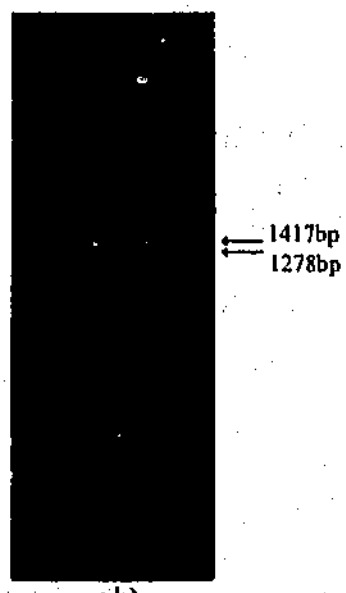
Other markers, except *hsr-omega* repeat-length, were genotyped by PCR using genomic DNA extracted from adult flies (Appendix C for cycling conditions). For genetic markers scored by PCR, DNA was extracted using the proteinase K method described by Gloor and Engels (1992). A single fly was ground in 50 µl of squishing solution (10mM Tris HCl pH 8.2, 1mM EDTA, 25mM NaCl and 200ug/ml proteinase K) and incubated at 37°C for 30 min. Proteinase K was inactivated by heating to 95°C for 2 min.

The *hsr-omega*$^{L/S}$ 8 bp indel polymorphism in exon I was scored using a PCR technique (Anderson *et al* 2003). Primer pairs 5` GCA GTC TGA GGC AGT TAT CC 3` and 5`CAA TCT TTC AAA ATC CGC AG 3` bind to a region surrounding the polymorphism and amplify a product of either 86 bp (*hsr-omega*^{S}) or 94 bp (*hsr-omega*^{L}). The polymorphism was visualised on a 3% ethidium bromide agarose gel run at 100v for 2 h 30 min (Figure 3.3a).

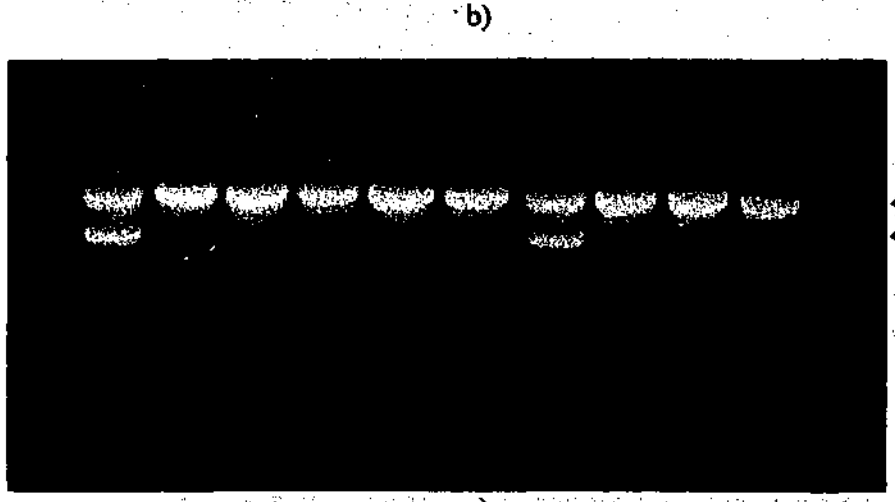
The *hsp 70*$^{56118/122}$ 149 bp indel polymorphism was scored by PCR as previously described (Bettencourt *et al* 2002). Primer pairs flanking this region, 5` CAT CCC AAA AAT CTG TAA AGC 3` and 5`ACT GTG TTT CTG GGG TTC AT 3`, amplified a product of 1278bp (149 bp insert absent, *hsp70*^{122}) and 1417bp (149 bp insert, *hsp70*^{56118}). The polymorphism was visualised on a 1% ethidium bromide agarose gel run at 100v for 1 h 30 min (Figure 3.3b).

In(3L)Payne (*In(3L)P*) was scored using a PCR technique that used three primers: 5` CAG TCA GCA GAC ATC CTT 3`, 5` CGC ACC TGA AAG CGA AAT 3` and 5` GTT CAA TAG ACT CAC TTT GAG 3` (Primers 8-, 13+ and 14+ respectively from (Wesley and Eanes 1994)) that amplify a PCR product of either 1075 bp or 1350 bp (inverted and standard chromosome arrangement respectively). The reagent concentrations used were as follows; 2mM $MgCl_2$, 0.2mM dNTPs, 0.2pmoles/µl each primer and 1.5units *Taq* polymerase in a 50 µl reaction. The polymorphism was visualised on a 1% agarose gel containing ethidium bromide run at 100v for 1 h (Figure 3.3c).

a)



b)



c)

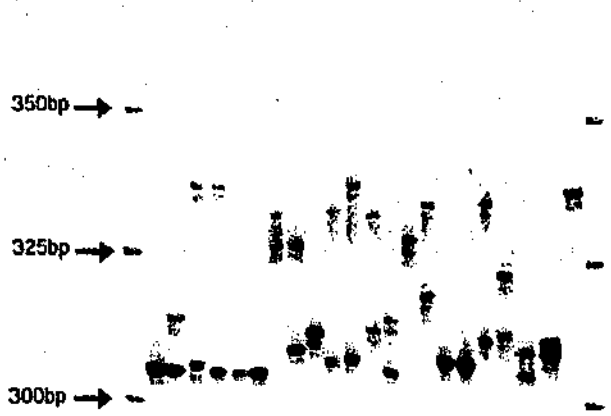
Figure 3.3 Agarose gel UV images of *hsr-omega*$^{1/5}$, *hsp70*$^{56H8/122}$ and *In(3L)P* alleles. Each lane, other than the molecular weight markers, is the genotype of a single fly for the genetic marker. a) *hsr-omega*$^{1/5}$ lane 1 is the 100 bp molecular weight marker (Promega). This gel was double loaded so that '1' and '2' indicate loads 1 and 2 respectively. The 100 bp marker was loaded with load 1. Load 1 lane 2 is homozygous for the long allele (LL) at 92 bp, load 1 lane 10 is homozygous for the short allele (SS) at 86 bp and load 1 lane 3 is heterozygous (LS). b) *hsp70*$^{56H8/122}$ lane 1 is MBI GeneRuler 1kb DNA ladder. Lane 2 a 122 allele homozygote (149 bp deletion, 1278 bp fragment), lane 3 is a 56H8 allele homozygote (149 bp insert, 1417 bp fragment), lane 4 is a 122/56H8 heterozygote. c) *In(3L)P* lanes 1 and 12 are 100 bp molecular weight markers (Promega). Lane 3 indicates a homozygous for standard arrangement at 1350 bp and lane 2 indicates a heterozygote chromosome for the standard (band 1350 bp) and inverted (band 1075 bp) arrangement. Due to the nature of the cross a homozygote for the inverted arrangement was not encountered.

Microsatellite markers (Gockel *et al* 2001) and *Clk* (Allada *et al* 1998) polymorphisms were scored by PCR using infrared labelled (IRDye) primers (see Appendix C for primers). The reaction reagents include; 2mM $MgCl_2$, 0.2mM dNTPs, 0.2pmoles/μl each unlabelled primer, 8nmoles/μl labelled primer and 0.5units *Taq* polymerase in a 25 μl reaction. The PCR products were run at 1500v for 1h 45 min using Seqagel on Li-Cor Global IR^2 that detect the IRDyes (Figure 3.4). Genotyping these markers was aided by Li-Cor $SAGA^{GT}$ software (SAGA Generation 2 version 3.0.0).

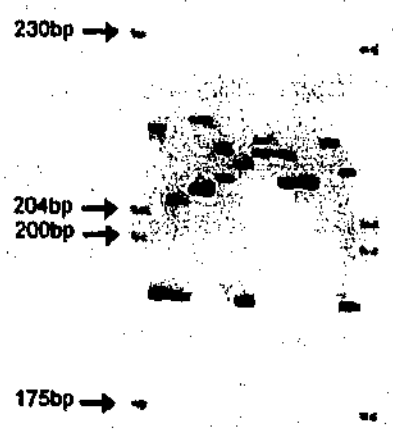
Hsr-omega repeat-length and *Tpi* polymorphisms were scored using the methods described in Chapter 2 and 5 respectively.

Trait marker associations using family lines

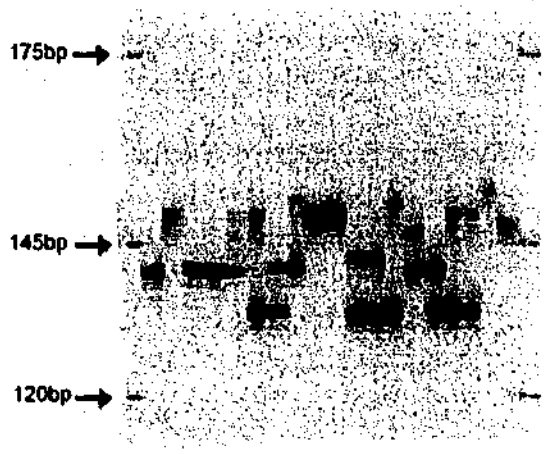
A subset of lines reported in Weeks *et al* (2002) were genotyped for the *hsr-omega* repeat-length polymorphism. These lines were derived from one hundred *D. melanogaster* isofemale lines collected from Coffs Harbour, Australia (30°19'00 S 153°08'00 E), in March 2001 (lines independent of those used above). To avoid inbreeding, F1 progeny of field collected fertilised females were used to set up 200 single pair matings between different pairs of lines (family lines), to maximise the number of trait/marker combinations. These were genotyped for *hsr-omega* repeat-length using the method described in Chapter 2 (long gels). Raw data for three traits (chill coma recovery, heat resistance and wing size) and genotype data for $hsr\text{-}omega^{L/S}$, on these family lines were obtained from Weeks *et al* (2002) and used for association analysis with *hsr-omega* repeat-length variation.



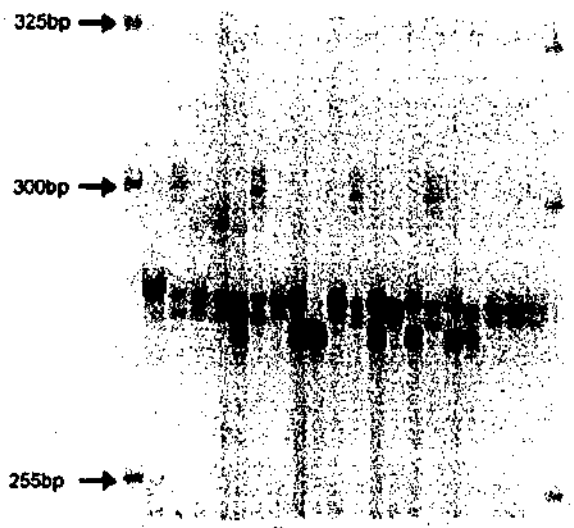
a)



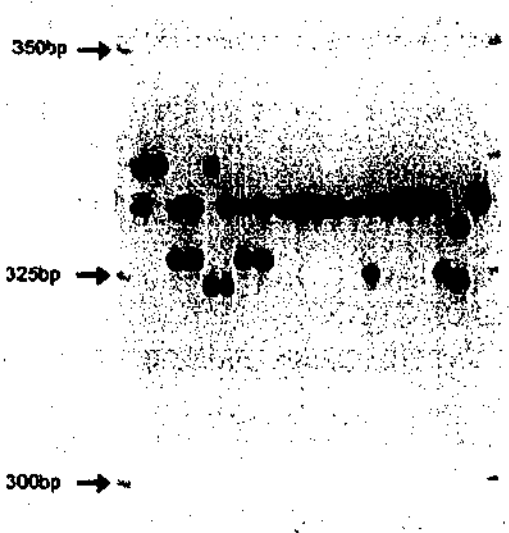
b)



c)



d)



e)

Figure 3.4 Electrophoretic Li-Cor images of markers a) *DMTRXIII*, b) *ACO08193* c) *DMU25686* d) *DMU14395* and e) *Clk*. The first and last lanes contain the 50-350 size standard. Each other lane is the genotype of a third chromosome line for that marker.

Analysis

The strength of the linkage association between pairs of clinal genetic markers was given by the standardised linkage disequilibrium coefficient D` (Hedrick 1987; Hedrick 2000). D` values range from zero (random association) to one (maximum gametic associations) and are independent of allele frequencies as D` has the same range regardless of allele frequency (Hedrick 1987). D` values were calculated using the program Haploxt (Abecasis and Cookson 2000) and significant linkage disequilibrium was detected using Fishers exact test (Raymond and Rousset 1995). An association was considered significant if the P-value from Fishers exact test was less than 0.05. The P-values were adjusted using the sequential Bonferroni method (Rice 1989) to account for multiple comparison. Linkage disequilibrium (D`) was also estimated between the two *hsr-omega* polymorphisms (*hsr-omega*^{US} and *hsr-omega* repeat-length) in the family line study and significance was measured using ANOVA (SPSS version 11.5 for Windows).

To look for associations between *hsr-omega* repeat-length variation and traits in the family line study, data were analysed using Spearman's coefficient of rank correlation. *Hsr-omega* repeat-length associations were analysed using allele groups frequencies as described in Chapter 2. Alleles were binned into 6 groups B1 (15.5 to <19kb), B2 (12 to <15.5kb), C (9 to <12kb), D1 (7.5 to <9kb) D2 (6 to <7.5kb) and E (<6 kb). Two new allele groups were created for family line associations by pooling, since some of these groups contained too few alleles. The long-repeat allele group (*hsr-omega'55'*) had an average repeat number of 55 and was created by pooling the B1 and B2 allele group frequencies. The short-repeat allele group (*hsr-omega'21'*) with an average repeat number of 21, was created by pooling the D2 and E allele groups frequencies.

Associations between wing size and chromosome 3R markers in the linkage disequilibrium lines were analysed using independent-samples t-tests (SPSS version 11.5

for Windows). For this analysis, markers with multiple alleles were pooled into two groups, that is, most common allele (MCA) and all other alleles.

3.3 Results

Linkage disequilibrium

Linkage disequilibrium was determined from the set of linkage lines as distinct from the family lines derived by Week *et al* (2002), from the same central population. The linkage lines were created to allow the haplotype of third chromosome genetic markers to be determined (raw haplotype data Appendix D) and used for linkage association analysis. The degree of linkage association for pairwise comparisons between the eleven chromosome three markers was measured by the linkage disequilibrium, D` values, and evaluated using Fisher's exact test (Figure 3.5). Fifty-five pairwise comparisons were investigated and seven comparisons (seven highest D` measurements) were found to be significant at the 0.05 level before correction for multiple comparisons. Only three pairwise comparisons remained significant after Sequential Bonferroni correction (Figure 3.5). The *hsr-omega* repeat-length polymorphism was not strongly associated with any of the clinal markers. However, it was mildly associated with *hsr-omega*^{US} (D`= 0.57) which was significant (P= 0.013) only prior to Bonferroni correction. A positive association occurred between *hsr-omega*$^{L}$ (the 8 bp insertion at the 5` end of the gene) and short-repeat allele group (*hsr-omega'21'*) in this central population.

Highly significant linkage disequilibrium was observed between *hsr-omega*^{US}*/In(3R)P* (D`= 0.72 P<0.001), *hsr-omega*$^{US}$*/DMU25686* (D`= 0.77, P<0.001) and *DMU25686/In(3R)P* (D`= 0.62, P= 0.01). Considering the three markers in significant disequilibrium, two main haplotypes were segregating in this population (Table 3.2).

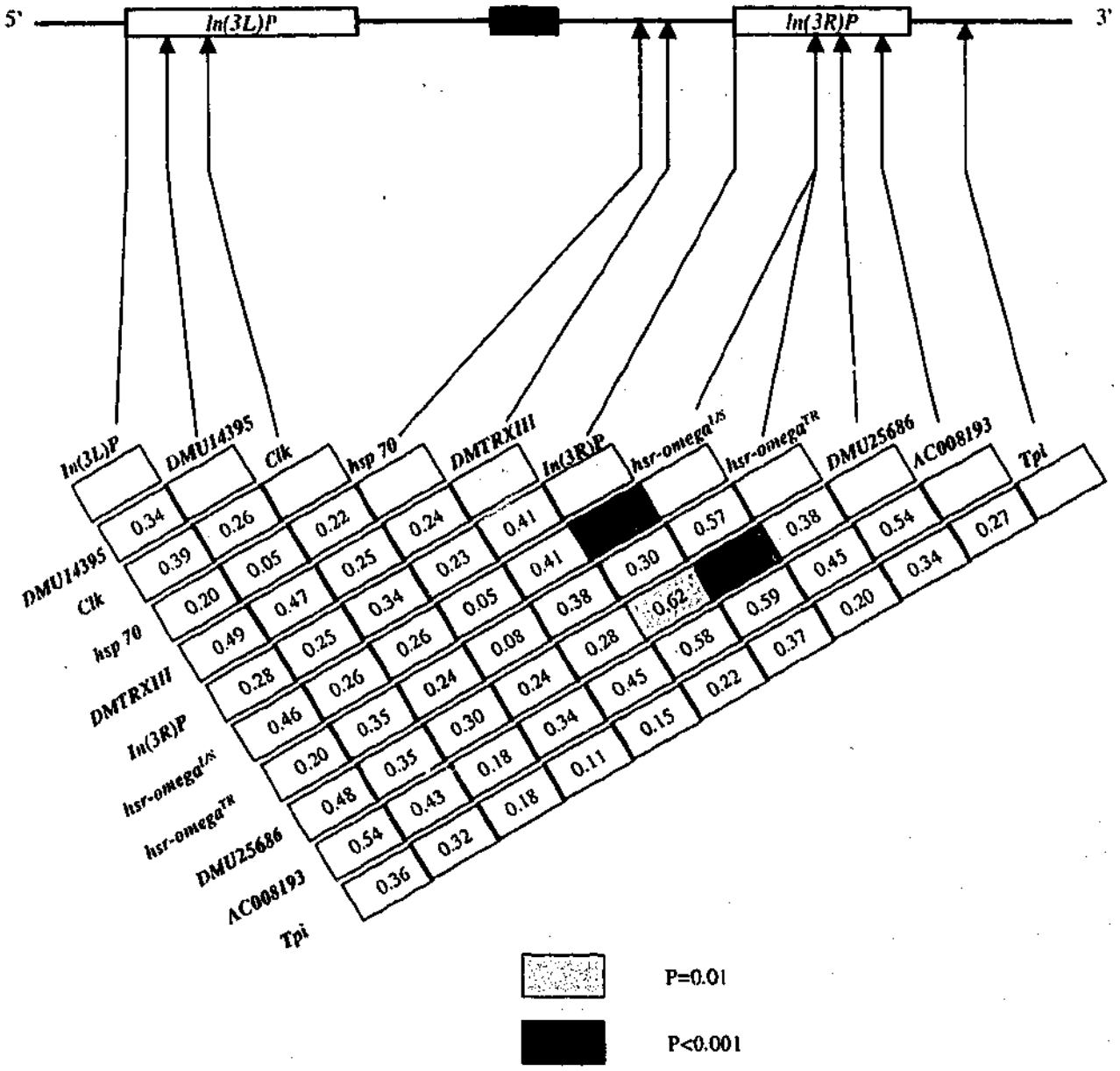


Figure 3.5 Schematic diagram of chromosome three and the relative positions of all markers, the black box indicating the centromere (above). Triangular matrix of pairwise comparisons between all markers (below). The degree of linkage disequilibrium (D`) is shown in the rectangles. Statistical significance was determined by Fishers exact test (with Bonferroni correction) and is indicated by shading (Note *hsr-omega*^{TR} = *hsr-omega* repeat-length).

Approximately 53% of the chromosomes were segregating as haplotype 1 (*hsr-omega*^{L}, *In(3R)P* standard orientation and all other *DMU25686* alleles) and 26% were segregating as haplotype 2 (*hsr-omega*^{S}, *In(3R)P* and *DMU25686* MCA).

Table 3.2 Distribution of observed haplotype numbers among the three markers showing significant D` values.

Haplotype	Observed Number
L/INV/5	1
L/INV/1	3
L/STD/5	1
L/STD/1	31
S/INV/5	15
S/INV/1	3
S/STD/5	3
S/STD/1	1
Total	58*

S= *hsr-omega*^{S} (8 bp deletion)
L= *hsr-omega*^{L} (8 bp insertion)
INV= *In(3R)P*
STD= standard chromosome arrangement
5= *DMU25686* MCA (5 repeats)
1= *DMU25686* all other alleles
* While 78 independent lines were isolated only 58 lines gave data for all three markers.

The crossing scheme used in the family line data set allowed us to make an independent estimate of association between the two *hsr-omega* polymorphic sites in the same Coffs Harbour population. Among the 138 family lines for which genotype data of both *hsr-omega* polymorphic sites were available, 22 families (88 chromosomes) were pure breeding for the *hsr-omega*^{L} allele and allowed the *hsr-omega'21'* repeat-length association to be quantified, giving a D` value of 0.31. Once again, *hsr-omega* short-repeat allele group, *hsr-omega'21'*, was positively associated on chromosomes with *hsr-omega*^{L} (Figure 3.6).

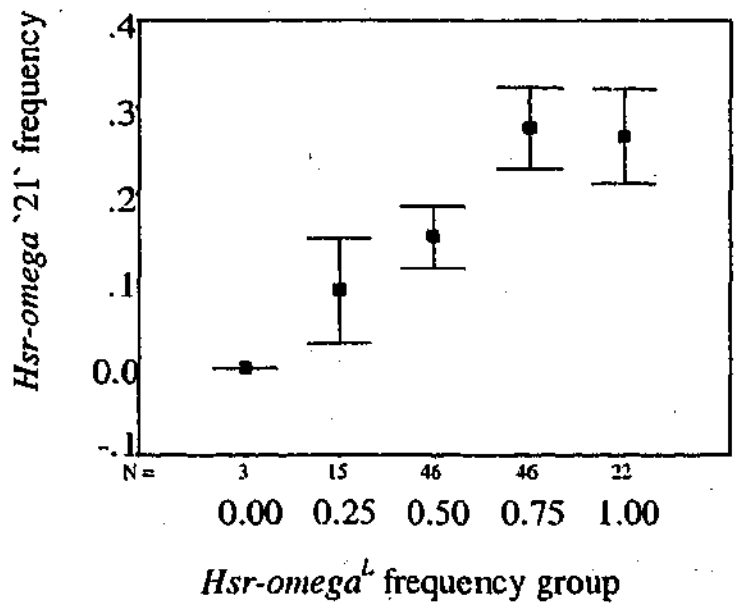


Figure 3.6 Allele frequency associations between *hsr-omega*^{US} and *hsr-omega* repeat-length among the Coffs Harbour population family lines. N gives the number of family lines in each *hsr-omega*^{L} frequency category. The mean frequency of the *hsr-omega'21'* allele group (± S.E) is given on the vertical axis. ANOVA indicates a significant effect of *hsr-omega*^{L} ($F_{(4, 127)} = 2.69$, $P < 0.05$).

Trait associations in the central population

In the family line study, *hsr-omega* repeat-length variation was assessed for association with chill-coma recovery time, heat resistance and body size. There was a negative association between the frequency of *hsr-omega'21'* (the short-repeat allele group) and chill-coma recovery time (Figure 3.7), but no association of *hsr-omega'21'* with heat resistance (Table 3.3). For the long-repeat allele group, *hsr-omega'55'*, there was no indication of an association with either chill-coma recovery time or heat resistance (Table 3.3).

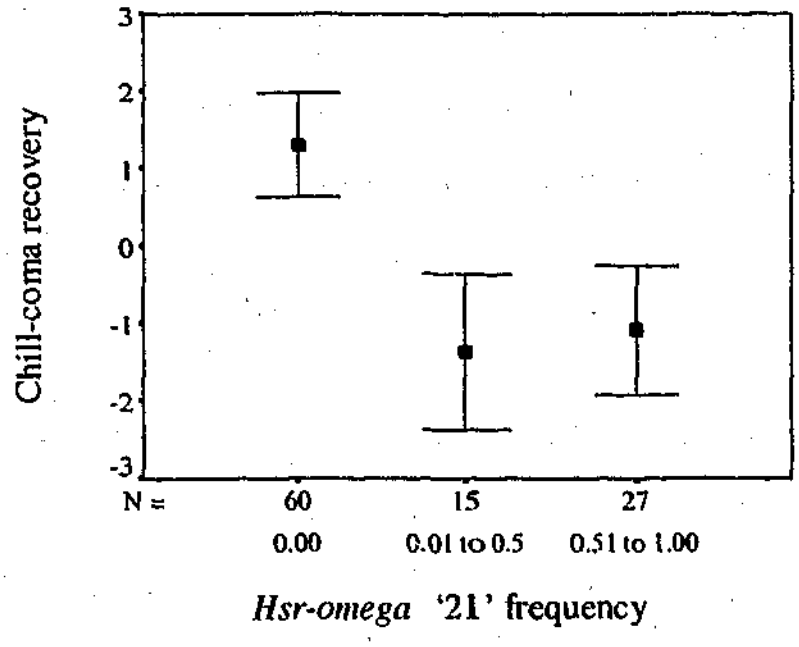


Figure 3.7 Association between *hsr-omega* repeat-length variation (lines grouped into three *hsr-omega'21'* frequency categories) and chill coma recovery (standardised recovery time ± S.E.). ANOVA indicates a significant effect of *hsr-omega'21'* frequency category ($F_{(2,99)} = 3.32$, $P<0.05$).

Table 3.3 Association statistics (Spearman rank correlation coefficients) between *hsr-omega* alleles and clinal traits for Coffs Harbour family lines.

Trait	*hsr-omega'21'*	*hsr-omega'55'*	*hsr-omega*^{S} ≠
Heat knockdown resistance ≠	- 0.057 (101)	- 0.043 (101)	- 0.085 (104)
Chill-coma recovery ≠	- 0.224 * (102)	0.085 (102)	- 0.037 (104)
Wing size ≠	0.234 * (94)	- 0.148 (94)	- 0.472 *** (96)

* P <0.05, *** P < 0.001 Numbers in parenthesis = number of family lines compared ≠ Data from Weeks *et al* (2002)

We also examined *hsr-omega* repeat-length variation for association with wing size variation (a measure of body size) in the family line study. The earlier report on these data by Weeks *et al* (2002), prior to obtaining the *hsr-omega* repeat-length data, found *hsr-omega*^{S} to be associated with wing size (Table 3.3). A positive *hsr-omega'21'*/wing

size association was evident in the current analysis (Table 3.3). However, this was not robust since a partial correlation between *hsr-omega'21'* and wing size after controlling for *hsr-omega*^{S}, was not significant (r = 0.104, df 88) whereas the partial correlation between *hsr-omega*^{S} and wing size, controlling for *hsr-omega'21'*, remained highly significant (r = -0.42, df 88 P<0.001). For *hsr-omega'55'*, there was no indication of any association with wing size (Table 3.3).

Wing measurements were also carried out on the lines derived for the linkage disequilibrium estimates. This allowed an independent analysis of the association of *hsr-omega* repeat-length variation (and other chromosome three markers) with wing size variation. Since body size variation has been mapped predominantly to the right arm of chromosome three (Gockel *et al* 2002), only right arm genetic markers were investigated for associations with wing size. *Hsr-omega* repeat-length variation was not associated with wing size in this data set, nor did wing size associate with any other chromosome 3R markers in these lines (Table 3.4).

Table 3.4 T-test between clinal markers (common allele versus other alleles) and wing size for Coffs Harbour linkage lines.

	Female wing size		**Male wing size**	
Marker	df	t	df	t
DMTRXIII	24	1.16	22	0.62
hsp70	26	1.05	26	-0.10
In(3R)P	28	-1.42	30	-0.32
hsr-omega^{US}	30	1.25	30	0.83
hsr-omega^{TR}	30	-0.22	30	0.89
DMU25686	28	-1.90	28	-0.84
AC008193	23	1.63	23	1.28
Tpi	30	-1.94	30	-0.83

hsr-omega^{TR} = *hsr-omega* repeat-length

3.4 Discussion

The cosmopolitan inversions *In(3R)P* and *In(3L)P* are retained in populations in a polymorphic state which suggests that they carry some sort of selective advantage (Mukai et al 1974; Knibb 1982). Generally, recombination is suppressed within and around the break points of inversions and this lack of recombination is known to keep blocks of genes together in these regions, whose 'supergene' content may confer an adaptive advantage in natural populations (Alvarez et al 1997; Andolfatto et al 2001). However, in the middle of large inversions recombination generally occurs more frequently (Hasson and Eanes 1996). The *hsr-omega* gene is situated within *In(3R)P* and it is of interest to know to what extent this gene is part of a supergene adaptive 'block' complex.

In this study, *hsr-omega*^{US} associated strongly with *DMU25686* and this is consistent with previous findings (Weeks *et al* 2002). However, the inversion is the most likely reason for this association as both of these markers also associated strongly with *In(3R)P* in this single population. Although there is evidence that *hsr-omega*^{US} and *DMU25686* are part of a block of genes in linkage disequilibrium, the data suggest that *hsr-omega* repeat-length variation was largely independent of *In(3R)P*, and of other clinally varying markers on chromosome three including those inside the right arm inversion.

Mild linkage disequilibrium, however, was detected between *hsr-omega* repeat-length variation and *hsr-omega*^{US}. This association was in the expected direction, given that *hsr-omega* short-repeat allele group (*hsr-omega'21'*), is found more common at high latitudes in Australian populations (Chapter 2), which is positively associated with *hsr-omega*^{L}, that also occurs at greater frequencies at higher latitudes (Anderson *et al* 2003). There is a concern that pooling of *hsr-omega* repeat-length alleles into a small number of groups, might have underestimated linkage disequilibrium, especially as sample size in

this study has been small (Zouros *et al* 1977). A more powerful technique for scoring and analysing *hsr-omega* repeat-length is needed but would be difficult to develop unless an expedient alternative to Southern blotting could be developed to score repeat-length variation.

The data suggests an association between cold tolerance variation, measured by chill coma recovery time, and *hsr-omega* repeat-length variation. The short-repeat allele group, *hsr-omega'21'*, was associated with greater cold tolerance (100-chill coma recovery), which is consistent with the clinal increase with latitude in cold resistance (Hoffmann *et al* 2002) and higher frequency of *hsr-omega'21'* in Australian eastern coast populations. This association has highlighted the potential importance of *hsr-omega* repeat-length in clinal cold tolerance variation. However, this association was not highly significant and needs further investigation.

Although a significant correlation was observed between *hsr-omega* repeat-length and body size (measured as wing size) in the family study, this association was not robust as it disappeared under partial correlation analysis. This was likely due to chromosomal associations with other markers, such as *hsr-omega*^{LS}, that are more closely associated with genes that affect body size. Additionally, body size did not associate with *hsr-omega* repeat-length in the independent third chromosome linkage disequilibrium lines. However, body size did not associate with any of the other clinal chromosome three markers in these lines, even with markers previously shown to associate with body size, such as *hsr-omega*^{LS}, *DMU25686* and *AC008193* (Gockel *et al* 2002; Weeks *et al* 2002). There was a reduced number of lines available for body size measurements in these linkage lines and sample size may have been too low to detect significant associations.

There is evidence to suggest that *hsr-omega* repeat-length variation is not contributing significantly to heat resistance. McKechnie *et al* (1998) reported that

although *hsr-omega* polymorphic variation at the 5` end of the gene (including *hsr-omega*^{US}) associated with heat resistance (among heat-resistant lines derived from populations where *In(3R)P* was rare), this trait did not associate with *hsr-omega* repeat-length variation. While natural heat resistance variation and population size was greater in the earlier study, similarly, a lack of association was observed between *hsr-omega* repeat-length variation and heat resistance in the family lines. Interestingly, there was also a lack of association between heat resistance and *hsr-omega*^{US} 5` polymorphic variation among these same family lines. These results, however, may have been confounded by the strong linkage association between *In(3R)P* and *hsr-omega*^{US} observed at this central population, where *In(3R)P* was at relatively high frequency within these lines.

Strong linkage disequilibrium was evident between three markers on chromosome 3R; *hsr-omega*^{US}, *DMU25686* and *In(3R)P*. A much larger proportion of *hsr-omega*^{S} alleles segregated with *In(3R)P* inverted arrangement. This relationship is consistent with previously published latitudinal clines where both the frequency of the *hsr-omega*^{S} allele and *In(3R)P* decrease with latitude. A large proportion of the *hsr-omega*^{S}*/In(3R)P* haplotypes were found to be segregating with *DMU25686* MCA (5 AT repeats). A strong correlation between *hsr-omega*^{US} and *DMU25686* has been previously reported across family lines (Weeks *et al* 2002). However, the previously reported negative latitudinal cline in *DMU25686* MCA was observed for '15 AT repeats' (Gockel *et al* 2001; Gockel *et al* 2002). From the available data it can not be determined if scoring here is consistent with that reported in Gockel *et al* (2001) and hence whether these associations are consistent in direction with clinal variation. It could be that what was scored here as the '5 AT repeats' allele is equivalent to the '15 AT repeats' allele in the Gockel *et al* (2001) study. Sequencing of the *DMU25686* alleles in question would resolve this discrepancy. Future, experiments need to determine if *In(3R)P* carries the same *hsr-omega*^{US} and

DMU25686 alleles in association, regardless of which part of the species range they are drawn from.

No significant linkage disequilibrium was observed with or among markers on chromosome arm 3L. Markers *DMU14395* and *Clk* that map close to each other inside *In(3L)P*, were independent of the inversion and each other in this population sample. *DMU14395* and *Clk* are located near to the middle of the inversion and therefore may be within a region of the inversion were genetic exchange occurs more frequently (Hasson and Eanes 1996).

3.5 Summary

This study has provided some insight into how *hsr-omega* repeat-length variation is structured within the genome. Population haplotype data suggest that *hsr-omega* repeat-length is largely independent of *In(3R)P* and other clinally varying markers on chromosome three. However, a mild association was detected between *hsr-omega* repeat-length and the *hsr-omega*^{US} polymorphic variation. In a study of a set of single pair mating families, *hsr-omega* repeat-length variation was not associated with body size or heat tolerance variation, however, the data suggested a relationship between *hsr-omega* repeat-length and cold tolerance, which needs to be further investigated.

Strong linkage disequilibrium was identified between *hsr-omega*^{US} and a closely linked microsatellite *DMU25686*, and these markers also strongly associated with *In(3R)P* in the clinally central Coffs Harbour population. However, *hsr-omega* repeat-length variation was not strongly associated with this same complex of linked variation within the inversion at this population. These associations need to be further investigated in other populations to see if these relationships are consistent over time and if they are geographically widespread.

CHAPTER 4

Altitudinal variation in traits and polymorphic markers that are candidates for climatic adaptation in *D. melanogaster*

4.1 Introduction

Altitude gradients

In *Drosophila*, altitudinal gradients have been studied less frequently than latitudinal gradients, however, altitudinal transects may be more interesting. Altitude changes occur over relatively small distances and there is generally a more rapid change in environmental conditions, especially temperature, compared to equivalent latitudinal gradients (Heath and Williams 1979; Baur and Raboud 1988). As a consequence, for a given degree of climate change, higher gene flow is more likely over shorter distances in altitude (Blanckenhorn 1997). Any altitudinal genetic differentiation is therefore less likely to be an effect of genetic drift and can be more easily attributed to natural selection, with temperature being a strong candidate selective agent.

Generally, populations at high altitude are more tolerant of environmental variability (they obey altitudinal Rapoport's rule (Stevens 1992)). Altitudinal variation occurs in many organisms including *Drosophila* (see Chapter 1 for review). In this genus,

altitude differentiation is known for wing shape, wing size, development time, body size and various behavioural adaptations (Stalker and Carson 1948; Kimura *et al* 1978; Ichijo *et al* 1982; Louis *et al* 1982; Kimura and Beppu 1983; Bitner-Mathe *et al* 1995; Beppu *et al* 1996; Dahlgaard *et al* 2001; Norry *et al* 2001). At the genetic marker level altitudinal variation in *Drosophila* species has been observed in gene arrangements and chromosome inversions (Dobzhansky 1948; Brncic and Koref-Santiraez 1965; Levitan 1978; Levitan and Scheffer 1993; Dahlgaard *et al* 2001; Aulard *et al* 2002). Previous altitudinal studies in *Drosophila* generally involve a few sites along a single elevation gradient. Such patterns of altitudinal variation could be the result of either adaptation or the product of chance historical population processes. The importance of these explanations need to be resolved

This Project

If temperature is an important selective pressure upholding *D. melanogaster* latitudinal clines then one would predict similar clines in the same traits and genetic markers across an altitudinal gradient, where temperature changes are steeper. Here we focus on thermotolerance traits, on fitness-related traits and on nucleotide variation, all of which are known to latitudinally cline in *D. melanogaster* populations from the eastern coast of Australia, and examine altitudinal variation.

Latitudinal clines in *D. melanogaster* have been reported in cold tolerance, heat resistance, ovariole number, development time and body size along the eastern coast of Australia (James *et al* 1995a; James and Partridge 1995b; Azevedo *et al* 1996; Hoffmann *et al* 2002a). Some of these traits also show parallel clines on other continents and in other *Drosophila* species which suggests that these traits are under climatic selection (Watada *et al* 1986; Capy *et al* 1993; Starmer and Wolf 1997; Van't land *et al* 1999;

Hallas *et al* 2002). Additionally, thermal laboratory selection experiments have strongly implicated temperature as the agent of selection for variation in clinal body size and development time (Partridge *et al* 1994a; Partridge et al 1994b; James *et al* 1995b). Although latitudinal differentiation in egg viability has been observed (Tantawy and Mallah 1961), clinal variation in this trait has not yet been reported. In this study altitudinal variation will be investigated in the six above-mentioned traits.

At the genetic level there are several molecular markers on chromosomes 2 and 3 in *D. melanogaster*, that are known to show latitudinal variation. These mark blocks of genes that are potentially involved with climatic adaptation. Markers on chromosome three, especially on the right arm, are of particular interest as this region contains genes that are involved with clinal thermotolerance variation and with body size variation (Lindquist 1986; Lakhotia 2001b; Gockel *et al* 2002; Anderson *et al* 2003). Along the eastern coast of Australia, polymorphic genetic markers on chromosome 3R that show a latitudinal cline include; *hsr-omega* repeat-length, *hsr-omega*^{US}, *hsp70*, *DMU25686*, *DMTRXIII* and *AC008193* (Gockel *et al* 2001; Bettencourt *et al* 2002; Anderson *et al* 2003; Chapter 2). These six markers will be investigated for altitudinal variation.

4.2 Materials and Methods

Collection sites

Isofemale lines were set up from inseminated females collected from ten locations in February and March 2002, covering 1000m of altitude and 2200km of latitude. The females were collected from high and low altitude from five different latitudes along the eastern coast of Australia (Figure 4.1 and Table 4.1) using banana bait traps (Tidon and Sene 1988).

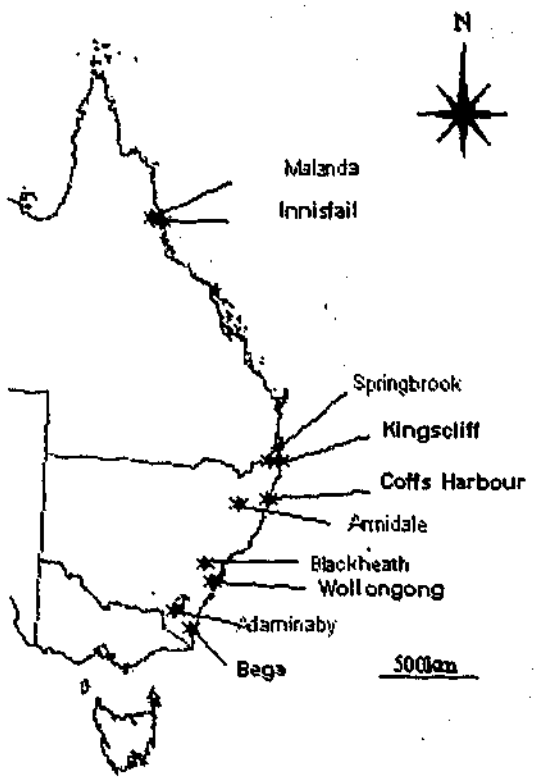


Figure 4.1 Collection sites showing the five paired latitude sites. Text in bold font style are the low altitude sites.

Table 4.1 Five paired latitudinal collection sites for the altitudinal study.

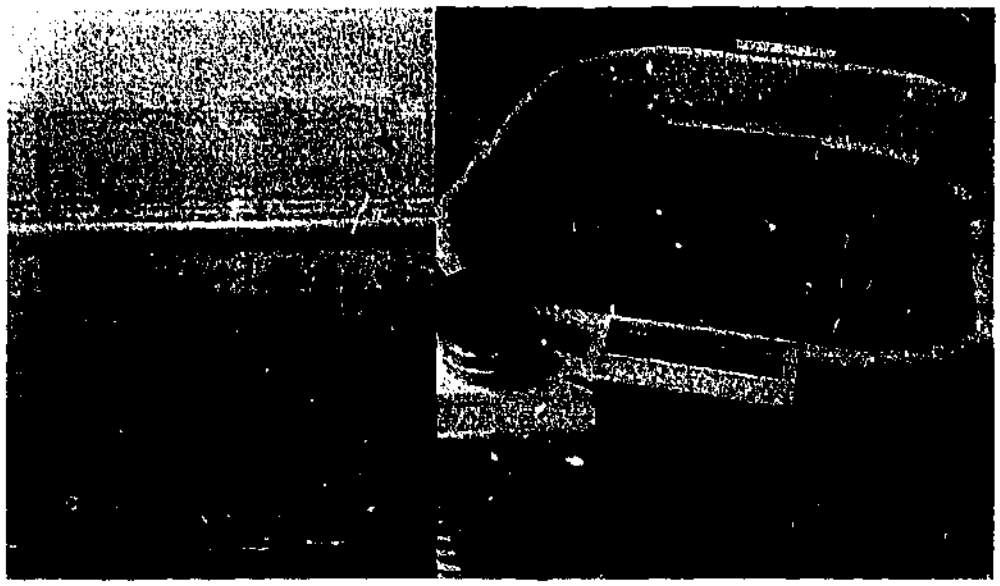
Latitude	Locality		Altitude (m)	Latitude	Longitude	Date	n(l)
1	1	Malanda	800	17°19'S	145°31'E	Mar-02	138(69)
	2	Innisfail	10	17°30'S	145°60'E	Mar-02	138(69)
2	3	Springbrook	1031	28°14'S	153°16'E	Mar-02	68(17)
	4	Kingscliff	10	28°17'S	153°07'E	Mar-02	172(46)
3	5	Armidale	1296	30°31'S	151°41'E	Mar-02	134(67)
	6	Coffs Harbour	14	30°22'S	153°06'E	Mar-02	106(53)
4	7	Blackheath	1046	33°38'S	150°17'E	Mar-02	96(48)
	8	Wollongong	10	34°25'S	150°52'E	Mar-02	96(48)
5	9	Adaminaby	1025	35°59'S	148°46'E	Feb-02	102(53)
	10	Bega	60	36°41'S	149°50'E	Feb-02	72(36)

n= number of alleles scored for genetic marker analysis. l = number of isofemale lines collected

Flies were reared under the same constant temperature/humidity conditions for a number of generations in the laboratory so that environmental induced phenotypic plasticity was diminished and genetic differences could be observed (Berven 1982b). The lines were maintained on potato medium at 18°C (Appendix A). Seven generations after establishing the lines, 10 mass bred populations were founded by pooling 17 isofemale lines (15 flies from each line) for each collection location. The progeny from the isofemale lines and the mass bred populations were used to score a series of latitudinally varying traits and genetic markers for altitudinal differentiation.

Thermotolerance traits

Cold recovery was measured using a chill coma recovery assay similar to that described in Gilbert and Huey (2001). This was carried out on mass bred populations reared at low density (25 females and 25 males), which were allowed to oviposit for 4 days at 18°C. Four to six days after eclosing females were collected for chill coma. Ten females were placed in empty vials from each population (Figure 4.2a) with two replicates for each (single 'run'). The vials were placed in a 0°C water bath, containing ethylene glycol, for 4 h (Figure 4.2b). Vials were removed from the water bath and allowed to recover at 25°C. Recovery was scored every minute for each fly. A fly was considered recovered when it was able to stand. Four replicate runs were carried out.



a) b)

Figure 4.2 Chill coma recovery assay. Flies were placed in capped vials a) and these vials were placed in racks. The racks where submerged, to a depth that did not cover the caps of the vials, in a cold bath b) set to 0°C which contained antifreeze for 4h.

Heat tolerance was measured by heat knockdown in a water bath. Plastic response to short-term sub-lethal heat shock (hardening) as well as unhardened (naive) flies were measured. This was carried out on mass bred populations reared at low density. Approximately 50 males and 50 females, 2 days old, were allowed to oviposit overnight in bottles containing *Drosophila* medium at 25°C. Three days after eclosing and the day before heat knockdown, females were collected from each of the ten populations and placed in vials containing fresh *Drosophila* medium. On the day of heat shock, a few flies were collected from each vial population and hardened at 37°C for 1 h in vials containing *Drosophila* medium. To obtain maximum resistance to heat shock, flies were allowed to recover for 6 h at 25°C after hardening (Krebs and Loeschcke 1994). Replicate heat hardening was carried out one hour apart to allow for replicate heat knockdown on a single day. Two hardened and two non-hardened flies from all ten populations were scored within a single heat knockdown replicate (single 'run'). Single females were placed in small vials that were then placed in racks and submerged in a 39°C water bath

(Figure 4.3). Heat knockdown was scored every minute and flies were considered knocked down if unable to stand. Eighteen replicate runs were carried out.

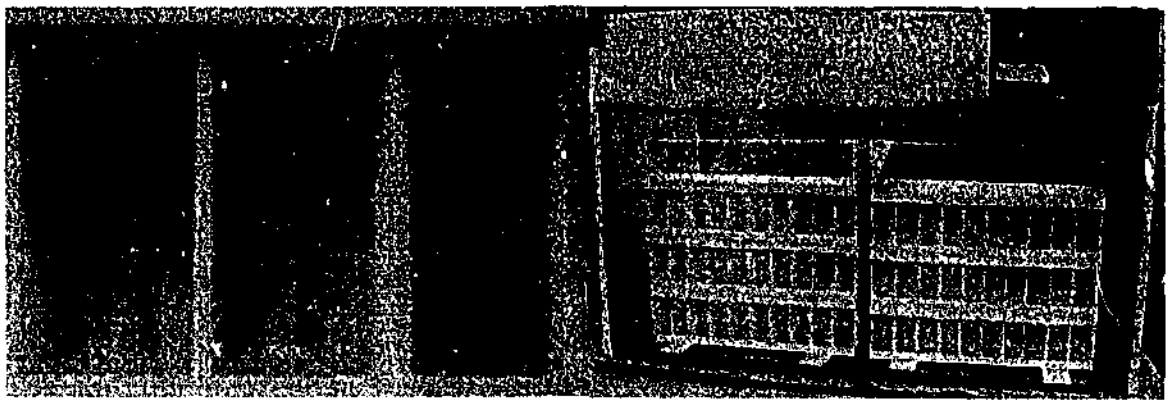


Figure 4.3 Heat knockdown experiment. Flies were placed in capped vials a) and these vials were placed in racks. The racks were submerged in a warm bath b) set to 39°C.

Fitness-related traits

Five isofemale lines from each of the 10 populations were chosen at random and used to measure ovariole number. Lines were reared at 18°C under low population densities. Two vials, containing *Drosophila* medium, were set up from each isofemale line. The first vial contained 4 virgin females and the second vial contained 4 females with 4 males. This allowed for a comparison of mated versus non-mated (virgin) ovariole number. After 5 days the females were collected and frozen at –20°C until dissection. Both ovaries were dissected out in Becker Ringer's solution (6.5g NaCl, 0.14g KCl, 0.2g $NaHCO_3$, 0.12g $CaCl_2$, 0.01g NaH_2PO_4 and made up to 1L) and stained in saturated potassium dichromate for 4 min (Coyne *et al* 1991; Carlson *et al* 1998). Excess stain was removed using Becker Ringer's solution and the ovarioles from both ovaries were dissected out under a dissecting microscope and counted (Figure 4.4).

Figure 4.4 Ovary of *D. melanogaster* (main image) and a single ovariole (insert).

Egg to adult development time and egg viability was determined using mass bred populations, two generations after establishment. Each mass bred was allowed 8 h to oviposit on a petri dish with *Drosophila* medium (Appendix A) at 19°C. From each of the ten populations, one hundred eggs were collected from each petri dish and groups of ten eggs were placed in a vial containing *Drosophila* medium. The eggs were allowed to develop at 19°C. Emerging flies were collected, counted and sexed every 24 h until the end of emergence. Emergence data was used to calculate development time. Egg viability was estimated as the percentage of flies obtained from the initial number of eggs transferred.

Wing size was measured from flies that emerged in the development time experiment. Wing size has been shown to be an indirect measure of body size due to the consistent high level of association between the two traits (David *et al* 1977). Ten males and ten females were chosen at random from each of the ten populations. The right wing from each fly was mounted on a glass microscope slide. A digital image was taken of each wing using a Wild M38 Heerbrugg dissecting microscope (40x) together with a PixeLINK digital camera (PL-A642). Ten landmarks were placed on the digital image (Figure 4.5) and x/y co-ordinates where determined using tpsDig V1.23 and tps utility

program V 1.05 written by James. F. Rohlf (2000). The x and y coordinates of the ten landmarks were used to calculate wing size using Procrustes analysis (square root of the sum of the squared inter landmark distances) described in Rohlf (1999), which is effectively a measure of body size. Sizes were recorded in pixels and converted into mm^2.

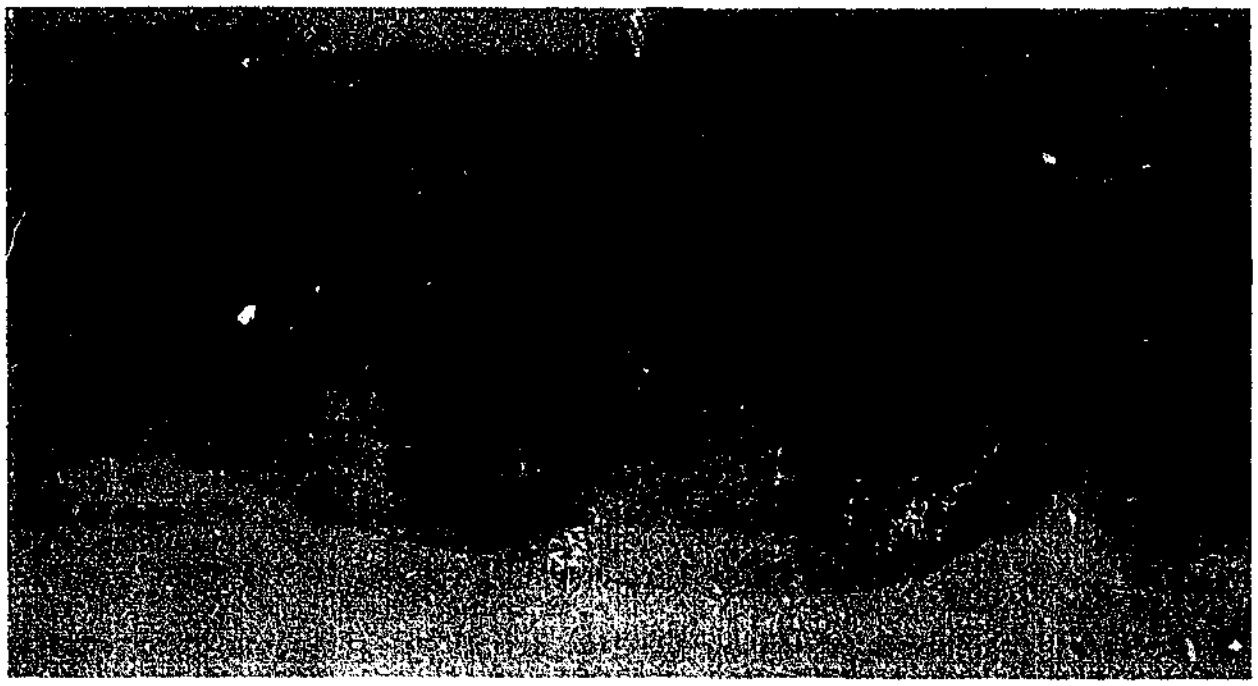


Figure 4.5 Wing of *D. melanogaster* showing the ten landmarks used for analysis of wing size.

Genetic markers

Six genetic markers on the right arm of chromosome three were scored for altitudinal variation (Table 4.2). Markers, $hsp70^{5618/122}$, *DMTRXIII*, $hsr\text{-}omega^{US}$, *DMU25686* and *AC008193* were genotyped using the methods described in Chapter 3. To score the *hsr-omega* repeat-length variation, DNA was extracted from pooled isofemale lines (five isofemale line per extraction) using the Southern blot method described in Chapter 2 for the 2002 collection. *Hsr-omega* repeat-length alleles were binned into 5 allele group sizes (A to E allele bin groups) and the average number of repeats was calculated as previously described.

Table 4.2 Genetic marker location, polymorphism type and alleles.

Locus	Cytological location	Genetic location	Polymorphism type	Allele size	Number of segregating alleles
hsp 70	87A5..7(3R)	3-51	149 bp indel	1278 or 1417 bp	2
DMTRXIII	88B3 (3R)	3-54.2	CT repeat	1 to 25 repeats	25
hsr-omega^{US}	93D6-7 (3R)	3-71	8 bp indel	86 or 94 bp	2
hsr-omega^{TR}	93D6-7 (3R)	3-71	280 bp repeat	18 to 68 repeats	5*
DMU25686	93F (3R)	3-73	AT repeat	1 to 20 repeats	20
AC008193	94D (3R)	3-77	TG repeat	0 to 16 repeats	17

hsr-omega^{TR} = *hsr-omega* repeat-length * number of pooled allele groups

Analysis

Phenotypic traits were analysed by ANOVA, testing for a main effect of latitude and altitude as well as testing for an interaction between altitude and latitude. Genotypic markers were assessed for altitudinal associations and interactions with latitude using a three-way χ^2 analysis of allele numbers (Cochrans-Mantel-Haensel and Breslow-Day test (Sokal and Rolf 1995)). These statistical analyses were carried out using SPSS 11.5 for Windows.

4.3 Results

Thermotolerance traits

Analysis of chill coma recovery variation indicated significant effects of altitude, latitude, run and an altitude by latitude interaction (Table 4.3). Generally, populations from higher altitudes were more cold tolerant (Figure 4.6) as were populations from higher latitude. Populations from the five different latitudes differed in the extent to which chill coma recovery was affected by altitude. This interaction is illustrated in Figure 4.6 where higher

altitude had greater cold tolerance at temperate latitudes but was not apparent at tropical latitudes. There was a significant effect between different runs of this treatment, however, no two-way interactions involving run occurred (data not shown).

Table 4.3 Analysis of variance of thermal tolerance traits in *D. melanogaster*. Testing the effect of run, latitude, altitude and interaction between latitude and altitude (Alt/Lat).

	Chill coma recovery			Heat resistance - non-hardened			Heat resistance - hardened		
Source of Variation	df	MS	F	df	MS	F	df	MS	F
Run	3	18605.15	47.84***	17	65.81	4.24***	17	120.97	5.91***
Latitude	4	1009.48	2.60*	4	89.09	5.74***	4	7.52	0.37***
Altitude	1	2023.58	5.20*	1	64.03	4.13*	1	85.57	4.18
Alt/Lat	4	1200.21	3.09*	4	26.28	1.70	4	15.42	0.75
Error	746	388.91		331	15.51		323	20.46	

* P<0.05, ** P<0.01, *** P<0.001

Analysis of non-hardened heat knockdown resistance indicated a significant effect of run, latitude and altitude (Table 4.3). While there was a significant effect of run in non-hardened and hardened flies no two-way interactions of run with altitude or latitude (data not shown) occurred. Non-hardened heat knockdown decreased with latitude and flies from lower altitude were less heat resistant compared with high altitude populations (Figure 4.6). The effect of altitude on heat knockdown was consistent across different latitudes (there was no interaction effect). Compared to non-hardened flies, heat resistance was increased by heat hardening. A significant amount of variation in hardened heat knockdown was explained by the main effects of run and latitude. Flies from different latitudes did show significantly different responses to hardened heat knockdown, however, there was no effect of altitude nor was there an interaction between altitude and latitude (Table 4.3 and Figure 4.6).

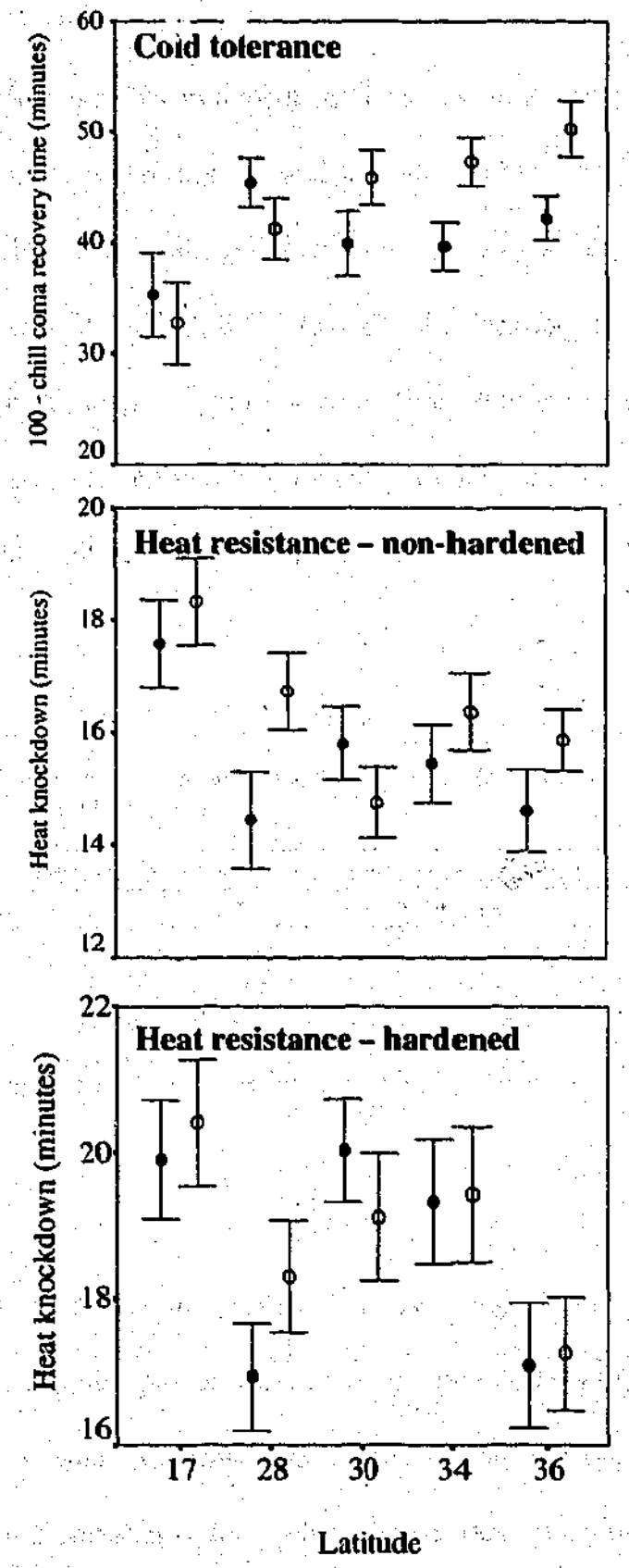


Figure 4.6 Variation in thermal tolerance traits across latitude and altitude (±SE). Low altitude: closed circles; high altitude: open circles. Note that 100-chill coma recovery is a measure of cold tolerance.

Fitness-related traits

Analysis of ovariole number in non-mated females indicated a significant effect of altitude and an interaction between altitude and latitude (Table 4.4). As indicated in Figure 4.7, non-mated females at low altitude had more ovarioles per ovary, compared with high altitude populations (except at 37° latitude). As latitude increased the effect of altitude decreased and so the effect of altitude was dependent on latitude (interaction effect). In sharp contrast ovariole number in mated females did not show any main effects or interactions (Table 4.4 and Figure 4.7).

Table 4.4 Analysis of variance of ovariole number and egg viability in *D. melanogaster*. Testing the effect of latitude, altitude and the interaction.

	Ovariole Number (non-mated)			Ovariole Number (mated)			Egg viability		
Source of Variation	df	MS	F	df	MS	F	df	MS	F
Latitude	4	4.82	1.30	4	13.15	1.29	4	17.22	7.58***
Altitude	1	66.50	17.96***	1	0.52	0.05	1	4.41	1.94
Alt/Lat	4	11.98	3.24*	4	1.56	0.15	4	8.39	3.69*
Error	40	3.70		40	10.18		90	2.27	

* P<0.05, ** P<0.01, *** P<0.001

Analysis of development time indicated significant main effects of proportion female, latitude, altitude, and an interaction between altitude and latitude (Table 4.5). As development time was different between the sexes and the proportion of females that emerged differed significantly for each replicate, development time was corrected for sex using an analysis of covariance, with proportion female as the covariate. Development time was generally faster at high altitude compared to low altitude (Figure 4.7) but the interaction effect suggests that the extent of this depends on latitude, there being less difference between altitudes at middle latitudes.

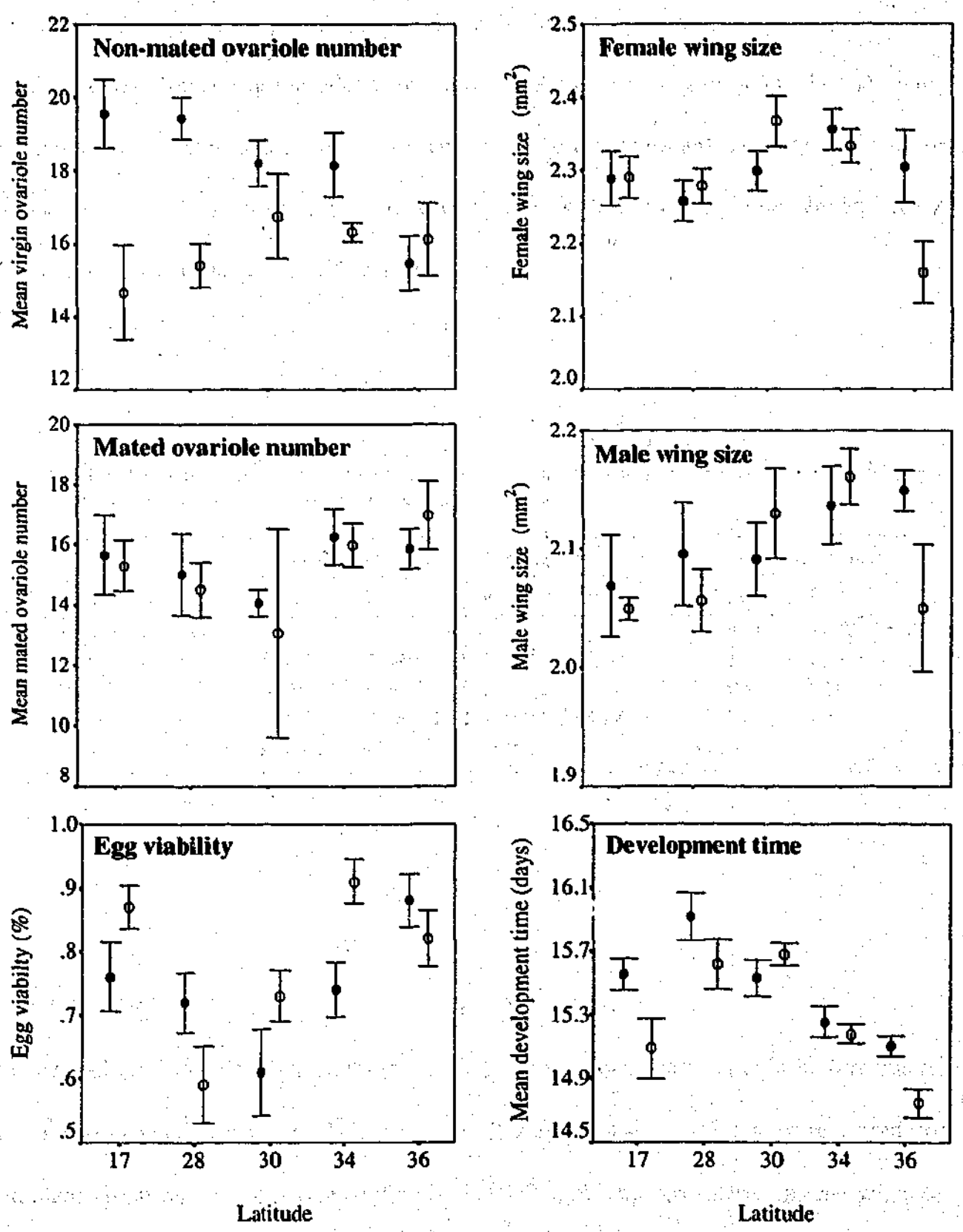


Figure 4.7 Variation in fitness-related traits across latitude and altitude (±SE). Low altitude: closed circles; high altitude: open circles.

There was no overall effect of altitude on egg viability or wing size as illustrated in Figure 4.7. A significant proportion of the variation in egg viability was explained by latitude and an altitude by latitude interaction (Table 4.4 and Figure 4.7). Female wing size indicated a significant effect of latitude and a significant altitude by latitude interaction (Table 4.5). Variation in male wing size could not be explained by any of the main effects or the interaction (Table 4.5).

Table 4.5 Analysis of variance of wing size and development time in *D. melanogaster*. Testing the effect of latitude, altitude and the interaction.

	Wing size (female)			Wing size (male)			Development time		
Source of Variation	df	MS	F	df	MS	F	df	MS	F
Proportion female	-	-	-	-	-	-	1	2.11	18.46***
Latitude	4	0.04	3.88**	4	0.02	2.02	4	1.97	17.22***
Altitude	1	$6x10^{-3}$	0.54	1	$9x10^{-3}$	0.78	1	0.77	6.70*
Alt/Lat	4	0.03	2.88*	4	0.02	1.28	4	0.29	2.52*
Error	90	0.01		90	0.01		89	0.11	

* P<0.05, ** P<0.01, *** P<0.001

Genetic markers

Five latitudinal pairs of high and low altitude populations were examined for genetic marker associations with altitude using a multiway χ^2 test. Markers were tested for altitudinal variation and only one of the six, *DMU25686*, showed interesting associations with altitude (Table 4.6 and Figure 4.8). There was a significant effect of altitude, after controlling for latitude, on the frequency of the most common *DMU25686* allele (5 AT repeats) (χ^2_{CMH}=7.81 df 1 P<0.01, estimated common odds ratio = 1.52) and no interaction between altitude and latitude (χ^2_{BD}= 6.52 df 4 P>0.05). The frequency of *DMU25686* (5

AT repeats) decreased with latitude and was significantly lower at high altitude, compared to low altitude, across all latitudes except at 17° latitude where the frequency was similar (Figure 4.8).

A possibly interesting, but here non-significant, trend occurred for the *hsr-omega*$^{L/S}$ polymorphism. The frequency of the L allele, that shows a strong positive association with cooler high latitude populations (Anderson *et al* 2003), was at higher frequency at high altitudes but only at the higher latitude populations (Figure 4.8).

Table 4.6 Summary of Cochrans-Mantel-Haensel test (CMH), Mantel-Haensel odd ratio (MH) and Breslow-Day test (BD) to determine the effect of altitude on allele frequency.

	CMH		MH	BD	
Markers	df	χ^2	OR	df	χ^2
hsp70	1	0.05	-	-	-
DMTRXIII	1	0.34	-	-	-
hsr-omega$^{L/S}$	1	0.01	-	-	-
hsr-omega^{TR}	1	0.65	-	-	-
DMU25686	1	7.81**	1.52	4	6.52
AC008193	1	0.19	-	-	-

hsr-omega^{TR} = *hsr-omega* repeat-length, OR = estimated common odds ratio ** P<0.01

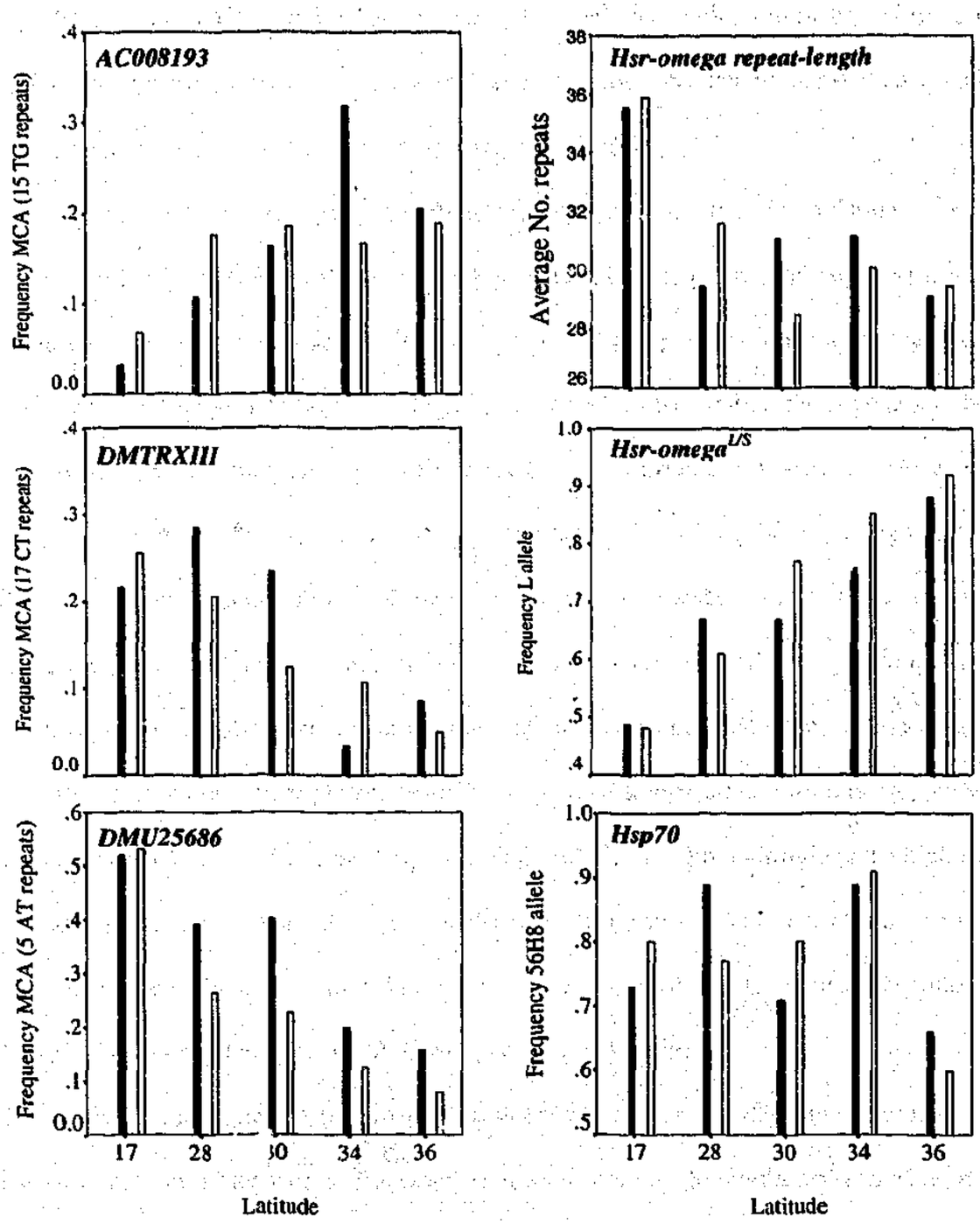


Figure 4.8 Distribution of allele frequency for six molecular markers at low (solid bar) and high (open bar) altitude at five different latitudes.

4.4 Discussion

Significant altitudinal differentiation was observed for cold tolerance (chill coma recovery), heat tolerance in non-hardened flies, ovariole number of non-mated females, development time and for a molecular marker, *DMU25686*.

Cold tolerance was higher at high altitude and in high latitude populations as might be expected if this trait is adaptive and is consistent with previously reported positive latitudinal associations of this trait (Hoffmann *et al* 2002). This altitudinal relationship was weaker at the lower latitudes as might be expected, as tropical latitudes generally have high average yearly temperatures with relatively low climatic variability, even at high altitudes (Janzen 1967). Heat resistance in non-hardened flies was higher at high altitude, which was inconsistent with a simple adaptive hypothesis, unlike cold tolerance. However, these results are consistent with the altitudinal Rapoport's rule (Stevens 1992). For survival in variable climatic conditions at high altitude along the eastern coast of Australia, populations have physiologically adapted to temperature extremes by evolving greater thermal tolerance ranges. Unhardened populations at high altitude had a high level of cold resistance and were also more resistant to heat stress. Thermal tolerance ranges have also been shown to increase with increasing altitude in species outside the *Drosophila* genus (Chen *et al* 1990; Gaston and Chown 1999).

Altitudinal variation in ovariole number was present in non-mated females. In sharp contrast, no trend with altitude was observed in ovariole number of mated females. These results suggested that a higher ovariole number is favoured in low altitude environments but only for unmated females. Ovariole number of non-mated females has been shown to be higher than mated females in single populations of *D. melanogaster* (Bouletreau 1978; Carlson *et al* 1998), however, comparisons along environmental gradients have not been previously reported. While positive correlations of ovariole

number with latitude in *D. melanogaster* mated females are known (Capy *et al* 1993; Azevedo *et al* 1996), latitudinal variation in non-mated female ovariole number has not been reported. The lack of concordance observed here between non-mated and mated ovariole number over latitude and altitude suggests that reproductive status (non-mated or mated) has a major effect on this trait. Ovariole number is a highly variable trait even within populations (Delpuech *et al* 1995) and phenotypic plasticity of ovariole number at different developmental temperatures is well documented (David and Clavel 1967; Delpuech *et al* 1995). Further research is needed to better understand the effects of temperature on genetic variation in ovariole number between mated and non-mated females, by investigating ovariole number responses to different developmental temperatures using populations from diverse latitudinal and altitudinal origins, and by conducting selection experiments on ovariole number at different temperatures. Additionally, this study needs to be repeated on different continents and over larger ranges of altitudes to see if this trend in ovariole number is a general effect. These types of experiments might help untangle the adaptive significance of the observations made here on larger differences in ovariole number between mated and non-mated females.

Development time was faster at higher altitude compared to low altitude in these populations. The results from this study are consistent in direction with findings of altitudinal differentiation in other populations of *D. melanogaster* (Louis *et al* 1982) and organism outside the *Drosophila* genus (Berven *et al* 1979; Berven 1982a; Berven 1982b; Dingle *et al* 1990). This altitudinal differentiation is consistent with laboratory selection experiments in *D. melanogaster* where rapid development has evolved at low rearing temperatures (Anderson 1966; Partridge *et al* 1994b; James and Partridge 1995b; James *et al* 1997). These results are also consistent with the widespread *D. melanogaster* latitudinal cline for development time, where faster development occurs at more temperate

higher latitude (James and Partridge 1995b; Van't land *et al* 1999). This study provides further evidence that faster development time is the result of natural selection at lower rearing temperatures.

Increased body size in *D. melanogaster* is selected by low temperature environments. This is evidenced by parallel positive latitudinal clines in body size across different continents and species, and by thermal laboratory selection experiments resulting in larger body size at lower rearing temperature (Anderson 1966; Cavicchi *et al* 1985; Cavicchi *et al* 1989; Partridge *et al* 1994a; James *et al* 1995a; Van't land *et al* 1999). However, no altitudinal differentiation in body size, measured as wing size, has been detected in this study. There was no main effect of altitude suggesting that there had been no genetic adaptation of body size. Perhaps average temperature differences between the approximately 1000m of altitude in this study have not been large enough to select for differences in body size, or perhaps selection on this trait is relatively weak and gene flow levels preclude differentiation.

In this study the frequency of the most common *DMU25686* allele (5 AT repeats) decreased with altitude (and latitude) associating negatively with cooler environments. If the *DMU25686* marker proves to be associated robustly with altitude in future studies, as it has with latitude (Gockel *et al* 2001; Gockel *et al* 2002), it will be of major interest in the context of climatic adaptation. Previous studies have shown that *DMU25686* strongly associates with body size variation, a trait under strong temperature selection (Gockel *et al* 2002; Week *et al* 2002). While *DMU25686* showed altitudinal differentiation, body size did not. However, the direction of the *DMU25686* correlations with altitude and latitude suggest that cold tolerance may be a candidate trait to look for association with this microsatellite marker, as this trait shows similar latitude (Hoffmann *et al* 2002) and altitude associations (unlike heat tolerance which was higher at low tropical latitude

(Hoffmann *et al* 2002) and high altitude). While these geographical patterns in *DMU25686* suggest the action of selection, this microsatellite locus may be only an indirect target of selection. Chapter 3 provided evidence that *DMU25686* is part of a block of genes held together by *In(3R)P*, as strong linkage disequilibrium was observed at a single population among three markers that included, *DMU25686* and *In(3R)P*. Unfortunately *In(3R)P* was not scored in this altitude study. Nevertheless, both altitudinal and latitudinal variation in the *DMU25686* microsatellite indicate it is a genetic hitchhiker with a block of genes under interesting differential geographical selection.

The *hsr-omega*^{US} polymorphism in which *hsr-omega*^{L} is more common at more temperate high latitudes (Anderson *et al* 2003) did show an interesting, although non-significant, association with altitude. There was a higher frequency of the *hsr-omega*^{L} allele at high altitude, but only at a cool temperate latitudes, where the effects of altitude are likely to be more extreme (Janzen 1967). This altitudinal effect at high latitudes in this marker is consistent in direction with the latitudinal cline and needs further investigation in the context of thermal adaptation.

Significant altitudinal differentiation was observed for four traits, cold tolerance, non-hardened heat resistance, non-mated ovariole number and development time, and for one genetic marker, microsatellite *DMU25686*. Smaller physical distance associated with a change in altitude suggests that strong cool temperature-related natural selection is at work on these traits, counter-acting any homogenising effects of gene flow.

CHAPTER 5

Latitude and altitude associations of a molecular marker in the Triosephosphate isomerase gene (*Tpi*) in *D. melanogaster*

5.1 Introduction

As discussed in earlier chapters, variation on the right arm of chromosome three has been associated with traits that vary clinally with latitude and that are likely to be important in climatic adaptation. This study was undertaken as the Triosephosphate isomerase gene (*Tpi*) maps to the very end of chromosome 3R at cytological position 99E (Shaw-Lee *et al* 1991), close to the *hsr-omega* gene and close to the 3` breakpoint of *In(3R)P*. *Tpi* may prove useful in studies to elucidate the genetic nature of climatic adaptation in *Drosophila*, both as a mapping tool and in understanding of metabolic adaptation along a climatic gradient. Triosephosphate isomerase (TPI) is an enzyme that is part of the glycolytic pathway of carbohydrate catabolism (Shaw-Lee *et al* 1991) and it catalyses the interconversion of glyceraldehyde-3-phosphate and dihydroxyacetone (Hasson *et al* 1998). In *D. melanogaster* a TPI allozyme polymorphism associates with latitude in Australasia, but not with altitude, and it shows seasonal variation associated with rainfall levels (Oakeshott *et al* 1984; Nielsen *et al* 1985). Many sequence polymorphisms occur in this gene, including an uncharacterised 3 bp TTA indel located within an intron near the 5` slice consensus sequence (Shaw-Lee *et al* 1991; Hasson *et al* 1998). An exploratory study

will be undertaken to assess geographical variation in this indel polymorphism. Both latitudinal and altitudinal variation will be surveyed along the eastern coast of Australia.

5.2 Materials and Methods

Isofemale lines of *D. melanogaster* were established from field caught females collected from sixteen low-altitude collection sites from a latitudinal transect along the eastern coast of Australia using banana-bait traps (Figure 5.1). At the same time, five high-altitude collections were made inland from 5 different low altitude sites (Figure 5.1). The number of isofemale lines and number of flies sampled per site are indicated in Table 5.1.

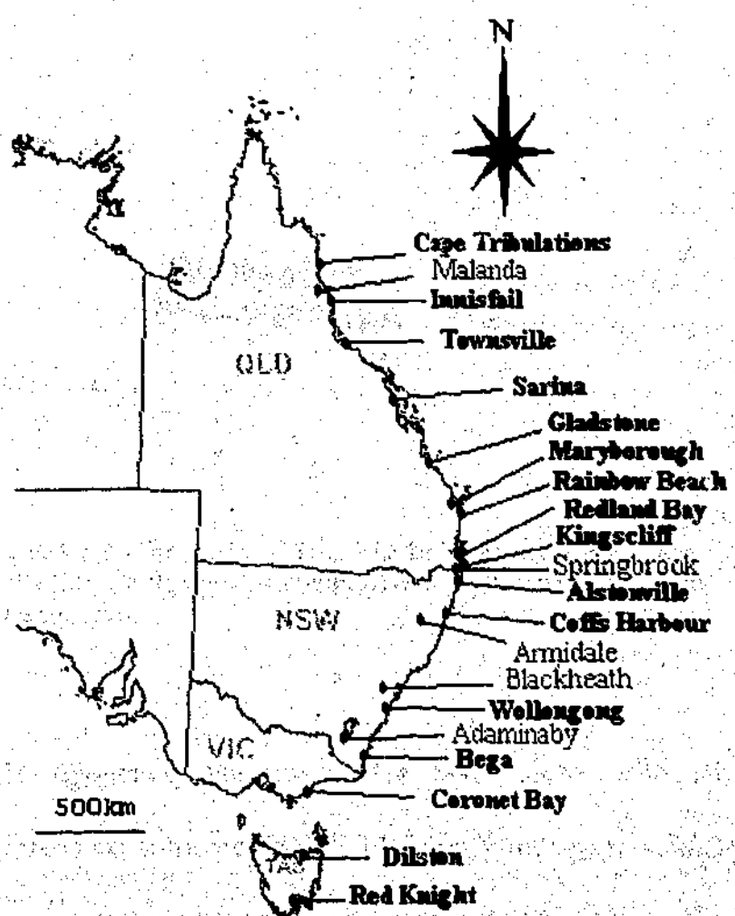


Figure 5.1 Map of collection sites for the latitude and altitude survey (black text = low-altitude; grey text = high-altitude).

DNA was extracted using the proteinase K method (Gloor and Engels 1992) and used as a template for PCR amplification of the *Tpi* gene polymorphism. Primer pairs 5` GGA CTG GAA GAA CGT GGT GG 3` and 5` GAA GCG TGG ACC TCT TGA GC 3` flanking the indel polymorphism were designed from sequence given in Hasson *et al* (1998). These primers together with an IRDye labelled primer (5' GGA CTG GAA GAA CGT GGT GG 3'), were used to amplify fragments of 152 bp (TTA insert absent: Tpi^D) or 155 bp (TTA insert present: Tpi^I). The PCR cycling conditions were as follows: initial denaturing at 95 °C for 2 min; 35 cycles of 95°C for 1 min, 57°C for 1 min, 72°C for 1 min; 72°C for 2 min. The product was run on Li-Cor Global IR^2 with Seqagel at 1500v for 1h 45 min (Figure 5.2). Genotyping was aided by Li-Cor $SAGA^{GT}$ software (SAGA Generation 2 version 3.0.0.).

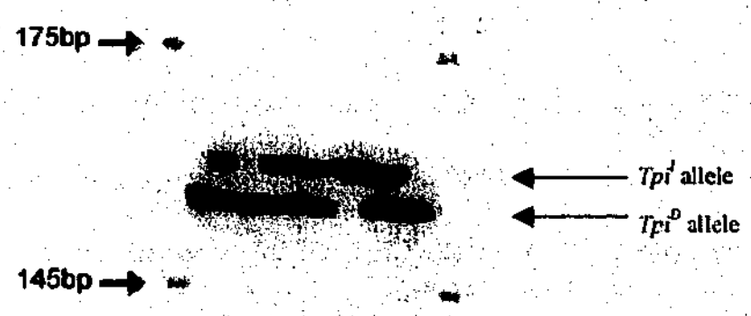


Figure 5.2 Li-Cor image of *Tpi*. The first and last lanes contain the 50-350 size standard. All other lanes represent the alleles from isofemale lines.

Tpi^D allele frequency was tested for associations with latitude by Pearson correlation using standard software (SPSS 11.5 for Windows). Climatic data (based on averages typically over 100 years) were acquired for the nearest meteorological station for each latitudinal collection site from the Australian Bureau of Meteorology website (www.bom.gov.au). For latitudinal associations six climatic variables were chosen:

average daily maximum temperature for the hottest month (Tmax), average daily maximum temperature for the coldest month (Tmin), average annual number of days above 30°C (T>30), average annual number of days below 2°C (T<2), mean monthly rainfall for the wettest month (Rmax), mean monthly rainfall for the driest month (Rmin). These climatic variables were initially tested for associations with latitude and *Tpi*^{D} allele frequency by Pearson correlation. Partial correlations were performed to look for associations between allele frequency and climatic variables while controlling for latitude. The five paired high/low altitudinal sites from 5 latitudes were assessed for altitudinal association using a three-way χ^2 analysis of allele numbers (Cochrans-Mantel-Haensel and Breslow-Day test (Sokal and Rolf 1995)).

5.3 Results

The frequency of the *Tpi*^{D} allele was calculated for each site (Table 5.1) and low altitude populations were investigated for latitudinal associations. Data from the 16 low altitude sites indicated that the frequency of the *Tpi*^{D} allele was positively correlated with latitude (r= 0.67, df 14 P= 0.004). The *Tpi*^{D} allele frequency was higher in more temperate southern locations (Figure 5.3a). Three of the six climatic variables, Tmin, T>30 and T<2, that significantly associated with the *Tpi*^{D} allele frequency among these populations (r= -0.66 P<0.01, r= -0.52 P<0.05 and r= 0.50 P<0.05 respectively) also associated with latitude (r= -0.79 P<0.01, r= -0.80 P<0.01 and r= 0.62 P<0.05 respectively). As latitude and climatic variables were associated with *Tpi*^{D} allele frequency variation, partial correlations controlling for latitude were performed to determine the effect of the climatic variables. Partial correlation analysis between *Tpi*^{D} and these climatic variables (while controlling for latitude) did not result in any significant correlations (Table 5.2).

Table 5.1 Collection sites and frequency of *Tpi*^{D} allele along the eastern coast of Australia.

Population	Latitude	Altitude (m)	n(l)	Allele Frequency*
Cape Tribulation	16°00'	<100	64(64)	0.555
Malanda	17°19'	800	69(69)	0.674
Innisfail ~	17°30'	<100	86(74)	0.657
Townsville	19°09'	<100	17(5)	0.647
Sarina	21°15'	<100	48(48)	0.615
Gladstone	23°50'	<100	43(43)	0.674
Maryborough	25°31'	<100	58(58)	0.741
Rainbow Beach	25°54'	<100	60(60)	0.708
Redland Bay	27°39'	<100	70(70)	0.686
Springbrook	28°14'	1031	34(17)	0.721
Kingscliff ~	28°17'	<100	106(51)	0.703
Alstonville	28°50'	<100	30(30)	0.717
Coffs Harbour ~	30°22'	<100	126(126)	0.635
Armidale	30°31'	1296	67(67)	0.761
Blackheath	33°38'	1046	48(48)	0.740
Wollongong ~	34°25'	<100	74(74)	0.736
Adaminaby	35°59'	1025	51(51)	0.912
Bega ~	36°41'	<100	110(110)	0.864
Coronet Bay	38°15'	<100	16(16)	0.750
Dilston/ Tamar ridge	41°00'	<100	19(5)	0.632
Red Knight	43°00'	<100	18(5)	0.889

n= number individuals scored, l= number isofemale lines, * Tpi^D allele frequency
~ coastal low altitude sites used in comparison with high altitude

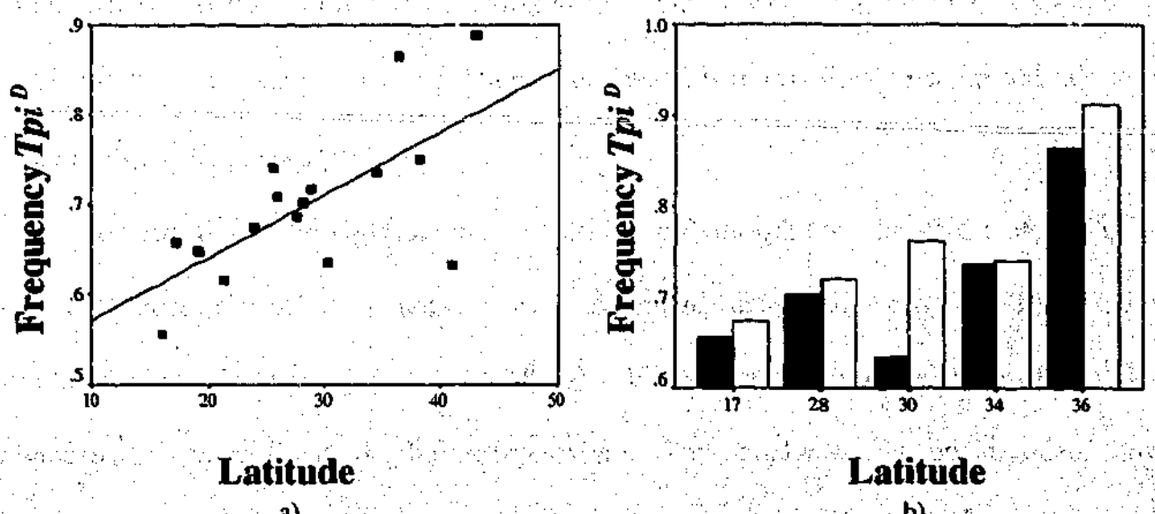


Figure 5.3 Geographical variation in the *Tpi*^{D} allele frequency. a) significant latitudinal cline (r= 0.67, df 14 P= 0.004), b) significant altitudinal differentiation between low and high altitude (solid bar and open bar respectively) at five different latitude (χ^2_{CMH}= 4.31 df 1 P<0.05).

Table 5.2 Correlation between climatic variables and *Tpi*. Partial correlations with latitude are also given.

	Correlation (r)		
Climatic Variable	**Latitude**	***Tpi*^{D} allele**	**Partial correlation~**
Tmax	-0.92**	-0.48	0.48
Tmin	-0.79**	-0.66**	-0.29
T>30	-0.80**	-0.53*	0.03
T<2	0.62*	0.50*	0.14
Rmax	-0.42	-0.52*	-0.35
Rmin	0.19	0.05	-0.25

*P<0.05 ** P<0.01

~ between *Tpi*^{D} and climatic variables that controls for latitude

(Tmax) = average daily maximum temperature for the hottest month

(Tmin) = average daily maximum temperature for the coldest month

(T>30) = average annual number of days above 30°C

(T<2) = average annual number of days below 2°C

(Rmax) = mean monthly rainfall for the wettest month

(Rmin) = mean monthly rainfall for the driest month

This *Tpi* polymorphism also showed significant altitudinal differentiation across 5 pair latitudinal sites. The frequency of the *Tpi*^{D} allele was significantly higher at high altitude across all five latitudes as illustrated in Figure 5.3b (χ^2_{CMH}= 4.31 df 1 p= 0.038, estimated common odds ratio= 0.77) and there was no interaction between altitude and latitude (χ^2_{BD}= 3.96 df 4 P>0.05).

To investigate the significance of this *Tpi* polymorphism, the effect of the TTA indel on secondary RNA structure, was examined using the program RNA fold (http://rna.tbi.univie.ac.at/cgi-bn/RNAfold.cgi). Fifty nucleotides either side of the *Tpi* polymorphism were used in the comparison. This analysis did not indicate any conspicuous effect of this indel polymorphism on secondary RNA structure.

5.4 Discussion

Generally molecular markers evolving at a selectively neutral rate do not show meaningful geographical variation such as clines (Gockel *et al* 2001). This *Tpi* indel polymorphism was expected to be selectively neutral since it occurs in the intron of the gene (Hasson *et al* 1998) and might not be expected to affect gene expression levels or enzyme efficiency. Data presented here clearly indicate that variation in the frequency of the 3 bp indel polymorphism of the *Tpi* gene, is associated with latitude and altitude on the eastern coast of Australia. These parallel results strongly suggest that natural selection is involved in affecting the population frequency of Tpi^D, however, the selective agent remains elusive. Obvious physical factors that might underlie these associations are temperature and rainfall levels as they vary along climatic gradients. The directions of the geographical associations are consistent with temperature being an important factor as for both latitude and altitude, Tpi^D is associated positively with cooler climates. However, Tpi^D frequency did not associate robustly with any of the climatic variables investigated and this analysis brings us no closer to identifying which climatic variables, if any, might be the more important factor.

Nonetheless, natural selection has been implicated as the cause of the geographical associations with Tpi^D. These associations may, however, be the result of linkage disequilibrium with some other segregating variation that is responding to natural selection. This indel marker is positioned close to the intron/exon boundary and occurs 32 bp downstream from the well-studied amino acid replacement polymorphism, which also associates with latitude (Oakeshott *et al* 1984; Hasson *et al* 1998). However, these two sites may not be in strong linkage disequilibrium as the allozyme polymorphism has not previously shown significant altitude differentiation, as was observed here for the indel polymorphism (Oakeshott *et al* 1984). Furthermore, *Tpi* is closely linked to the ACPH

allozyme polymorphism (genetic map position 3-101.1 and 3-101.3 respectively) that also shows latitudinal variation along the eastern coast of Australia (Oakeshott *et al* 1983). These linkage associations need to be assessed.

The *Tpi* gene maps just outside, although close to, the proximal breakpoint region of *In(3R)P* (breakpoint cytological position 96A18..19) and this inversion also clines along a latitudinal gradient (Knibb 1982). Molecular variations around the breakpoints of inversions are often in linkage disequilibrium with inversions (Hasson and Eanes 1996; Andolfatto *et al* 2001; Rodriguez-Trelles 2003). However, the *Tpi* marker is likely to be independent of *In(3R)P*, as strong linkage disequilibrium was not detected between these two polymorphisms at a single population (D`= 0.22 Chapter 3).

Although Tpi^D may be 'hitchhiking' with a block of genes that is responding to selection, it is possible that it is itself the subject of selection since intron polymorphisms that effect gene expression and that are not neutral, are known (for example Stam and Laurie 1996).

Traits that are associated with both latitude and altitude in *D. melanogaster* include body size, cold tolerance and heat resistance (Louis *et al* 1982; James *et al* 1995a; Hoffmann *et al* 2002; Chapter 4). Several genes involved with thermal tolerance have been mapped to chromosome three, while variation in body size has been predominantly attributed to genes on chromosome 3R (Tucic 1979; Gockel *et al* 2002; Anderson *et al* 2003). This right arm chromosome three *Tpi* genetic marker may therefore prove useful in future studies to map and isolate genes that contribute to adaptive variation in both thermal tolerance and body size. Initial observation suggest no association between *Tpi* variation and body size variation (Chapter 3 Table 3.4), however, this relationship needs to be more extensively investigated.

5.5 Summary

This exploratory study on a recently identified *Tpi* TTA indel polymorphism in *D. melanogaster* revealed a latitudinal cline and altitudinal differentiation in the frequency of Tpi^D. Geographical variation in latitude and altitude were similar in direction, since in both cases Tpi^D associated positively with cooler environments. Natural selection is likely to be involved and environmental temperature, acting either directly or indirectly, remains as a probable selective agent. Further geographical sampling is needed to see if these *Tpi* patterns occur on other continents. Additionally, detailed linkage disequilibrium studies are required, together with trait association studies, to uncover the selective si_nificance of the *Tpi* indel variation.

CHAPTER 6

Preliminary steps to characterise the *hsr-omega* gene in *D. serrata* and *D. simulans*

6.1 Introduction

There is evidence that natural variation in *hsr-omega* of *D. melanogaster* plays a role in heat tolerance variation (McKechnie *et al* 1998) and possibly cold tolerance variation (Anderson *et al* 2003; Chapter 3). To investigate the possibility that *hsr-omega* is important in other species we need to look closely at variation in this gene in related species for which relevant ecological and thermal tolerance information is available. Two excellent species for such a study are *D. simulans* and *D. serrata* in the *melanogaster* species group.

D. simulans (Sturtevant 1919) and its sibling species *D. melanogaster* (Meigen 1830) of the melanogaster species subgroup, recently evolved from a common ancestor between 2 and 3 million years ago (Cariou 1987). Both species are well adapted to climatic variability as they have cosmopolitan distributions, exhibit similar clinal patterns for many phenotypic traits and similar thermotolerance ranges (Tantawy and Mallah 1961; Stanley *et al* 1980; Capy *et al* 1993). At the genetic level these two species show quite high levels of nucleotide sequence homology, averaging 92-97%, regardless of the gene or sequence of interest (Beverley and Wilson 1984; Caccone *et al* 1988; Hey and Kliman 1993; Jeffs *et al* 1994). *D. simulans* exhibits chromosome puffing activity at the same

cytological locations as *D. melanogaster* in response to temperature stresses, including *hsr-omega* puffing on the right arm of chromosome three at position 93D (Ashburner 1970). In situ hybridisation studies suggest that *hsr-omega* sequence homology is likely to be high between the sibling species (Peters *et al* 1980; Pardue 1990).

In contrast, *D. serrata* (Malloch 1927) of the montium species subgroup, is restricted in distribution being endemic to Australia and Papua New Guinea (Mather 1955; Dobzhansky and Mather 1961; Ayala 1965; Bock 1972; Jenkins and Hoffmann 2001). This species has a reduced tolerance of environmental variability. For example, *D. serrata* is less resistant to cold and desiccation than both *D. melanogaster* and *D. simulans* (Hoffmann *et al* 1991a; Jenkins *et al* 1999). It would be of interest to identify the genetic differences underlying these differences in stress tolerance, as they are likely to be important in defining species distribution. The montium species subgroup and the melanogaster species subgroup diverged from a common ancestor between 18 and 30 million years ago (Powell and DeSalle 1995). In *D. serrata hsr-omega*-equivalent puffing activity is found on chromosome 2L, at cytological location 35 (Drosopoulou *et al* 1996). In situ hybridisation studies suggest that the *hsr-omega* sequence in *D. serrata* is likely to be divergent from that of *D. melanogaster* (Drosopoulou *et al* 1996).

This chapter will report the approach and results from an effort to obtain the *hsr-omega* sequence from *D. simulans* and *D. serrata*. These data will enable future assessment of polymorphic *hsr-omega* variation, to determine the role of this gene in thermal tolerance and for comparative functional analysis in these and other closely related *Drosophila* species. Natural population variation in *D. simulans hsr-omega* repeat-length will also be investigated and compared to that now known for *D. melanogaster*.

6.2 Materials and Methods

Sequencing *hsr-omega*

To obtain unknown *hsr-omega* sequence from *D. simulans* and *D. serrata*, PCR on genomic DNA from these species was conducted. Primers were designed in regions where sequence conservation exists between *D. melanogaster*, *D. pseudoobscura* and *D. hydei*, the only species for which enough *hsr-omega* DNA sequence is available (Peters *et al* 1984; Hovemann *et al* 1986; Ryseck *et al* 1987; Garbe *et al* 1989). Sequence is only weakly conserved between these three evolutionary diverged *Drosophila* species, however, short stretches of sequence conservation are scattered throughout the gene (Garbe *et al* 1986a; Garbe *et al* 1989). PCR was used to span regions of unknown sequences using different PCR primer pairs designed in regions of sequence conservation (detailed in Appendix E). *Hsr-omega* repeat monomers were sequenced from *D. simulans* genomic DNA using primers designed from the *D. melanogaster* repeat monomer sequence published in Hogan *et al* (1995).

All PCR reactions were carried out in 50 µl reactions using a proof reading enzyme, Expand High Fidelity (Roche Diagnostics), and the products were purified using Wizard SV PCR Preps DNA Purification System (Promega). Each PCR product was ligated into a pGEM-T vector using manufacturer instructions (Promega). Competent cells (Promega JM109) were transformed with the ligation mix by heat shock and spread onto LB plates containing ampicillin, IPTG and X-gal. Single white colonies were cultured and plasmids were purifed using Wizard Plus Minipreps DNA purification System (Promega). The purified plasmid was checked for the presence of a DNA insert by restriction enzyme digestion with *Sac II* and *Pst I* (Promega) and this digest was run on an agarose gel. Those plasmids containing the insert were prepared for sequencing by

labelling with Terminator Ready Reaction Mix (using standard protocols) under the following cycling conditions; 96°C (30 sec), 50°C (15 sec), 60°C (4 min) for 25 cycles. The labelled plasmid was precipitated in 3M sodium acetate and 95% ethanol before it was spun down to a pellet. The pellet was washed in 70% ethanol and dried for sequencing. All sequencing was carried out on the ABI 3730 automated sequencer by Microbiology oligonucleotide synthesis and nucleotide sequencing facility, Monash University.

Repeat-length variation

A small number of natural populations of *D. simulans* were investigated for *hsr-omega* repeat-length variation. In 2001, *D. simulans* was collected from four different latitudes (Table 6.1) down the eastern coast of Australia using banana bait traps for which isofemale lines were established on *Drosophila* medium (Appendix A) and reared at 18°C. These lines were genotyped for tandem repeat-length variation.

Tandem repeat fragments were visualised as described in Chapter 2 with some modifications. Southern blots were hybridised with *D. simulans* repeat unit probes. The purified PCR product used to make the repeat probes consisted of repeat monomers amplified from *D. simulans* DNA, using primers designed from the *D. melanogaster* repeat monomer sequence (primer sequences given in Chapter 2).

Table 6.1 *D. simulans* collection sites and number of isofemale lines scored for *hsr-omega* repeat-length variation.

Location	Latitude	No. isofemale lines
Innisfail	17°30'	2
Kingscliff	28°15'	10
Coffs Harbour	30°19'	10
Red Knight	42°45'	25

Analysis

Sequence data were aligned using DiAlign professional Release 2.7.5. (Genomatix Software GmbH 1998-2004, http://www.genomatix.de/cgi-bin/dialign). The level of *hsr-omega* sequence homology was determined using Alion: pairwise alignment (Nevill-Manning *et al* 1997 http://motif.stanford.edu/alion/).

6.3 Results

A proportion of the *hsr-omega* gene was sequenced from both *D. simulans* and *D. serrata*. *D. simulans* was sequenced from the TATA box to the repeat region (2931 bp), which included both exons, the intron and four repeat monomers (Figure 6.1b). In *D. serrata*, sequence information was obtained from the 5` intron splice junction to the first polyadenylation site (1524 bp), which included the intron and exon II (Figure 6.1c). The sequence data for both species can be found in Appendix F and G.

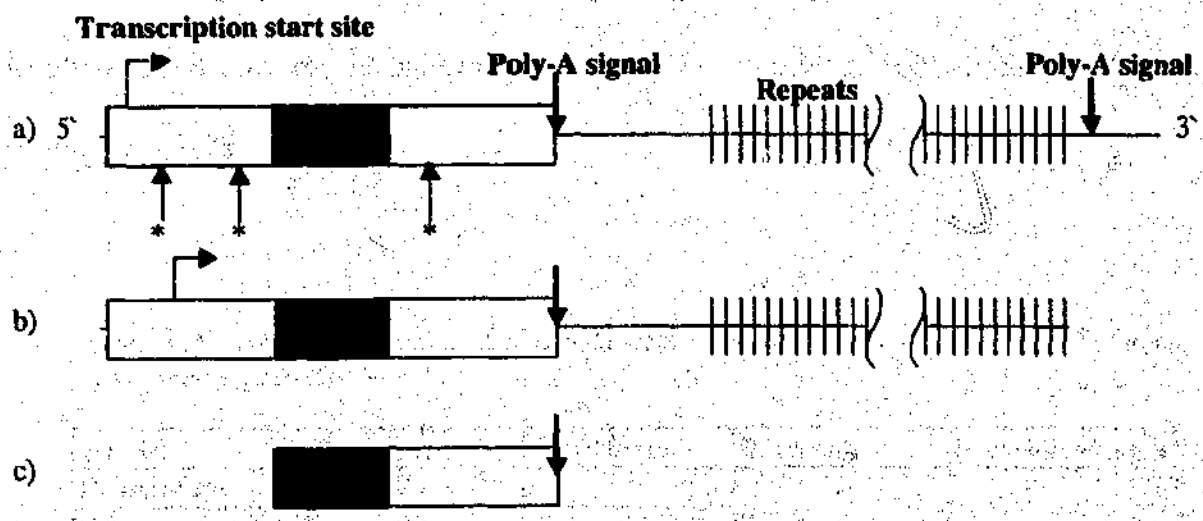


Figure 6.1 a) Schematic diagram of the *hsr-omega* gene in *Drosophila* species. The bent arrow indicates the transcription start site. The black box indicates the intron and open boxes the exons. The first and second poly-a signals are required for termination of *omega-c* and *omega-n* respectively. The repeats at the 3` end are truncated. New regions of sequence homology identified in this study across *Drosophila* species are indicated (*). b) & c) Indicate the regions of *hsr-omega* sequence obtained in *D. simulans* and *D. serrata*, respectively (note that only 4 repeat monomer clones were obtained in *D. simulans*).

Non-repeat 5` portion of the gene

Hsr-omega sequence data for four other *Drosophila* species (*D. melanogaster*, *D. triauraria*, *D. pseudoobscura* and *D. hydei*) were obtained from Genbank and aligned with *D. simulans* and *D. serrata* sequence (Appendix F). This comparison highlighted three new short stretches of conserved sequence across all species (Figure 6.1a and Appendix F). The longest of these conserved sequences was twenty-nucleotides in length, positioned at 224 bp downstream from the 3` intron splice junction in exon II of *D. melanogaster*. A standard NCBI BLAST search was conducted to see if this sequence exists elsewhere in the *D. melanogaster* genome, however, this failed to retrieve a match. The second small conserved stretch was a 13-nucleotide motif positioned 149 bp (in *D. melanogaster*) downstream from the TATA box, the third was a 12-nucleotide motif positioned 377 bp (in *D. melanogaster*) downstream from the TATA box.

The level of *hsr-omega* sequence homology among five *Drosophila* species is indicated in Table 6.2. This matrix indicates that interspecfic *hsr-omega* nucleotide sequence divergence is high, except for sequence comparisons between the two most closely related species, *D. melanogaster* and *D. simulans*, for which average sequence homology is 95%.

Table 6.2 Average nucleotide *hsr-omega* sequence homology (%) between different *Drosophila* species.

Species	*D. melanogaster*	*D. simulans*	*D. serrata*	*D. pseudoobscura*
D. simulans	95			
D. serrata	59	59		
D. pseudoobscura	58	57	51	
D. hydei	45	46	42	46

Repeat portion of the gene

Hybridisation of Southern blots detected different levels of homology for *D. simulans* and *D. serrata hsr-omega* repeat sequence, using the *D. melanogaster* repeats probe. While there was about a 50% reduction in hybridisation signal for *D. simulans*, no signal was observed in *D. serrata*. The lack of *D. melanogaster* repeat probe hybridisation with *D. serrata* repeat DNA indicates a high level of repeat monomer sequence divergence between these species.

Using *D. melanogaster* repeat monomer primers to amplify from genomic *D. simulans* DNA, four repeat monomer clones were obtained from *D. simulans* and these were all sequenced (Appendix G). *D. simulans* repeat monomer alignments showed that repeat sequence homology ranged from 81 to 96% with an average of 88%. A consensus sequence was determined from the four *D. simulans* repeat monomer sequences and is given in Appendix G. Repeat monomer sequence alignments between *D. simulans* and *D. melanogaster* (GenBank Acc. No. M14556, M14578, M14579, M14580, M14581, M14582 and M14583), showed that interspecific repeat monomer sequence homology was relatively high among the sibling species, which averaged ~84% and ranged from 81 to 87%. The nine-nucleotide motif (nanomer) that is conserved across published *D. melanogaster* and *D. hydei* repeat monomer sequence (Garbe *et al* 1986a), was also evident in *D. simulans* (Appendix G). As with *D. melanogaster*, the nanomer was present twice per repeat in *D. simulans*. A single base pair substitution was observed for a nine-nucleotide motif in one *D. simulans* repeat monomer sequence, however, substitutions are also present in the motifs of *D. melanogaster* and *D. hydei* (Garbe *et al* 1986a).

PCR on *D. serrata* DNA failed to amplify a repeat monomer using *D. melanogaster* repeat monomer primers, even under conditions of reduced specificity.

Repeat-length variation in *D. simulans*

Using the repeat monomer sequence obtained for *D. simulans*, repeat-length variation was investigated in natural populations of this species. *D. simulans* was polymorphic for repeat-length and 8 bands (repeat-length alleles) were resolved (on long gels) that ranged in size from ~9 to ~19 kb of repeating monomers (Figure 6.2a). In contrast, *D. melanogaster* across a similar geographical range resolved a larger number of bands, ranging from ~6 to ~19 kb of repeat monomers (Figure 6.2b). Additionally, there was no indication of a clinal pattern in *D. simulans* repeat-length over a latitudinal gradient. Altitudinal variation in *D. simulans* was also briefly investigated in four mass bred populations from different altitudes in Argentina, however, data was uninformative and only 2 bands were resolved at ~11 and ~15 kb.

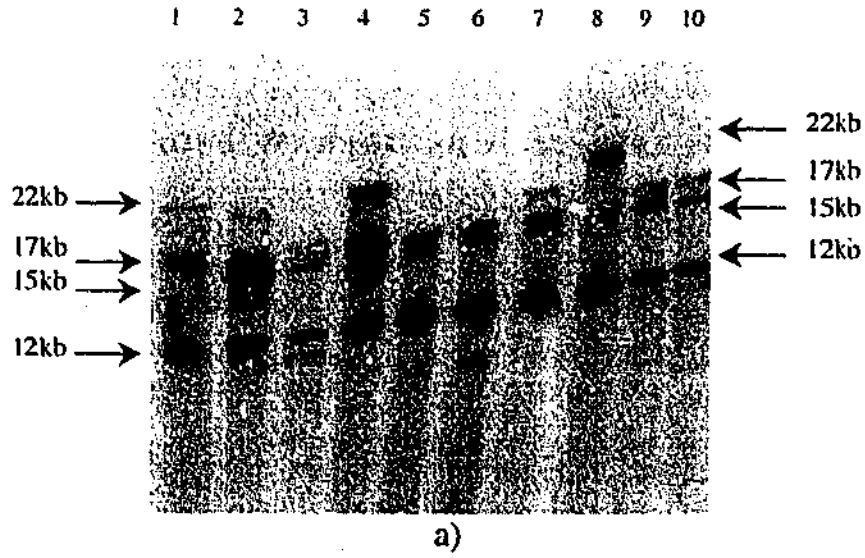


Figure 6.2 Autoradiographs of Southern blots for *hsr-omega* repeat-length polymorphism in *a*) *D. simulans* and b) *D. melanogaster*. Each lane represents the repeat-length variation from 5 pooled isofemale lines (except *D. simulans* lane 1, which contains genomic DNA from only 2 isofemale lines). Samples are organised in order of increasing latitude (left to right). *D. simulans* populations a) lane 1 Innisfail; lanes 2 & 3 Kingscliff; lanes 4 & 5 Coffs Harbour; lanes 6 to 10 Red Knight. *D. melanogaster* populations b) lane 1 to 10 are from equivalent latitudes as in a). Repeat size is indicated (includes ~2.5 kb flanking region).

6.4 Discussion

Partial sequence of the *hsr-omega* gene was obtained for *D. simulans* and *D. serrata*, close relatives of *D. melanogaster*. However, sequence was not attained for the entire *hsr-omega* gene in both species. Still needed is sequence 5` of the intron and 3` of the first polyadenylation site including the repeats in *D. serrata*. Needed for *D. simulans* is sequence 5` of the TATA box and 3` of the last repeat. However, the now available data will provide valuable foundations for ongoing efforts in our laboratory to characterise genetic variation in *hsr-omega* in natural populations of these species.

The *hsr-omega* sequence alignment revealed three additional small regions of conservation, not previously identified. These conserved regions were not within or immediately adjacent to any of the significant features of this gene to indicate possible functional significance. However, these regions may prove useful in future studies of this gene.

The level of interspecific *hsr-omega* sequence homology among the five *Drosophila* species was reflective of the time since evolutionary divergence of the species (Beverley and Wilson 1984; Cariou 1987; Powell and DeSalle 1995). The sibling species, *D. simulans* and *D. melanogaster*, showed low sequence divergence (5%) and this level was consistent with previous estimates by Blackman and Meselson (1986) in non-coding sequences other than *hsr-omega*. However, species outside the *melanogaster* species group including *D. serrata* show high levels of *hsr-omega* sequence divergence to this species group. These sequence results parallel previous in situ hybridisation studies, that found outside the sibling species relationship the *hsr-omega* gene sharply diverges (Garbe *et al* 1989; Drosopoulou *et al* 1996).

Within *D. melanogaster*, *hsr-omega* repeat-length is highly polymorphic and the latitudinal cline in repeat-length (Chapter 2) suggests that environmental selection may be

involved. However, tandem repeat-length variation was less polymorphic in *D. simulans* and the data suggest the absence of a latitudinal cline. Previous studies indicate that *D. simulans* is generally a less variable species compared to *D. melanogaster*. For example, *D. simulans* is less polymorphic for a variety of enzymes (O'Brien and MacIntyre 1969; Berger 1970; Begun and Aquadro 1994), genes (Choudhary and Singh 1987) and chromosome inversions (Ashburner and Lemeunier 1976; Aziz and Mbarak 1987). At the phenotype level, *D. simulans* is also less tolerant to desiccation, alcohol and thermal extremes, and geographical clines for many traits are less obvious (Tantawy and Mallah 1961; Parsons 1975; Stanley *et al* 1980; Davidson 1990; Capy *et al* 1993; Morin *et al* 1999; Kojima and Kimura 2003). The decreased level of repeat-length variation in *D. simulans* may be related to the reduced level of environmental adaptability in this species, however, this is yet to be investigated thoroughly.

6.5 Summary

Hsr-omega sequence variation has proven to be involved with thermotolerance in *D. melanogaster*. However, it is not known if this feature is widely conserved. Most of the *hsr-omega* sequence is now available for *D. simulans* and *D. serrata* to begin investigating the importance of this gene in these species. Interestingly, initial observations of repeat-length variation did not detect a parallel latitudinal cline in *D. simulans* compared to *D. melanogaster* (Chapter 2). These sequences will provide a strong foundation for future investigations of the role of *hsr-omega* polymorphisms in thermal tolerance variation especially in the *montium* species subgroup, species that are endemic to Australasia, such as *D. serrata* and *D. birchii*.

CHAPTER 7

Conclusion

7.1 Overview

In this chapter I bring together the major findings and address some of the important implications of this research, including a consideration of directions for future studies. This thesis makes a significant contribution to our understanding of climatic adaptation in *D. melanogaster* through four major observations;

- Robust *hsr-omega* repeat-length latitudinal associations
- Significant linkage associations among a set of genetic markers that vary climatically including the cosmopolitan inversion, *In(3R)P*
- Significant altitudinal associations of several clinal traits and a genetic marker
- *Tpi* latitudinal and altitudinal associations

7.2 The hsr-omega gene repeat-length polymorphism

One of the major objectives of this study was to provide some preliminary insight into the hyper-variable *hsr-omega* repeat-length polymorphism in natural populations. A latitudinal cline in repeat-length variation was observed over three consecutive years which suggests that natural selection was acting on the repeat-length polymorphism along the eastern coast of Australia (Chapter 2). While natural selection on *hsr-omega* repeat-length latitudinal variation in *D. melanogaster* seems likely, further evidence for selection

is needed. Repeat-length variation needs to be investigated on independent latitudinal gradients to see if this cline is geographically widespread over different continents. It may also be valuable to investigate seasonal variation in repeat-length, as an association would further support the action of natural selection.

The observed significant association between the *hsr-omega* repeat-length polymorphism and cold tolerance variation in the family line study (Chapter 3) is consistent with the direction of the clinal data for this marker and trait over a latitudinal gradient. Although this association between cold tolerance and repeat-length needs extensive further investigation the data offers a first clue as to a trait that repeat-length variation might be directly or indirectly effecting at the phenotype level. However, while selection pressures at altitude extremes did generate differentiation in cold tolerance, no altitudinal effect was observed for repeat-length variation in *D. melanogaster* (Chapter 4). Repeat-length clinal variation is unlikely to be upheld by a single climatic factor but rather a combination of complex environmental parameters. More research is needed to identify other significant environmental parameters that might be selecting for repeat-length variation, and to find other traits that this variation may be influencing.

The *hsr-omega* sequence data obtained here, for *D. simulans* and *D. serrata* (Chapter 6), will provide strong foundations for future investigations of the role of *hsr-omega* polymorphisms in thermotolerance variation in these species and close relatives. Preliminary data indicated that *D. simulans* was less polymorphic for repeat-length and that latitudinal variation was not obvious in this species in contrast to *D. melanogaster* (Chapter 6). Further comparative studies between these two closely related sibling species may help reveal more about the importance of repeat-length variation.

To determine the effects of repeat-length variation on phenotype, future experiments should involve creating transgenic strains that differ in repeat-length only, so

that effects of repeat-length can be unambiguously identified. Transgenic lines of differing repeat-length assessed for thermal tolerance and a variety of life history traits may help elucidate the functional and adaptive significance of repeat-length variation.

7.3 Linkage disequilibrium

Hsr-omega repeat-length may be hitchhiking with linked gene(s) under selection, however, repeat length variation did not strongly associate with any of the clinally varying polymorphic markers investigated including *In(3R)P*. While the main focus of the linkage disequilibrium study was to assess linkage between repeat-length and other candidate markers, we did observe other markers in significant linkage disequilibrium with each other at this single Coffs Harbour population. *Hsr-omega*^{LS} and *DMU25686* showed significant linkage disequilibrium with each other and with the inversion, *In(3R)P*, in which they are situated. These single population associations may have arisen from natural selection or neutral forces and so linkage associations need to be assessed in other populations to see if these allele combinations occur together regardless of the population they are drawn from. Only then can we speculate on the role of natural selection in maintaining these associations. Furthermore, *hsr-omega* repeat-length linkage associations need to be assessed at other populations to be confident that clinal variation in repeat-length is largely independent of the ten clinal genetic markers investigated here.

7.4 Altitudinal variation

Altitude is generally thought to parallel latitude and result in similar adaptations at high latitudes and high altitudes. Although results were confounded by latitude, altitudinal

trends were demonstrated for cold tolerance, non-hardened heat resistance, non-mated ovariole number, development time and two molecular marker, *DMU25686* and *Tpi*. Environmental variables correlated with altitude and latitude are likely to be important in shaping this variation, for which temperature is a strong candidate selective agent. However, altitudinal effects were not detected for two traits and five markers for which previous latitudinal variation has been observed. Selection pressures that shape latitudinal variation in these traits and markers may have been too weak between 1000m of altitude to select for genetic differentiation.

An interesting effect of female reproductive status on ovariole number was observed. Significant altitudinal differentiation was observed for ovariole number in non-mated females but not mated females. This contrast between mated and non-mated ovariole number in natural populations has not previously been reported. Follow up work is needed to better understand the observed difference in trend, which should begin by replicating the experiment to see if these patterns persist.

The altitudinal and latitudinal variation in the polymorphic *Tpi* molecular marker observed here, strongly suggested the action of natural selection and future experiments might look to see if these patterns are paralleled on other continents. Although selection does seem probable it may not be acting directly on this polymorphism. Further work is needed to investigate *Tpi* linkage associations with other candidate clinal polymorphisms (such as the cytologically nearby TPI and ACPH allozyme polymorphisms) as the TTA indel might mark a selected block of adaptively significance genes. The *Tpi* TTA indel is an attractive marker in future studies to map and isolate genes that contribute to adaptive variation in traits such as body size, thermal tolerance and metabolism.

This thesis has revealed patterns of geographical variation that will contribute to a better understanding of climatic adaptation in nature and has opened up new avenues of research. Most importantly, this study has advanced our understanding of *hsr-omega* repeat-length variation in natural populations and the importance of linkage in the structure of this variation. This work has also provided preliminary insights into a phenotypic trait that may be influenced by *hsr-omega* repeat-length variation.

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APPENDIX A

Drosophila medium

Potato mash 1.97% w/v
Sugar 2.96% w/v
Agar 0.63% w/v
Yeast 3.14% w/v
Water 89.77% w/v
Nipagin (10%) 1.08% w/v
Propionic acid 0.45% w/v

APPENDIX B

Raw hsr-omega repeat allele group frequencies

	Average allele size														
	A			B			C			D			E		
Location	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Cooktown		0.000			0.486			0.514			0.000			0.000	
Cape Tribulations		0.000	0.000		0.452	0.323		0.463	0.525		0.085	0.152		0.000	0.000
Mossman	0.000	0.000		0.311	0.673		0.487	0.327		0.202	0.000		0.000	0.000	
Innisfail	0.000	0.017	0.000	0.397	0.641	0.574	0.533	0.341	0.426	0.070	0.000	0.000	0.000	0.000	0.000
Townsville		0.000	0.000		0.420	0.367		0.190	0.171		0.391	0.462		0.000	0.000
Bowen	0.000			0.367			0.185			0.448			0.000		
Sarina	0.031	0.000	0.000	0.391	0.278	0.385	0.197	0.150	0.309	0.382	0.226	0.306	0.000	0.347	0.000
Rockhampton	0.000	0.000	0.000	0.609	0.310	0.584	0.246	0.627	0.173	0.000	0.063	0.242	0.145	0.000	0.000
Gladstone			0.000			0.255			0.242			0.503			0.000
Miriam Vale	0.000		0.000	0.187		0.580	0.419		0.161	0.244		0.259	0.149		0.000
Maryborough	0.000		0.000	0.254		0.107	0.249		0.109	0.497		0.785	0.000		0.000
Rainbow Beach	0.000		0.000	0.377		0.319	0.254		0.346	0.369		0.335	0.000		0.000
Redland Bay	0.000		0.000	0.295		0.000	0.475		0.713	0.230		0.287	0.000		0.000
Kingscliff		0.026	0.000		0.479	0.409		0.219	0.464		0.276	0.126		0.000	0.000
Duranbah	0.000			0.375			0.503			0.122			0.000		
Alstonville	0.000		0.000	0.198		0.245	0.133		0.529	0.194		0.163	0.474		0.064
Coffs Harbour	0.000	0.000	0.000	0.324	0.360	0.126	0.254	0.247	0.773	0.422	0.272	0.101	0.000	0.121	0.000
Port Macquarie			0.000			0.341			0.461			0.198			0.000
Belmont			0.000			0.125			0.524			0.174			0.177
Gosford	0.000			0.112			0.451			0.437			0.000		
Thirlmere															
Wollongong			0.000			0.083			0.374			0.542			0.000
Cobargo		0.063			0.259			0.678			0.000			0.000	
Bega	0.000	0.000	0.000	0.222	0.220	0.114	0.430	0.441	0.562	0.289	0.339	0.324	0.059	0.000	0.000
Yan Yean	0.000			0.083			0.333			0.485			0.099		
Wandin	0.000	0.000		0.000	0.036		0.579	0.592		0.421	0.372		0.000	0.000	
Coronet Bay			0.000			0.033			0.494			0.473			0.000
Pomona		0.000			0.217			0.179			0.208			0.396	
Tamar Valley	0.000	0.000		0.078	0.292		0.417	0.355		0.505	0.353		0.000	0.000	
Windara		0.000			0.048			0.400			0.375			0.178	
Dilston			0.000			0.067			0.442			0.491			0.000
Red Knight			0.000			0.238			0.363			0.398			0.000
Huonville	0.000			0.215			0.434			0.351			0.000		
Scenic Road	0.000			0.070			0.379			0.551			0.000		
Franklin		0.013			0.194			0.430			0.362			0.000	
Panorama		0.000			0.091			0.408			0.364			0.136	

1= 2000 2= 2001 3= 2002

APPENDIX C

PCR Cycling Conditions

Hsr-omega$^{L/S}$

95 °C (5min) 1 cycle
94 °C (20 sec) 57 °C* (30 sec) 72 °C (30 sec) 4 cycles
95 °C (20 sec) 49 °C ^ (30 sec) 72 °C (30 sec) 5 cycles
94 °C (20 sec) 44 °C (30 sec) 72 °C (30 sec) 15 cycles
72 °C (1 min) 1 cycle
*decrease annealing temperature 2 °C per cycle
^ decrease annealing temperature 1 °C per cycle

Hsp70

95 °C (2min) 1 cycle
92 °C (1 min) 54°C (1 min) 72 °C (90 sec) 35 cycles
72 °C (5min) 1 cycle
(I note that differences in *hsp 70*$^{S6H8/H22}$ fragment size (1417 and 1278 bp respectively) reported by Bettencourt *et al* (2002) does not equal a 149 bp indel as published)

In(3L)P

94 °C (5min)
94°C (1 min) 64°C *(1 min) 72 °C (90sec) 10 cycles
94°C (1 min) 54°C (1 min)72 °C (90sec) 30 cycles
72 °C (10min)

* decreasing annealing temperature 1°C per cycle

Microsatellites and Clk

95°C (2min) 1 cycle
95°C (1min) #°C(1min) 72°C (1min) 35 cycles
72°C (2min) 1 cycle

Marker	Annealing Temperature # (°C)	Primers	References
Clk	55	5` ACT GAA CTC GGT CGC AGA TGC TT ~ 5` GGA CAG ATT GAG AAA GGC GTC T	(Saleem *et al* 2001) (McKechnie unpublished)
DMU14395	58	5` GGG CAG AGG AAA AGC ACT CA ~ 5` TCG GTG AGA CCG TAA TCT GC	(Gockel *et al* 2001)
DMTRXIII	55	5` TTT ACC TTT TGC GCT TGC TT ~ 5` AGA CAA TCG GCC AAC AAA AC	(Gockel *et al* 2001)
DMU25686	54	5` CGA TAA TTT ACT CTG TGC TCC ~ 5` CAG CTC ACA CAA AAG GCA AA	(Gockel *et al* 2001)
AC008193	54	5` AGG TGG GGT TTG CTT TTT CT ~ 5` CAA ACT CCG GAT CGT GGT A	(Gockel *et al* 2001)

~ primer supplemented with infrared labelled primer (Li-Cor IRDyes)

APPENDIX D

Linkage disequilibrium haplotype table

Line	*hsr-omega*^{TR}	*hsr-omega*^{UK}	*In(3R)P*	*DMU25686*	*DMU1439595*	*DMTRXIII*	*AC008193*	*Clk*	*Tpi*	*In(3L)P*	*hsp70*
1a	5	L	I	5	11	18	13	26	D	S	122
1b	6	S	I	5	19	18	10	31	I		122
2a	4	L	S	8	11	28	14	30	D	S	56H8
3a	4	L	S	18	11	18	3	28	D	S	56H8
4a	6	L	I	13	16	33	14	30	D		122
5a	4	S	S	5	11	18	3	30	D	S	56H8
6a	6	S	S	5		20	12	25	I	I	56H8
6b	5	L	S	5	11	28	13	30	D	S	56H8
5b	5	L	S	13	11	28		30	D	S	56H8
7a	4	L		9	11	19	14	30	D	S	122
8a	4	L	S	13		34	14	27	D	S	56H8
9a	2	L	I		11	28	10	30	D	S	
9b	2	L	S	8	11	28	10	30	D	S	56H8
10a	5	L	S	7	11	35	11	33	D	S	56H8
10b	4	S	I	5		24	10	33	I		56H8
11a	5	L	I		16	25	10	30	D	S	56H8
11b		S			16	25	10	30	D		
12a	9	L	S	13	16	20			I		56H8
13a	4	L	S	9	16	18	14	24	D		56H8
13b	6	S	I	5	16	18	3	30	D	I	56H8
14a	9	L	S	13	16		16	30	I	S	56H8
15a	4	S	I	10	9	19	10		D	I	56H8
15b	5	L	S	13		18	15	30	D	I	122
16a	4	L	S	10	19	21	14	30	D		56H8
16b	4	S	I	5	7	18		30	D	S	122
17a	1	L		10	11	34		30	I		
17b	4	S	I	5	11	18	10	28	I		56H8
18a		L		13		18		30	L		
18b		S		5	11	18	10	30	I		
19a	4	L	S	10	11				I	I	122
20a	10	L	I	13	11	34		30	D		56H8
20b	6	S	I	5	11	19	15	30	I		56H8
21a	8	L	S	13	11	34	15	27	I	S	56H8
21b	4	S	I				3	25	I	I	122
22a	8	L	S	13	11	18	15	25	I	S	122
22b	4	S	I	5	13	20		30	I	I	56H8
23a	8	L	S	11	11	28	15	25	I		
24a		L		10	11	34	12	30	I		
24b		S			11		11	30	I		
25a	4	S	I	5	11	25	12	30	I	I	122
26a	4	L	I	8	11	18	13	33	I	S	56H8

Line	*hsr-omega*^{TR}	*hsr-omega*$^{1/5}$	*In(3R)P*	*DMU25686*	*DMU1439595*	*DMTRXIII*	*AC008193*	*Clk*	*Tpi*	*In(3L)P*	*hsp70*
26b	4	L	S	8	11			33	D	S	56H8
27a	4	S	S	5	11			30	I	S	56H8
27b		L		13	11	18	5	30	D		
28a	6	L	S	8	11		14	24	D	S	56H8
29a		L		13	11	18	14	30	I		
30a		L		9		34	15	26	I		
31a	2	L	S	9	11	18	12	30	I	S	56H8
31b	4	S	I		13	18	3	30	I	I	
32a	4	S	I	5	11		14	30	I		56H8
32b	4	S	I	5	11	21		30	I		56H8
33a	4	S	I	5	11		3	30	I	S	56H8
33b	2	L	S	7	11		10	25	I	I	56H8
34a	4	S	I	5	11	18		30	I		56H8
34b	6	L	S	10		18	11	23	D		56H8
35a	6	L	S	9			14		I		56H8
36a	4	L	S	9	19	35	16	30	I	S	56H8
36b	5	S	I	8	19	17	14	30	I		
37a		S	S	13	13	18	15	30	D		
38a	4	L	S	13		33	11	30	D		56H8
38b	4	S	I	5		20	3	28	D		122
39a	10	L	S	9	13	18	11	24	D	S	56H8
40a	6	L	S	13		21	14		D	S	122
41a	4	S	I	5	11	18		26	I	S	56H8
41b		L		13	11	34	14	30	D		122
42a		S		5	11	18	3	25	I		
42b	8	L	S	13	11	28	11	30	D	S	56H8
43a		L		13		18	5	25	D		56H8
44a	6	S	I	10	13	18	10	30	D		
44b	6	L	S	14	11		15		I	S	56H8
45a	4	L	S	9	11	34	15	30	D	S	122
45b		S		10	11	18		30	D		
46a	5	L	S	8	11		15	30	D	I	56H8
47a	4	S	I	5	11	18		30	I	S	56H8
48a	4	L	S	9	11	35	15	26	D		56H8
49a		L		13	13	18	14	25	D		
50a	9	L	S	13	11		15	25	I		122
50b	4	S	I		11	18		30	D	S	56H8

hsr-omega^{TR} = *hsr-omega* repeat-length where numbers indicate bin group (1= long-repeat group 10= short-repeat group) which are ~1.5 kb apart e.g. group 5 is 10.5 to <12 kb (binning aided by the Southern standard). Group 8, 9 and 10 are equivalent to *hsr-omega*'21'

Numbers (except for *hsr-omega*^{TR}) = the number of repeats

For inversions, S= standard and I= inverted arrangement

APPENDIX E

D. simulans and D. serrata PCR conditions

All PCR reactions were carried out in 50µl reactions containing 1 unit Expand high fidelity enzyme (Roche Diagnostics), 5µl Expand high fidelity buffer, 0.2mM dNTPS and 30ng each primer.

Exon I †
Primers - A and B
$MgCl_2$ concentration - 2.5mM
DNA- 1µl proteinase K extraction
Cycling conditions - 94 °C (5 min) 1 cycle: 94 °C (30 sec), 44 °C (30 sec), 72 °C (1 min) for 35 cycles

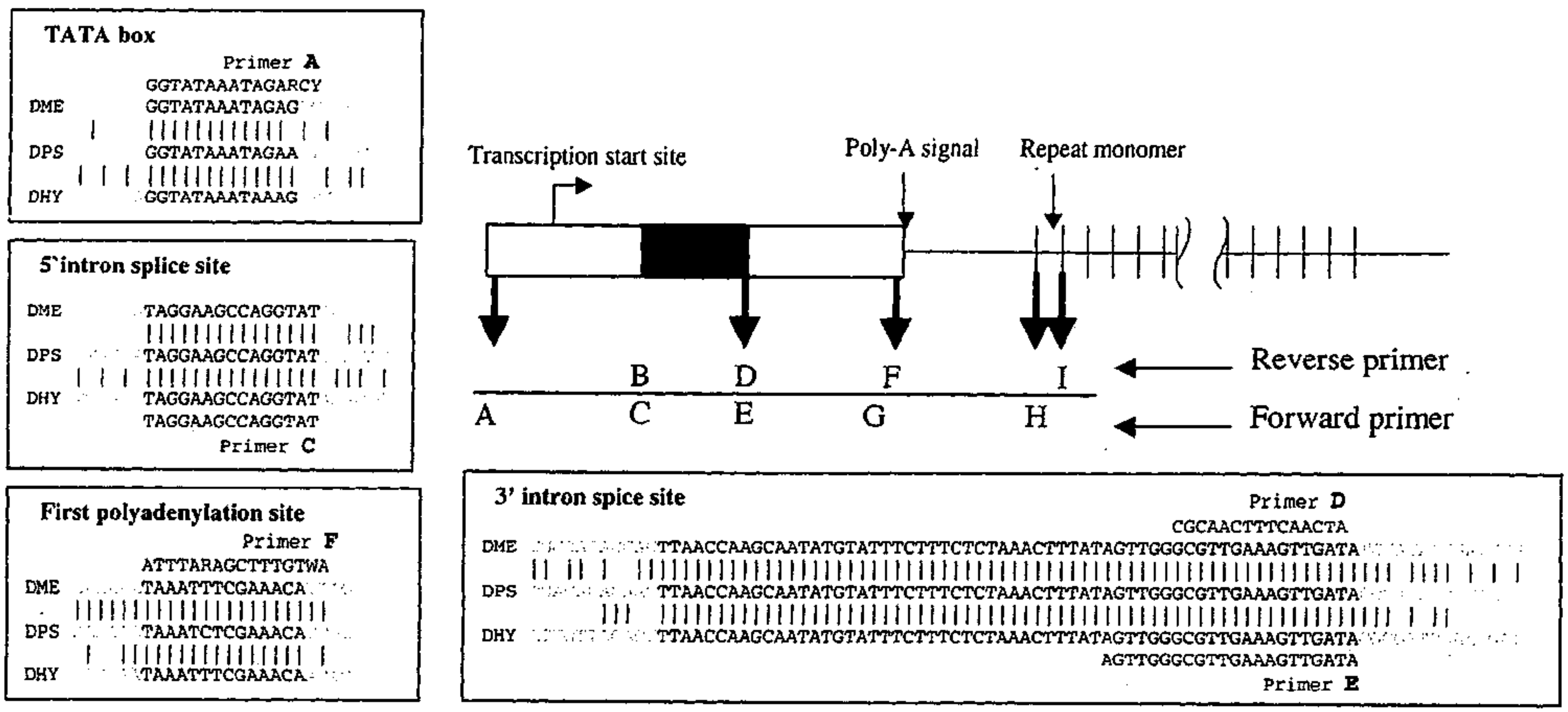
Intron
Primers – C and D
$MgCl_2$ concentration - 1.5 mM (*D. simulans*) 2.5 mM (*D. serrata*)
DNA - 1µg (*D. simulans*) 0.5µg (*D. serrata*) phenol chloroform DNA extraction
Cycling conditions - 94 °C (5 min) 1 cycle: 94 °C (30 sec), 45 °C (30 sec), 72 °C (30 sec)* for 35 cycles
* *D.serrata* 45 sec extension time and final 5 min extension

Exon II
Primers – E and F
$MgCl_2$ concentration – 2 mM
DNA –3µg phenol chloroform DNA extraction
Cycling conditions - 94 °C (5 min) 1 cycle: 94 °C (30 sec), 43 °C (1 min), 72 °C (1 min) for 35 cycles

First polyadenlyation site to 3` first repeat monomer †
Primers – G and I
$MgCl_2$ concentration – 2.5 mM
DNA – 1µg phenol chloroform DNA extraction
Cycling conditions - 94 °C (1 min) 1 cycle: 94 °C (15 sec), 40 °C (30 sec), 72 °C (1 min 30 sec) for 35 cycles: 72 °C (5 min)

Tandem repeat monomer †
Primers – I and H
$MgCl_2$ concentration – 1.5mM
DNA – 10µg phenol chloroform DNA extraction
Cycling conditions - 94 °C (5 min) 1 cycle: 94 °C (30 sec), 43 °C (1 min), 72 °C (1 min) for 35 cycles

† *D. simulans* only



The gene was sequenced in sections, the intron, exon I, exon II, poly-A signal to the repeats and repeat monomers. The black box indicates the intron and the open boxes indicate the exons. Primers used to sequence the *hsr-omega* gene in *D. simulans* and *D. serrata* are indicated above. Primers A, C, D, E and F were designed from sequence homology between *D. melanogaster* (DME), *D. pseudoobscura* (DPS) and *D. hydei* (DHY). Primers H and I were designed from the published *D. melanogaster* repeat sequence (Hogan *et al* 1995) and are given in chapter 2. Primer B (5` TGC TGT GTA TGT ATA CCT GGC TT 3`) was designed from the *D. simulans* sequence following sequencing of the intron. Primer G (5` TAC ACT TCA CCA AWT AAG AAT TAC 3`) was designed from alignments of Exon II sequence from *D. melanogaster*, *D. simulans* and *D.serrata*.

APPENDIX F

Alignment of Drosophila hsr-omega sequences (non-repeat)

Sequence alignment of the *hsr-omega* gene (TATA box to 5` of first repeat) in all *Drosophila* species sequenced to data. Sequence data for *D. melanogaster* (MEL), *D. triauraria* (TRI), *D. pseudoobscura* (PSEUDO) and *D. hydei* (HYD) was obtain from GenBank (Acc. No.U18307, AB111909, X16337 and M14558 respectively) and aligned with *D. simulans* (SIM) and *D. serrata* (SER). Sequence homology is indicated by a star symbols under alignment. Previously identified regions of high sequence conservation are indicated by boxed in sequence (solid line). The intron slice junctions are marked by arrowheads. New sequence conservation is identified by the boxed in sequence (dashed line) and primer sequence indicated by ◆⟷◆ .

```
MEL          1   TATAAATAGA GCCGCCTCAG TCCGGTCAC- GTCACTCTCA AATGAAAAGT
SIM          1   ◆--------◆ GCCGT-TCAG TCCGGTCAC- GTCACTCTCA AATGAAAAGT
SER          1
TRI          1
PSEUDO       1   TATAAATAGA ACTGG-TCGA ACCAGATACT GCCATTCGCA AAAAAAAAGT
HYD          1   TATAAATAAA GGTGACTGCC TGGACTGCA- GTCATTCGAA AAAAATAAGT
                 ******** *                       * ** **  * **  * ****

MEL         50   GTTCAAGTGC ATTCAA--AG TGAAGCTGAA AAAAT----- -AACCAGTTA
SIM         49   GTTTAAGTGA AGTCAA--AG TGAAGCTGAA AAAAATCGAA AAACCAGTTA
SER          1
TRI          1
PSEUDO      50   GTTCAAGTTT AGTCGC--AG TGCCTATGGA AAAAA----- -AGTTAT---
HYD         50   GTTGAAGTGA AGTTCTCTAG TGAAACTTAA AACTGTTTGA AGAATA----
                 *** ****   * *      ** **

MEL         92   AAAATAGTAC AAAGAAATTT TCTTTCTTGC AATTTGCAAG CAGTAGCTAC
SIM         97   AAATTAGTAC AAAGAAATTT TCTATCTTGC AATCTGCAAG CAGTAGCTTT
SER          1
TRI          1
PSEUDO      89   AAAATTGTAA AAAAAAGTAT TCATTATTGC TGGCTAGCAT ATATAATCAT
HYD         96   --------AA ATATATTTCT GGATAAGTGC TGTTTTGCAT TGTTTCTACA
                        *  * * *  * *         ***     *   *

MEL        142   AACC--AAA- --------AA TGGAAAAGTG TAAAAATCGT GTCCCAGCAG
SIM        147   AACC--GAA- --------.. .......... .AAAAATCGT GTCCCAGGAG
SER          1
TRI          1
PSEUDO     139   CAACGAAAA- --------.. .......... .GGAAAACGT GTCGTAAATG
HYD        138   TAAATAATAT ACAACTGC.. ....G..... .ACAGTATTT GTTGCGAAGG
                             ** **** ***** *  *      * **        *

MEL        181   ACGAGCAGCA GCAGTACGAG TATTGCAAAA TGCAGGGGCA AGGGCCCACG
SIM        186   GCGAGCAACA G---TACGAG TATTGCAAAA TGCAGGGGCA AGGGCCCACG
SER          1
TRI          1
PSEUDO     180   CGAAACAAAG GCCATATTCC AGCATGGCAA CAAC------ ---GCCAACG
HYD        188   CGAGATTGCG GAGCTATAAT ATGTCACGTC GACTGAAATA ATTGCTAG--

MEL        231   TAGTATTTTT CCACGTCGGG CATTTAATGC TCTCGAGTTG GAAACAATGA
SIM        233   TAGTATTTTT CCACGTCGGG CATTTAATGC TCTCGAGTTG GAAACAAAGA
SER          1
TRI          1
PSEUDO     221   TAGTCTTTCT CAATGTCGGG CAATATTTGC TCATAAGTT- GAAACAAAGA
HYD        236   ---------- ------CGCA CCGTTCAAGC TGTTTTGCTG TGAACAAAGA
                             **    *  *    ** *            ***** **
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MEL          281  AACCATACGC AAACCCCCTG GAAAAGATGT GATTAGTCAT CGATTTTGCT
SIM          283  AACCAAATGC AAACCCACTG GAAAAGATGT GATTAGTCAT CGATTTTGCT
SER            1
TRI            1
PSEUDO       270  AATACG---- -------CTG GAAAAGTGT- -ATGAGTCAT CGATTTCACC
HYD          270  AAATAAAATC TGCGTCATCG TCGTGGCCAG TTTTCGGCCG TTTT------
                  **

MEL          331  GGTCAGCGTC GGGGAATTTC GCAATGCAGC AGGC------ ----------
SIM          333  GGTCAGCGTC GGGGAATTTC GCAAAGCAGC AGGC------ ----------
SER            1
TRI            1
PSEUDO       307  -GTCAGCTTC GGGGCATTTC ACAATGCGGA AGCAACTGGA AAGGCGCAG-
HYD          314  ---------- ---GCATTTG AGGAAATTGG GGCAACACGA AAGCAGCGGC
                             * ****     *   *   *

MEL          365  --AGTTTTCT CAAATTTGGC TAGAAAGTGA CCCAC-TAGG CAGTCTGAGG
SIM          367  --AGTTTTCT CGAG..... .....GTGA ACCAC-TAGG CAGTCTGAGG
SER            1
TRI            1
PSEUDO       355  -----TTTCT CGTT..... .....GTAA CTCCC-TAGG CAGGATGAGG
HYD          351  CAAGTTTTTC AACA..... .....AATT GCACTGTAGG CAGGCTGAGC
                       ***    ****** ******          **** ***  ****

MEL          412  CAGTTATCCA GGATGTAAGG ATGTGTGCAG T-----ACTC TCTGTGTCGA
SIM          414  CAGTTATCCA GGATGTCAGG ATGTGTGCAG T-----ACTC TCTGTGTCGA
SER            1
TRI            1
PSEUDO       399  C---TATCCA GGAGGTTCAG GAAACTCTCG AAACAGACTC AGTCGGGTGC
HYD          401  AGATG----- ---------- ---------- ---------- ----------

MEL          457  ATGGCGATGG CCCTGACCTG CGGATT---- ----TTGAAA GATTGAAA--
SIM          459  ATGGCGATGG CCCTGACCTG CGGATT---- ----TTGAGA AATTGAAA--
SER            1
TRI            1
PSEUDO       446  AATGCGGCAG CCCAGACCTG CAGTGTACGT GGAATTGATA ATTTGGGA--
HYD          406  ---------- ------CCAG GAGTTTGCGA TAAATTGATG AGGCAGGAAA
                             ** *   *  *        ****         *

MEL          497  -------TAG GAAGCCAGGT ATACATACAC AGCAACGAAT GCAGCGTGTT
SIM          499  -------... .◆------◆ACATACAC AGCAACGAAT GCAGCGTGTT
SER            1  -------... .◆------◆ACA--CAC AGCAGCGACT TCAGCGTGTT
TRI            1
PSEUDO       494  -------... .......... ..TAA--C-- AGCAGCAAAT CAAGAGTGTC
HYD          490  TTGGAGT... .......... ..AAA--CCG AAACGGCGAA ACCAGCTTCG
                  ***  ********** **   *  *

MEL          540  CTATATTCT- --------TA ATTCGTAAGC -CAAA-CCTA TGTTTAAGAC
SIM          542  CTATATTCT- --------TA ATTCGTAAGC -CAAA-CCTA TGTTTAAGAC
SER           42  CTCTTTTTC- ---------T ATTCGTAAGC -ACAA-CCTA AGCCTAAGAT
TRI            1
PSEUDO       533  ATCTTTCAAT GCACCTTGTA ATTCGTAAGA -AAAAACGTA GTCCTAAGAA
HYD          538  T--------- --ACCTTATT ATACGTAAGC GAAAA-TCTA AGCATAAGGC
                             ** ******     **   **     ****

MEL          579  GTAGTTTATA TAACCGTA-C ATATGTATGT AGCGAAACAC GTTTTCAAAC
SIM          581  GTAGTTTATA TAACCGTACG -TATGTATGT AGCGAAACAC GTTTTCAAAC
SER           80  GTAGTTTTAT AAAC-----C AATCTACGGA ----AAAAAC GTGTTCGAAC
TRI            1
PSEUDO       582  CTAGATAATT GTCGACAGAC GATATGCAGA ----AAAAAC CTTTTCTAAC
HYD          576  CTAGTTTTAT TACT-----C AACTATATGC AATTTAAAAC GTTTTCAAGC
                   *** *                    *       ** **  * *** * *

MEL          628  GTCGCATCGA AGCCATTTCT TCTGCA---- ---------- ----------
SIM          630  GTCGCATCGA AGCCATTTCT TCTGCA---- ---------- ----------
SER          121  CTCATCCGTA GGTTACTTTT TTCTTCGCTG AAAACGTCA- ----------
TRI            1
PSEUDO       628  GTTGGCCGAT GCTCCATTTC ---------- ---------- ----------
HYD          621  CTGACGTCAG CGTAATTTTT GTTTTTCGAG TAAACGTTTA TACTCTGAAA
                   *
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```
MEL          654  ---------- ---------- ---------- ---------- ----------
SIM          656  ---------- ---------- ---------- ---------- ----------
SER          160  ---------- ---------- ---------- ---------- ----------
TRI            1
PSEUDO       648  ---------- ---------- ---------- ---------- -GTCATCACT
HYD          721  TTTCGAAGCT TCTAGCACTA TTTCCACCCC ATGCAAAGCA TGTCTTCCCA

MEL          654  ---------- ---------- ---------- ---------- ----------
SIM          656  ---------- ---------- ---------- ---------- ----------
SER          160  ---------- ---------- ---------- ---------- ----------
TRI            1
PSEUDO       657  CGTTTTTTGT GGCCTTTTCC AATGCAAAAT CTATAAGTCG GA---------
HYD          771  TCTATCTTTC GGCCATTTCG AGTAAATTCT CGACTGTTCT TTGTTTAATC

MEL          654  ---------- ---------- ---------- ---------- --------GC
SIM          656  ---------- ---------- ---------- ---------- --------GC
SER          160  ---------- ---------- ---------- ---------- ----------
TRI            1
PSEUDO       699  ---------- ---------- ---------- ---------- ----------
HYD          871  TGCATGTACA TATATATAAA TCTCGTATAC ACGTATTCTC TCTCGCTTGC

MEL          656  CATTGCAATT TCATCGTGTT GCGTAATCGC AAATTGTAAA TG--------
SIM          658  CATTGCAATT TCATCGTGTT GCGTAATCGC GAATTGTAAA TG--------
SER          160  ----GCAATT TCGCCACGTT GCGTAACGAG AAATACTTCC CCATTTCATA
TRI            1
PSEUDO       699  -----CTCTT GCAGCCCATT GCGTAATATG GAATTGTTCC AGAACAACGC
HYD          921  ACTTGCGCTT ACATGCCGTT GCGTGGC--- ---------- ----------
                       *  **  *      ** ****

MEL          698  ---------- ---------- ---------- ---------T GTCCCTACTA
SIM          700  ---------- ---------- ---------- ---------T GTCCCTACTA
SER          206  ---------- ---------- ---------- ---------- ----------
TRI            1
PSEUDO       794  CCGTGGCCAC CACCAGGCCT GCTCTAAAAA TACCCCTCTT TTCACTTACA
HYD          948  ---------- ---------- ---------- ---------- ----------

MEL          709  CTTCACATTT GTGTGTATCC AAGTCGAAAG TTCTCGAACT ATTCTCGTGA
SIM          711  TTTCACATTT GTGTGTATCC AAGTCGAAAG TTCTCGAACT ATTCTCGTCA
SER          206  ---------- --------CC CAGTCGAATG TTCTCGATTC TCTCTTTGTT
TRI            1
PSEUDO       844  TTTTACATGT ATGTATATTC ATCTCG---- ---------- ----------
HYD          948  ---------- ---------- ---------- ---------- ----------

MEL          759  G--------CA ACCCCCGGAT CGAAAGGCCA CCAACTGGCT CGTTCTCTCT
SIM          761  G--------CA ACCCCCGGAT CGAAAGGCCA CCAACTGGCT CGTTCTCTCT
SER          238  CTTCAACCCA ACCCCCGGAT TGAGAGGCCT CCCACTGGCT GCTCTATATA
TRI            1
PSEUDO       870  ---------- ---------- --------CT CCCACTTTTG GGATACGCCT
HYD          948  ---------- ---------- ---------- ---------- ----------

MEL          802  CTCCAGAAAC AGCTTACTCT TACGTTTACA ATTG------ ----------
SIM          804  CCCCAGAAAC AGCGTACGCA TACGTTTACA ATTG------ ----------
SER          288  TAGCGTATTG CTCATACGCC CCGGGTTACA ATTAGTTTCA T---------
TRI            1
PSEUDO       892  ATCTATACTG CATTCGGTTG TACTCACACT GACGTCAACA GCAGAGCAGC
HYD          948  ---------- ---------- ---------- ---------- ----------

MEL          836  ---------- ---------- --ACTCTCTA CCTATCGGGT ATACACATTT
SIM          838  ---------- ---------- --ACTCTCTA CCTATCTGGT ATACACATTT
SER          329  ---------- ---------- ---CTCGCTC CCACTCGGGG ATACACACCT
TRI            1
PSEUDO       942  TGCAAAAAGT ACTTGCGTGT ACACACTCTA CCCATGTGTT TACTCTCTCT
HYD          948  ---------- ---------- ---------- ---------- ----------
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MEL          864  TTATATATGT ATACACTCAG AGACACCCCA ATCCCCCACC ATACATATGG
SIM          866  TTATATATGT ATACACTCAG AGACACCCCA ATCCCCCACC ATACATATGG
SER          356  TAAAGTATTC AGA------- ---------- ----CCAGCT TTACATACAT
TRI            1
PSEUDO       992  TTATATATTA TGTACGCGTA CATTG----- ---------- ----------
HYD          948  ---------- ---------- ---------- ---------- ----------

MEL          914  CGAGATGTGT GAATGTATCG CAGTTGGACG AA-------- ----------
SIM          916  ACAGATGTGT GAATGTATCG CAGTTGGACG A--------- ----------
SER          385  ACATATGTAT GTATGATAAT GGACGTGGAT TTCTACATAC ATACGTATAT
TRI            1
PSEUDO      1017  ---TATGTAT GTATGTATTT TCCCACATGC TGGGTATAGG GAAAATTTAA
HYD          948  ---------- ---------- ---------- ---------- ----------

MEL          946  ---------- ---------- ---------- ---------- -----AACGG
SIM          947  ---------- ---------- ---------- ---------- ----CAACGG
SER          435  TATTCAGACC CCGTACATAT GTTTATACAT ACATATATAT ATGGCAATGG
TRI            1
PSEUDO      1064  CTGCTTTATG ---------- ---------- ---------- ----------
HYD          948  ---------- ---------- ---------- ---------- ------ATGG

MEL          951  CAGCTGCGCA GAAAACGCTG CGGCGTACAT ACAAACGAGC TCTTTGTTTG
SIM          953  CAGCTGTGCA AAAAACGCTG CGGCGTACAT AACACTGAGC TCTCTGTTTG
SER          485  CAGCTGCGCA AAAACTCAAT ACTGCCC--- ---------- --------TG
TRI            1
PSEUDO      1074  ---------- ---------- ---------- ---------- ----------
HYD          952  CAGCTGCGCG TAAACTCAGC CTCGTTGGTC GAGTTGTTCG ACCCACCTTT

MEL         1001  CCTTTCCCCA GTTTTGCTTC CCC------- ---------- -----ATACG
SIM         1003  CCTTTCCCTA GTTTTGCTTC CCC------- ---------- -----ATACG
SER          514  CCTTTCCCTC AGTCCCCTAC CCC------- ---------- -----ATA--
TRI            1
PSEUDO      1074  ---------- ---------- ---------- ---------- ----------
HYD         1002  GTCTTACAAT GTATTTGTGT GTTTCCATCT CTTCTCTCTA TACATATACG

MEL         1029  TATAACTGGT GTTTATCAGC GGTAATCTCG CCAGCCTGAC T---------
SIM         1031  TATAAATGGT GTTTATCAGC G-TAATCTCG CCAGACTGAC T---------
SER          540  ---------- --TTCGCAAT GGAAAACTTT CCAGTTTGAC GTCTTTCTTC
TRI            1
PSEUDO      1074  ---------- ---------- ---------- ---------- ---TTTCTTC
HYD         1052  TATAAGTATA AGACTACATC TACTTCAGAC AGTTTTCTCA AA--------

MEL         1070  ---T--TGTC CCTGTCGCTT CTATTGACAT TCTAAACTAG ATTG------
SIM         1071  ---T--TGTC CCTGTCGCTT CTATTGGCAT TCTAAACTAG ATCG------
SER          578  ATGT--TGTT TCTGTCGCTT CTCTTAGGC- -----ACTAG CTTG------
TRI            1
PSEUDO      1081  ATCCCCTGTT TCTGTCGCTT CTATTGGCAT CTTACCTACG ----------
HYD         1094  ---------- -CTGTCGCTT ATAATTGGC- -----ACATC TCTTAATAAC
                             *********  *  *

MEL         1109  ---------- ------TAGA CGTTTTAACA TTTACCTAGC TGTTAATCTT
SIM         1110  ---------- ------TAGA CGATTTAACA TTTACGTAGC TGTTAATCTT
SER          614  ---------- ------TAGA CGACTTAACC ATTTACTTAG CGTTAATTTT
TRI            1
PSEUDO      1122  ------ATAA GTTTCGTAGT CATTTTAACA TTTACATAGC TGATAATCTT
HYD         1127  TAAATCATAA GTTCTTTAGC CGTTTAAACC C------TAG TGTTAATCTT

                             ***  *   * ***                * **** **
MEL         1143  ATTTGTAAAC ATCTCTATAT GCAAC----- --CTGCCATA CCACTTAACC
SIM         1144  ATTTGTAAAC ATCTCTATAT GCAAC----- --CTGCCATA ..........
SER          648  ATTTGTAACC ATCTCTATAT ATGTATGCAA CTCTGCCACA T.........
TRI            1
PSEUDO      1166  ATTTGTAATA ATGCTT---- ---TATGCAA CTCTGACACA CA........
HYD         1171  ATTTATAAAC TGTCACTTGA TAATTGTTAC ACT------- ----......
                  **** ***                                   ******
```

```
MEL       1186  AAGCAATATG TATTTCTTTC TCTAAACTTT ATAGTTGGG- CGTTGAAAGT
SIM       1187  .......... .......... .......... .........G ◆———————
SER        698  .......... .......... .......... .......... ◆———————
TRI          1
PSEUDO    1209  .......... .......... .......... .......... ..........
HYD       1210  .......... .......... .......... .......... ..........
                 ********** ********** ********** *********  **********

MEL       1235  TGATATCGAT ATCGATCCGT GAA------- ---------- --------AA
SIM       1237  ———◆..... ATCGATCCGT GAA------- ---------- --------AA
SER        746  ———◆CGCGA A------CGT GAA------- ---------- --------AG
TRI          1     CGCGA A------CGT GAA------- ---------- --------AA
PSEUDO    1258  .....CGCAA A------CGC GTAGGGCATC GATCCAAGTA AAACTCTGAA
HYD       1259  .....CGCGA T------CGA ACGATGTTAA CTTG------ --------AA
                 *****       **                                     *

MEL       1260  GTCGATACCC TGCGCAAGCA TGGGGCGGCA TATGGGTGCT GAAAACGCAC
SIM       1262  ATCGATACCC TGCGCAAGCA TGGGGCGGCA TATGGGTGCT GAAAACGCAC
SER        765  CACGATACCC TGCGCCAGCC TGGGGCGGCA TATGGGTGCT GAAAATGCAC
TRI         15  CTCAATACCC TGCGCGAGCC TGGGGCGGCA TATGGGTGCT GAAAATGCAC
PSEUDO    1302  CTCTGTACCC TTCGCGAGCA ATAGGCCAGA AGAA-GTGCT GAAAATGCAC
HYD       1289  CGCAATACGT GAG------A AAAGGCCTTA CAAT-TAGCT AAAAATGCTG
                   *  ***              ***   *       ***  **** **

MEL       1310  TCGGC----- CC-GATCCCG AT----TGC- -----AGCGT TATTCGAAAG
SIM       1312  TCGGC----- CC-GATCCCG AT----TGC- -----AGCGT TATTCGAAAG
SER        815  TTTGC----- CCACTTGTCC CAGTGCTGC- -----TGCGT TCGGCATGGG
TRI         65  TCCGC----- CC-CGTGCCC CAGAGCTCCT GCGTTTGGTC AGGGCTGCGT
PSEUDO    1351  TTCGG----- CC-CATGTAC ---------- GCGTTTGGGC TACGCGAAAG
HYD       1332  ATTGTTCGGT CC-CATGTAG ---------- CCGTGAGAGT ATATCTAAA-
                           **   *

MEL       1344  CTGTGTCTGC GACCGTGACT GAGATCATAT ---GCGTACA TATATCTAAT
SIM       1346  CTGTGTCTGC GACCATGACT GAGATCATAT GCTGCGTACA TATATCTAAT
SER        854  CTGCGTCTGC GACAATGACC CGGGACAGAG ATAATACCGC CTGTGGTGTA
TRI        109  CTGCGACTGT GACCGTGACT GAGATAACGC CCTG------ -TGCGGTGTA
PSEUDO    1385  TTGTGGCTGC GACCCTGACT CAGCCTTAGT GT-------- -------TGAT
HYD       1370  CTGCGACTAA ATG------- ---------- ---------- ----------
                  **  *  **

MEL       1391  --------GTC CGGGGTCGTA GGCCAGCCAG G------GTG ------C-TC
SIM       1396  --------GTC CGGGGTCGTG GGCCAGCCAG G------GTG ------C-TC
SER        904  TCTGGGGGTCT CGTGGTCGCG GACCAGCCAG GCCGAGGGTG ------C-TC
TRI        152  TCCGGGGGTCT CGTGGTCGCG GGCCAGCCCA GCCCGCGGTG ------C-TC
PSEUDO    1421  --------TGT CGGGGTCGCG GACTGACCAG G------GTG ------CGTC
HYD       1383  --------TGT GTGTGTCGCG GCCTACACAG G------GGT ACTAATCGTC
                            ****   * *    *          *         * **

MEL       1421  GATTCTGTC- ---AGATTGA TTGTGCGGAT TGTGT----- TA-TAGGAAC
SIM       1426  GATTCTGTC- ---AGATTGA TTGTGCGGAT TGTGT----- TA-TAGGAAC
SER        947  GCGATTCAGT CAGAGATTGA TTGTGCGGAT TGTGT----- TAATAGGAAC
TRI        195  GCGATTCAGT ---------- ---------- ---------- ----------
PSEUDO    1452  GTTTCTATC- ---AGATTGA TTGTGCGGAT TGTGT----- TA-TAGGAAC
HYD       1420  GTTTTTATC- ---AGATTGT GATTGCGTGG ATTGAATATA TA-TAGGAAC
                 *    *       ******    ****                ** *******

MEL       1461  ACTGGTGTAT CGACTTCTCT GCT---CCAC TATGGGTGA- ----------
SIM       1466  ACT..... .......... ...---CCAC TATGGGTGA- ----------
SER        992  ACG..... .......... T..GCTCCAC TAAGGGTGA- ----------
TRI        205
PSEUDO    1492  TCC..... .......... ...---CTAC TAAGGGTGA- ----------
HYD       1465  C-...... .......... ...---CTAC TAAGACTTGT TCAAAAGAAA
                   ***** **********  **   * ** ** *

MEL       1497  ---------- ---------A GGATACCCTA CCGAAAAGGC CTTCTGTCGC
SIM       1502  ---------- ---------A GGATACCCTA CCGAAAAGGC CTTCTGTCGC
SER       1031  ---------- ---------G CCCTACA--- ---AAACGGC CTTAAGTTGC
TRI        205
PSEUDO    1528  ---------- ---------A CCCTACA--- ---ATG-GGC CTTCTGTTGC
HYD       1508  GGCGCCCAGT TACATATGTA CTATATA--- ---AAC-GGT CGTCTCTTAT
                                      **         *   **  * *   *
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MEL          1528   TTAC-TATCA TCGAACA--A GTTCC----- GTAAAGGGCA GACATACGTA
SIM          1533   TTAC-TATCA TCGAACA--A GTTCC----- GTAAAGGGCA GACATACGTA
SER          1056   TTAC-TATCA TCATCGAACA GTACC----- AAAGCGGGCA GACAGGCATA
TRI           205
PSEUDO       1552   TTACCTACCA TCGAACG--A ATACCAATAC ATACCGGGCA GATATACGTA
HYD          1551   TTACCTACCA TCGAACCGTT ATCTCAATAC TGATCGAATA GATACAACAC
                    **** ** ** **          *  *        *  *   * ** *

MEL          1570   CACGTGGCAG CATATTAC-- ---------- ---------- ----------
SIM          1575   CACGCGGCAG CATATTAC-- ---------- ---------- ----------
SER          1100   CATATGACTC TGTTCCAACA GCGATCAATC GGTTGAGCTT GGCAGTATTG
TRI           205
PSEUDO       1600   TA----ACTC TGTTCTGACA TCGATCG--- ---------- ----------
HYD          1601   TA-------- ----CTGACA ACGATCG--- ---------- ----------

MEL          1588   ---------- ---GTTCAAT GACACATCGT CTCTGGATTA GTAGTTGAAC
SIM          1593   ---------- ---GTTCAAT GACACATCGT CTCTGGATTA GTAGTTGAAC
SER          1150   GCGTATTGAA ACTGTTCAAT GACACATCGT CTATGGATTT AGGAATCGAA
TRI           205
PSEUDO       1623   ----ATTGAA ACTGTTC--- -ACACGTCGT CTTTGGAGTA GCAATTTAAC
HYD          1616   ----ATATAC ACATATC--- ------TAGT ATCTGGAGTA GCAACCGATT
                                             * **  * **** *

MEL          1625   CAACGAGCTC GAATATACCC TGCCAGTTGG TTGTAAATAT TAATTTTGTT
SIM          1630   CAACGAGCTC GAACATACCC TGCCAGTTGG TTGTAAATAT TAATTTTGTT
SER          1200   GCAGCGTCTC GA--ATGCCA AATCAGTTGG TTGTAAATAT TAATTTTGTT
TRI           205
PSEUDO       1665   CAACAAG-TC CA--AAACAA TGGCTGTTGC TTGCAAACAA TCTTTTCTGT
HYD          1653   GCTCGTTCGT TTCACAAAGC ACAAATTATA AGATACATCT TTCTTGTTAT

MEL          1675   CT-------- --TATATTCA GTTGTAAAGT CAATACGGAA GACATTTCCA
SIM          1680   CT-------- --TATATTCA GTTGTAAAGT CAATACGGAA GACATTTCCA
SER          1248   CCTGTACCCT TATTTATTCA GTTGTAAAGC CGAACCGGAA GACATTTCTT
TRI           205
PSEUDO       1712   GGC------- TATACATTTT GTGGTGAACT CTAAGCGGAA -ACAGTTCCA
HYD          1703   TTACTTA--- -ATATATTTT TTTCTTTGTT GTTAACTTAA -ACATTTACA
                              *  ***    *  *           *  **  *** **

MEL          1715   C-ACCAAC-C ATGTGTCACT TATGTTCTT- AA--ATACCA GAAACTGTTT
SIM          1720   C-ACCAAC-C ATGTGTCACT TATGTTCTT- AA--ATACCA GAAACTGTTT
SER          1298   TTACCAAC-A ATGTGTCACT TACAAATGTT CTTTATACCA GATACTCTTC
TRI           205
PSEUDO       1754   C-ACCAAC-A ATGTGT---- TAAGTTCTT- AA--ATATTT GAAATTGTTT
HYD          1748   AGTACACGAC AAGCGTGGCT TAAGTTCTTG AA--ATACCT GAAACTGTAA
                            * * **      **              ***     ** * ***

MEL          1760   AAA---T--- ---------- -----TTTT- -GTTCTTATA CACTTCACCA
SIM          1765   AAA---T--- ---------- -----TTTT- -GTTCTTATA CACTTCACCA
SER          1347   AAA---T--- ---------- -----TTTTT TGTTTTTGTA CACTTCACCA
TRI           205
PSEUDO       1795   AAACTGT--- ---------- -----TTTT- -GTTTTTGTA CACTCTACGA
HYD          1796   AAAAAAAAAA TACAAAAATT ATTTCTTCT- -GTTTTTGTA CATATCCAAG
                    ***                      ** *   *** ** ** **

MEL          1787   ATTAAGAATT ACCTGTACTG ATGTAAGTGT A----CCTGT TG--------
SIM          1792   ATTAAGAATT ACCTGTACTG ATGTAAGTGT A----CTTGT TG--------
SER          1376   AATAAGAATT ACTTGTACCG ATTTATACTT ACTTTCTTGT CG--------
TRI           205
PSEUDO       1825   CTTTATAATT ACTTGTACCG ATGTAAAATT A----CTTGT CG--------
HYD          1844   ATTTAAGAAA ACTTGTGTCC ATTTTTGTAA TTACAATTGT CCAATTCAGT
                      * *  *   ** ***

MEL          1825   ---------- ---------- AATCACAACA AAAA------ ---TAGAAAA
SIM          1830   ---------- ---------- AATCACAACA AAAA------ ---TAGAAAA
SER          1418   ---------- ---------- AAACAAAACA AAAA------ ---TTGAAAA
TRI           205
PSEUDO       1863   ---------- ---------- AAACAAAACA AAAAATAAGA AAATGGAAAA
HYD          1894   AATAGAAAAT GAGAAGAAAA AAATAATACG AAAA------ ---TGAAAAA
                                          **  *  **  ****          *  ****
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```
MEL        1846  AATGA----A TAACAAAAGA GCTCTAT-AA CAGAAAAGCC ---ACAG---
SIM        1851  AATGA----A TAACAAAAGA GCTCTAT-AA TAGAAAAGCC ---ACAG---
SER        1439  AAAATTAATA TGAAAAGAGC GCTCCTT-AA TGGAAAAAGA AAGCCACAGA
TRI         205
PSEUDO     1893  AATGC----T TAACAAAAGA GCTCATC-TA TAGAAAAGAA GCAACAA---
HYD        1935  GAAAA----A GAAAAAGAGA GCTCACCAAA CAGAAAAGAA GCTACAATCA
                   *           * ** **  ****     *    *****

MEL        1885  AAAAAGTGAA TAAAAATTCA AAAATTCAAA AAATAAATTT CGAAACATTT
SIM        1890  AAAAGTGAAT AAAAAATTCA AAAATTCAAA A..◆──────────◆CT
SER        1488  AAAAAGTGAA TAAAAATTCC AAGATTCCAA A..◆─-────────◆T-
TRI         205
PSEUDO     1935  AAAAGTGAAT --AAAATTCA ATAATTCCAA A.......C. .......TTG
HYD        1981  AAAAAAAGAA TAAAAGAAAT CTGAGACC-- -......... .......ATT
                   ****     *    ***              ***** * * ********

MEL        1935  GCTCGTGATA --TTTTTATT TCAAGAAAAG ATCC------ GATTTCAGTT
SIM        1940  GCTCGTGATA --......... .....AAGAG ATCC------ GATTTCAGTT
SER        1536
TRI         205
PSEUDO     1983  CTCCTCATAT TT........ .....CTTAA ATTC------ AATTTCAGTT
HYD        2028  GTTCGCAATA --........ .T...TTAAA ATTCTAAACA AATTTCAGTC
                             ******** * ***   *  ** *        ********

MEL        1977  ATACCATCTA GCTTAAATTT AGCGTTACGA GAATAGTCCC TCGGAGGAAA
SIM        1982  ATACCATCTA GCTTAAATTT AGCGTTACGA GAATAGTCCC TCGGAGGAAG
SER        1536
TRI         205
PSEUDO     2027  AGATCATCCA ACCGTTTTAC
HYD        2076  CAATCACCAT AC
                   * ** *    *          ********** ********** *********

MEL        2027  GGCCCTTTAT CAAGATGCCA ATATCGTTCG TCCTATACAC AAATTACTC-
SIM        2032  GGCCTTTTAT CAAGATGCCA ATAGCGTTCG TCCTATACAC AAATTACTCA
                   **** ***** ********** *** ****** ********** *********

MEL        2076  -ACGTTATGA AACCAAGTCG AAAAATCGGA CAAAGTGTCT ACAATTGAGG
SIM        2082  GACGTTATGA AACCCAGTCG AAAAATCGGA --AAGTGTCT ACCATTGAGG
                   ********* **** ***** **********   ******** ** *******

MEL        2125  AAAGGTCTAT TGATCTCCTG TGAGCGCAAA AAGTTTGTAT TCGAACGAGA
SIM        2130  AAAGGTCTCT TGATCTCCTT TGAGCGCTAA AAGTTTGTAT TGGAACGAGA
                   ******** * ********* ******* ** ********** * ********

MEL        2175  ATTTTAAGGT TTAAAATCAG CTGAAAAGTA CGTATATTCC GCATTTATTT
SIM        2180  ATATTAAGGT TTAAAATCAG CTGAAAAGTA CGTATATTCC GCATTTATTT
                   ** ******* ********** ********** ********** **********

MEL        2225  TTCTCCACAT TTTTAGGATC AGAACATCAG AACTATATTT TTTTGGAAAA
SIM        2230  TTCTCCACAT TTTTAGGATC AGAACATCAG AACTATATTT TT-TGGAAAA
                   ********** ********** ********** ********** ** *******

MEL        2275  ATCAACGGAT ATTATTTTGT TTGGAGGTCC CAAATTCTAT ACACCTTCAC
SIM        2279  ATCAACGGAA ATTATTTTGT TTGGAGGTCC CAAATTCTAT ACACCTTCAC
                   *********  ********** ********** ********** **********

MEL        2325  ACATATTTTT GCATACAAAA AAACCTAAAA ATAATA-TTA TTGAATGGCA
SIM        2329  GCTTATTCTT GCACACGAAA AAAACAATAA ATTATTTTTA TTGAATGACA
                   * **** ** *** ** *** *** * * ** ** **  *** ******* **

MEL        2374  AAAAAAACCA TTTATAACAT GAAAATTACT CGAAGTACTT CAATTTAATT
SIM        2379  AAAAAACACA TTTATAACTT GAAAACTATT ATAAGTACTT CACTTTAATT
                   ******  ** ******** * ***** ** *    ******** ** ********

MEL        2424  ACCGAACCAA AATATATACA CGTACGACTC GTGTTGGACG AGATTACATT
SIM        2429  ACCGAACCAA CATATATACA CATGCGACTC GTGTTGGACG AGATTACATT
                   **********  ********** * * ****** ********** **********

MEL        2474  AAGAGAACTT TGAAAAATAA TAAAAAAAGG TTTGAAATTG AAAACTGTAA
SIM        2479  AAGAGAACTG TGAAAAATAA AAAAAAAGGC TTACAATTAG AAAA-TGTAA
                   *********  **********  ****** *  **  ** * * **** *****

MEL        2524  AGTATAATAT TCGTAATAAG GAATTTATAA AGATAAATAA CCACAACAAA
SIM        2528  GGTACAATAT TCTTTATAAG GATTTTTAAA AAATAAATAA TAATAACAAA
                   *** ***** ** * ***** ** ***  ** * *********   * *******
```

```
MEL     2574  ATGAACCACA ATAATATGAA ACCATGAACA ATAGGTATGA TTTATGTCGA
SIM     2578  ATGAACCACA ATAATATGAT ACCTTCAACA ATAGGTATAA TTTATGTCGA
              ********** *********  *** * **** ******** * **********

MEL     2624  ---------- ---------- ---------T TAGCCTCTTG GTAAAATAAA
SIM     2628  TTTCCCCAAA TATAATCCGT CGAAAAGGCT TAGCCTCCTT GTAAAAAA
                                              * ******* *  **********

MEL     2645  CTTAAAATCC ATATAGGT-- GTGTGTTATA AAACTGCCCC AATTCAAAAT
SIM     2678  CTTAAAACCG TAAAGGTACG GTTTGATATT AAACTGTCCC CAAACACA-T
              ******* *    *  *     ** ** ***  ****** ***  *  ** * *

MEL     2693  TGCGTCTCGT AATAAACCAA AAGTACATCG TACAACTGCA ACATGACCCG
SIM     2727  CGCATCTAAT AATTAACTAA ATTATTAGCA ATTTAAAAGG TGTGTTTTAG
               ** ***  * *** *** ** *     * *      *               *

MEL     2754  ---------- ----ACTCTT TAACAATAGG TTTCATTTAC TCCGATTACC
SIM     2827  GTATGTATTA TATGACTCTG CAATAAAATG TATGATTTAT CTCGATAATA
                         *****   ** ** * * * * * *****    **** *
```

APPENDIX G

Alignment of D. simulans hsr-omega repeat monomer sequences

Aligned sequence of 4 repeat monomers of *D. simulans* and consensus sequence is also given. Sequences were aligned using DiAlign professional Release 2.7.5. (Genomatix Software GmbH 1998-2004, http://www.genomatix.de/cgi-bin/dialign). Primer sequence is indicated by ◆——◆ . Boxed in sequence indicates the 9-nucleotide conserved region. Sequence homology is indicated (*).

```
Clone 2          1  ◆————————————◆ GGTAAAATAA ACTTAATTTC CATATAGGTA
Clone 3          1  ◆————————————◆ .......... .......... ..........
Clone 4          1  ◆————————————◆ .......... .......... ..........
Clone 5          1  ◆————————————◆ .......... TT....AA.. .C........
                                   ********** **** ** * ********

Consensus           TCGAAAAGGC TTATCCTCTT GGTAAAATAA ACTTAATTTC CATATAGGTA

Clone 2         50  GGATGTCTTA TAAAAtT... CCACTTCAAA ATCTCGTCAC ATAATAAGCA
Clone 3         51  .......C.. .....CA... .......... .......... ..........
Clone 4         51  .......... .....C.... .......... .......... ..........
Clone 5         51  ..G...G... .....C.... ...A...... ...GA...TG G......A..
                    ** ***  ** ***** *** *** ****** ***  ***    ****** **

Consensus           GGATGTCTTA TAAAACTGTC CCACTTCAAA ATCTCGTCAC ATAATAAGCA

Clone 2        100  AAAAGTTTAA GCATCTTAAA AGGTATGATT TTTAGGACAT ATTAATTTAC
Clone 3        101  .......G.. .......... ....CG.... .........A ..A.......
Clone 4        101  .......G.. .......... .......... .......... .A........
Clone 5        101  ...T-..A.. ...A.GCG.. C.AA.G.... .......... .C...A...T
                    ***  ** ** *** *   **  *    **** *********  *  ** ***

Consensus           AAAAGTTGAA GCATCTTAAA AGGTAGGATT TTTAGGACAT ATTAATTTAC

Clone 2        150  TTAATAGGTA GGCATTGAAC ATACGGTATT TTT-TATATG ACTTATTTAA
Clone 3        151  .......... .......TT. .......... .......... ..........
Clone 4        151  .......... .......TT. .......... .......GGT T--...C...
Clone 5        150  .........A .......... .......... ...A...GGT .--...C...
                    ******** * ********   ********** *** ***       *** ***

Consensus           TTAATAGGTA GGCATTGAAC ATACGGTATT TTT-TATATT ACTTATTTAA

Clone 2        199  GCAACATCAA CATGA--CCC ACAATTCTAT GAAGTCTTTA ACAATAGGTT
Clone 3        200  .......... .......... .......... .......... ..........
Clone 4        198  .......... .......... .......... ......C... .......T..
Clone 5        198  A....TG... ...T.CT... ......A... ...C...... ....G.TA..
                     ****  *** *** *  *** ****** *** *** ** *** **** *  **

Consensus           GCAACATCAA CATGA--CCC ACAATTCTAT GAAGTCTTTA ACAATAGGTT

Clone 2        247  TTATTTAC ◆————————————◆
Clone 3        248  ........ ◆————————————◆
Clone 4        246  .......T ◆————————————◆
Clone 5        248  .......T ◆————————————◆
                    *******

Consensus           TTATTTATCC CGATTACCCC AATCACTAT
```

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Thermal tolerance trade-offs associated with the right arm of chromosome 3 and marked by the *hsr-omega* gene in *Drosophila melanogaster*

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Drosophila melanogaster occurs in diverse climatic regions and shows opposing clinal changes in resistance to heat and resistance to cold along a 3000 km latitudinal transect on the eastern coast of Australia. We report here on variation at a polymorphic 8 bp-indel site in the heat shock *hsr-omega* gene that maps to the right arm of chromosome 3. The frequency of the genetic element marked by the L form of the gene was strongly and positively associated with latitude along this transect, and latitudinal differences in L frequency were robustly associated with latitudinal differences in maximum temperature for the hottest month. On a genetic background mixed for genes from each end of the cline a set of 10 lines was derived, five of which were fixed for the L marker, the absence of *In(3R)P* and 12 kb of repeats at a second polymorphic site at the 3′ end of *hsr-omega*, and five that were fixed for the S marker, *In(3R)P* and 15 kb of *hsr-omega* repeats. For two different measures of heat tolerance S lines outperformed L lines, and for two different measures of cold tolerance L lines outperformed S lines. These data suggest that an element on the right arm of chromosome 3, possibly *In(3R)P*, confers heat resistance but carries the trade-off of also conferring susceptibility to cold. This element occurs at high frequency near the equator. The alternate element on the other hand, at high frequency at temperate latitudes, confers cold resistance at the cost of heat susceptibility.
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Keywords: heat and cold tolerance; tradeoff; latitudinal variation; *Drosophila melanogaster*; *hsr-omega*; polymorphism

Introduction

Research in Drosophila is providing a focus for understanding the physiological and genetic basis of thermal tolerance variation in ectotherms (Huey and Kingsolver, 1993; Duncker *et al*, 1995; Krebs and Loeschcke, 1995; Guerra *et al*, 1997; Ohtsu *et al*, 1998). Significant variation in resistance to temperature extremes are known among several groups of closely related Drosophila species and these are likely to be adaptive since they are related to the climatic zones where the species are found (Kimura, 1988; Jenkins and Hoffmann, 1994; Gibert *et al*, 2001). While thermal tolerance is an important factor limiting the distribution of species, some species such as the cosmopolitan *Drosophila melanogaster* successfully survive and reproduce over a broad range of climatic zones that cover a wide spectrum of thermal extremes. Heritable strain variation and regional variation in thermal tolerance have been well documented for this species (Parsons, 1977; Stalker, 1980); however, only recently has a firm basis been established for believing that an adaptive cause underlies this variation. Opposing latitudinal clines in resistance to both heat and cold temperature extremes occurred along a north–south transect of samples from the eastern coast of Australia (Hoffmann *et al*, 2002). In samples not confounded by variation in altitude, knockdown time when exposed to heat shock increased towards the tropics whereas recovery time following cold shock decreased towards temperate high latitude regions, as might be predicted under an adaptive scenario.

This Australian eastern coast transect for *D. melanogaster* has recently been shown to vary in a clinal fashion for a number of different quantitative traits, in addition to the heat and cold tolerance clines. Traits that change with latitude include body size (Partridge *et al*, 1999), egg size (Azevedo *et al*, 1996) and life-history traits (Mitrovski and Hoffmann, 2001). Clinal variation also occurs for numerous genetic markers, including the frequency of four common cosmopolitan inversions (Knibb *et al*, 1981), and allele frequencies of numerous polymorphic markers (Oakeshott *et al*, 1984) including five microsatellite markers (Gockel *et al*, 2001). Many of these variations are also 'generic', occurring in a latitudinally consistent manner on other continents (Knibb, 1982; Mettler *et al*, 1977; Inoue *et al*, 1994), and in related species (Oakeshott *et al*, 1984). A major question of interest is the relation between the quantitative traits and the genetic markers, and the extent to which we can understand the genetic and physiological/selective basis of the differential adaptation to climate along these latitudinal clines.

Towards this end, we have recently focused on thermal tolerance variation and its relation to polymorphic candidate gene variation (McColl *et al*, 1996). In

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particular, strong clinal variation in allele frequency of a polymorphic heat shock gene, *hsr-omega*, parallels the thermal tolerance clines down the Australian eastern coast (McColl and McKechnie, 1999). Further, in laboratory studies heat-tolerance variation has been associated with molecular variation in *hsr-omega* and with variation in two other heat shock genes, *hsp70* and *hsp68*, all of which map to the right arm of chromosome 3 (Figure 1) (McColl *et al*, 1996; Feder and Hofmann, 1999). The right arm of chromosome 3 harbours the large cosmopolitan inversion *In(3R)P* that also varies latitudinally (Knibb *et al*, 1981). Both *hsr-omega* and *hsp68* map within the bounds of *In(3R)P*. This linkage and latitudinal association of the heat-shock-protein markers and an inversion hints at a possible causal role of chromosome 3R in clinal thermal-tolerance variation. Intriguingly, heat shock genes may also play a role in recovery from cold stress (Guy *et al*, 1986; Chen *et al*, 1987; Burton *et al*, 1988), and *hsr-omega* in particular has been implicated in expression changes following cold exposure (Singh and Lakhotia, 1984; Lakhotia and Singh, 1985). A major effect of chromosome 3 on population variation in cold tolerance has been previously indicated (Tucic, 1979).

In this report, we use polymorphic variation in the *hsr-omega* gene as a marker for variation that occurs on the right arm of chromosome 3 and we ask about the relation between variation in this marker and both heat and cold tolerance variation. We chose two different ways of testing tolerance to these extremes, to see if the associations are of general significance. One of these heat-tolerance tests and one of the cold-tolerance tests are identical to those used to detect clinal variation along the eastern Australian transect (Hoffmann *et al*, 2002). Our data strongly suggest that the right arm of chromosome 3 is involved with genetic adaptation to both heat and cold stress and that fitness trade-offs are involved; heat resistance comes at the cost of cold susceptibility, and the cost of being cold tolerant is susceptibility to heat.

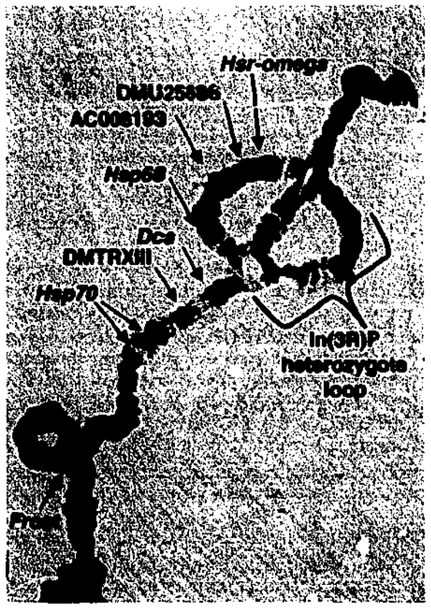

Figure 1 *Drosophila melanogaster* right arm of chromosome three. Depicted are cytological locations of *In(3R)P* (89C2-C3 – 96A18), *hsr-omega* (93D), *Dca* (88D), *Frost* (85E2), *hsp63* (95D), *hsp70a,b* (87A,C), microsatellites DMU25686 (93F), AC008193 (94D) and DMTRX3 (88B3) on a polytene chromosome spread of the right arm of chromosome 3 from an individual heterozygous for *In(3R)P*.

Materials and methods

Drosophila lines

A total of 160 *D. melanogaster* isofemale lines were collected from December 1999 to February 2000 along the east-coast of Australia. A minimum of four and maximum of 15 isofemale lines were collected from 19 sites and were reared in vials on treacle medium at 18°C with constant light in the laboratory. Two isofemale lines from the east-coast collection were used for the hybrid study. Line 11T was fixed for the *hsr-omega*^{L} allele (L) and was collected from Huon, Tasmania. Line13Q was fixed for the *hsr-omega*^{S} (S) allele and was collected from Weipa, in far northern Queensland. To produce hybrid lines 25 virgin females from each line were mated *en masse* with 25 males from the other line. Progeny from the two crosses were mixed at eclosion and allowed to interbreed *en masse* for five discrete generations in a large population cage at 25°C. More than 100 single-pair matings were set up from this cage and parents were genotyped for *hsr-omega*$^{L/S}$. A subset of 15 of these lines were selected for testing according to genotype; five lines fixed for the L allele, five for S and five H lines (50% for each allele). Hybrid lines were reared on treacle medium at 25°C with constant light.

Genotyping – *In(3R)P* cytology

Males from the derived 'hybrid' lines that were fixed for *L* and *S* were crossed to a line known to have the standard arrangement of chromosome 3R (Celera, virgin females) and larval progeny were genotyped for the heterozygous loop. Polytene squashes were carried out as described by Yoon *et al* (1973). Briefly, salivary glands were dissected from third instar larvae in Becker Ringer's solution and placed in 45% acetic acid for 10 s, then in 1 M HCl for 30 s, before staining in lacto-acetoorcein for 40 min. After staining, glands were transferred to lactoacetic acid to visualize the glands. The preparation was covered with a cover slip and squashed and examined with ×100 microscopy. The third chromosome was recognized by landmark banding patterns and the presence/absence of an inversion heterozygous loop was recorded.

Genotyping – *hsr-omega*

Single fly DNA extraction was performed using a modified proteinase K method (Saiki *et al*, 1987). Single flies were ground in 100 µl 10 mM Tris-HCl pH 8.2, 1 mM EDTA, 25 mM NaCl and 200 µg/ml Proteinase K. Homogenates were left at room temperature for 25–30 min and then heated to 95°C for 2 min to inactivate proteinase K. DNA (10 µl) was used as template in the PCR amplification (25 µl final volume). Two primers, 5′-GCA GTC TGA GGC AGT TAT CC-3′ and 5′-CAA TCT TTC AAA ATC CGC AG-3′, were designed using the EMBL database (DM18307) to give fragments of 86 and 94 bp for the *hsr-omega*^{S} and the *hsr-omega*^{L} alleles, respectively. The PCR products were electrophoresed through horizontal 10% thin-layer polyacrylamide gels or for large-scale genotyping, through 3.5% agarose-1000. For Southern blots, DNA extractions were carried out on 25 adults that were glass-pestle ground using a standard proteinase K/phenol/chloroform protocol. The DNA (~3.5 µg/µl) was digested with *Pst*I and *Hind*III (that cut outside but not within the tandem repeat segment) overnight at 37°C. A probe was produced from wildtype genomic DNA (RNase treated) using primers (5′-CGA AAA GGC TTA TCC TCT TGG, 5′-ATA GTG ATT GGG GTA ATC GGG) that bind to each end of the hsr-omega 280 bp tandem repeat unit. PCR amplification entailed 36 cycles of 94°C (5 min), 47°C (30 s) and 72°C (10 s). The product was purified using Wizard PCR Preps (Promega) and used in conjunction with a GIGAprime DNA Labelling Kit (Geneworks) to make the ^{32}P-labelled probe for hybridization.

Thermal tolerance

Thermal tolerance has been variously measured using mortality assays, knockdown assays or recovery assays (Parsons, 1977; McColl *et al*, 1996; Gibert *et al*, 2001). Often, for any one type of stress (eg heat) different physiological and genetic mechanisms have been implicated (Hoffmann *et al*, 1997), at least in part. In order to maximize our chances of detecting effects of mechanisms that may be relevant to natural populations, we have used two different knockdown assays to measure heat resistance, and both a mortality assay and recovery assay for cold tolerance.

Heat tolerance

Two measures of heat tolerance were applied. First, the set of 15 hybrid lines and their two parental isofemale lines were tested for heat knockdown as single flies (females) in small, capped, vials (5 mm by 20 mm) emerged in a circulating waterbath tank at 39°C with a clear glass side for scoring. Adults were reared at 25°C and aged in fresh-medium bottles for 3–4 days prior to testing. Knockdown time was scored in batches of six lines of 20 flies per line (10 nonhardened and 10 hardened). For hardening, flies were placed in a 37°C incubator for 1 h and aged for a further 6 h at 25°C before being tested. Two lines (one parental and one H line; both nonhardened) were included in all batches with the four test lines as internal standard to control for variation between batches. For analyses individual knockdown times were divided by the mean knockdown times of all control flies from the batch, and for ANOVA the data were square-root transformed.

Second, heat-knockdown was carried out in a large knockdown tube, 80 cm long, with an internal diameter of 9.5 cm. Adults were reared and hardened as for the small-vial heat-knockdown test. The tube was surrounded by a water jacket attached to a Ratek circulating bath set at 39°C. The temperature within the tube was 38.5–39°C. Each knockdown run involved a comparison of four lines (150 flies each; sexes mixed): three test and one control line. Lines in the same knockdown run were distinguished by marking them with different coloured (pink, orange, yellow, no dust) fluorescent dusts (Helecon, Hackettstown, NJ, USA). Flies were placed in empty vials with about 3.5 mg of fluorescent dust and allowed to recover from dusting for 24 h in fresh bottles, before testing. Flies were not anaesthetized at any time. After collection from heat knockdown in a chronological series of vials, flies were frozen and later sorted and counted according to dust colour under a dissecting microscope.

Tolerance for each line was recorded as average time taken for individual flies to knockdown (as a ratio of the control line) based on 150 flies in each of three replicate runs.

Cold tolerance

This was measured using a chill coma assay (Gibert *et al*, 2001) and a mortality assay (Hoffmann and Parsons, 1993). Briefly, for the chill coma assay single adult flies were exposed to 0°C for 16 h in glass vials. Flies were then transferred to 25°C and recovery time measured every minute. A fly was considered as having recovered when able to stand on its legs. For the mortality assay, cold tolerance was determined by exposing vials of 20 females to −2°C for 2 h and survivors were scored after 24 h. Tests on the hybrid-line set involved preculture at 25°C in constant light. Number of survivors were analysed by nested ANOVA.

Statistics

Climatic data, downloaded from the Australian Bureau of Meteorology website (www.bom.gov.au), were obtained from neighbouring weather stations closest to the collection site. The climatic variables were: average daily maximum temperature (°C) for the hottest calendar month; average daily minimum temperature (°C) for the coldest calendar month; mean monthly rainfall for the wettest month; mean monthly rainfall for the driest month; mean humidity levels at 9 am for the most humid month; and mean humidity levels at 9 am for the driest month. Temperature, rainfall and humidity averages were based on all the available data for the particular weather station usually exceeding 100 years. Associations between latitude were initially tested using linear regression (SPSS 7.0 for Windows). To test for associations between allele frequency, latitude and climatic variables, Mantel matrix comparison tests (and partial mantel tests using matrices of residuals based on standardized differences) were carried out (Fortin and Gurevitch, 1993). These tests minimize the complications of spatial autocorrelation (nonindependence of data points). Matrix comparisons utilized GENEPOP, version 3.1 (http://www.cefe.cnts.mop.ft/ Raymond and Rousset, 1995).

Results

The *hsr-omega* association with heat tolerance in laboratory studies and with latitude, previously reported (McColl *et al*, 1996), is for an A/T variation at the 5′ end of the gene that is detected by denaturing gradient gel electrophoresis (DGGE; Figure 2a, b). Since scoring large samples by DGGE is time consuming, and since linkage disequilibrium across many polymorphic sites has been indicated for this region (McKechnie *et al*, 1998), to expedite scoring of allelic variation, we developed a simple gel technique for an adjacent polymorphic site,

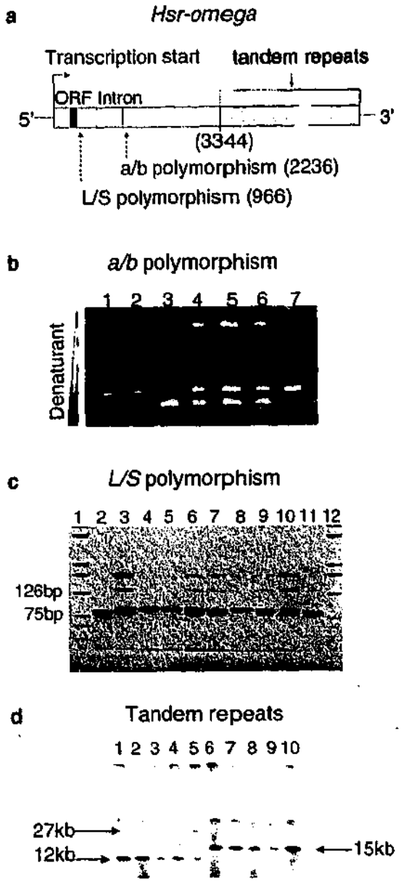

Figure 2 (a) *hsr-omega* structure and polymorphic sites. ORF marks a potential open reading frame. (b) Separation of *hsr-omega*^{a} and *hsr-omega*^{b} alleles by denaturing gradient gel electrophoresis. Lanes 1, 2 and 7: *hsr-omega*^{a} homozygotes, lane 3: *hsr-omega*^{b} homozygotes and lanes 4–6: *hsr-omega*$^{a/b}$ heterozygotes. Top bands in lanes 4–6 are heteroduplex bands. (c) Separation of *hsr-omega*^{L} and *hsr-omega*^{S} alleles by horizontal thin-layer polyacrylamide gels. Lanes 1, 12: pGem DNA marker (Promega); lanes 2, 11: *hsr-omega*^{S} homozygotes; lanes 3, 6, 7, 9, 10: *S/L* heterozygotes; lanes 4, 5, 8: *hsr-omega*^{L} homozygotes. (d) Southern blot of hybrid lines. Lanes 1–5: L1–L5 lines, respectively, lanes 6–10: S1–S5 lines, respectively.

an 8 bp indel polymorphism (Figure 2a, c). The insertion-bearing allele, L, occurred within chromosome sequences that encoded *hsr-omega*^{a} (McKechnie *et al*, 1998). We refer to the indel variants as the *hsr-omega* long (*hsr-omega*^{L} or L) and *hsr-omega* short (*hsr-omega*^{S} or S) alleles, respectively, indicating the presence (L) or absence (S) of the indel. More distally, at a 3′ position in the gene, extensive variation in the number of tandem repeats occur. Some laboratory strains have as few as 5 kb of a 280 bp repeat unit and others as many as 16 kb of these repeats (Hogan *et al*, 1995). We also detected the repeat-length variation at the 3′ end of the gene using Southern blots (Figure 2a, d).

To look for thermal phenotype differences associated with the chromosome 3 variation, we created a set of lines that were controlled for the chromosomal region tightly linked to the L/S variation but that had randomized genetic backgrounds for other genetic differences that occur between high and low latitude populations. We examined the set of five L and five S lines for the presence of *In(3R)P* by crossing each line to a stock known to be homozygous for the standard chromosome 3 arrangement and cytologically inspected the F1 larval salivary gland squashes. Without exception the derivation process resulted in fixation of the L lines for the standard arrangement and fixation of the S lines for the inverted arrangement. We also examined these 10 lines for the 3′ repeat-length variation of *hsr-omega* and found that the different sets were virtually fixed for different repeat-length alleles (Figure 2d). The S lines were each homogeneous for repeats totalling about 15 kb in length and the L lines for repeats with a total length of about 12 kb (except for one variable line that contained both 12 and 27 kb repeats). The data suggest that the original two north and south lines chosen for hybridization had been fixed for either the noninverted right arm of chromosome 3 (the southern L line) or the inverted (*In(3R)P*) right arm of chromosome 3 (the northern S line). L and S have acted as markers for linked blocks of genes in this region of chromosome 3. In this instance, S marked both the presence of *In(3R)P* and an allele of *hsr-omega* that contained about 15 kb of 3′ repeats and no 8 bp indel at the L/S site.

Using a small-vial tolerance test, the hybrid lines and their parental strains were tested for heat resistance, both with and without a prior mild-heat hardening treatment. The relative heat resistance, for both unhardened and hardened flies, as measured by average knockdown time is depicted in Figure 3a. No significant difference between the parental strains was detected. However, a significant effect of genotype was indicated by nested ANOVA when the analysis was carried out on the 10 lines marked by L and S ($F_{1,8} = 6.60$, $P < 0.05$), with the L lines being more heat susceptible than the S lines (and no suggestion of any interactions). Effects of hardening ($F_{1,8} = 1.029.2$, $P < 0.001$) and of line within genotype ($F_{8,180} = 3.81$, $P < 0.001$) were detected. A genotype by hardening interaction effect ($F_{2,12} = 5.51$, $P < 0.05$) was detected when the five H lines, which were each polymorphic for the L/S markers, were included in the analysis. This was because of the H lines displaying a relatively high, unhardened heat resistance like S lines, but a hardened heat resistance that was relatively low like the L lines. These data support the hypothesis that the chromosome 3 segment marked by the L/S variation is part of the genetics that underlies an individual's susceptibility to heat stress.

The different ways that an organism experiences thermal stress may challenge different physiological and genetic mechanisms. To examine the generality of any effect of this variation on heat resistance, a large-tube heat knockdown test was also carried out on batches of adults from four of the hybrid lines, two L lines and two S lines, both with and without prior hardening. Relative knockdown times for nonhardened lines did not indicate a significant genotype, or line within genotype, effect. However, relative knockdown times for the heat-hardened lines indicated a significant difference between

genotypes ($F_{1,2} = 55.33$, $P < 0.05$), with no effect of line (Figure 3b). This difference in sensitivity between hardened and nonhardened heat tolerance tests for detecting variation between L and S lines is consistent with previous results indicating that *hsr-omega a/b* variation has a larger differential effect for hardened resistance (McColl *et al*, 1996). The L lines, carrying the chromosome 3 genotypes that occur at higher frequency in cool-temperate Australia, were also the more heat susceptible in this alternate test of heat stress, having shorter relative knockdown times after hardening than the S lines. Thus, for two different measures of heat-resistance S lines outperformed L lines.

We also measured cold tolerance on the set of hybrid lines and their two parental strains using a mortality assay after a short exposure to −2°C. Significant variation occurred among genotypes ($F_{2,14} = 21.40$, $P < 0.001$) with no effect of line within genotype. L lines had a higher cold tolerance (Figure 3c), with a Tukey *post hoc* test indicating that only this genotype was clearly different to the S and H line groups. The parental line that originated from the southern cool-temperate region (that carried the L marker) clearly had a higher cold tolerance than the northern-derived parental line (that carried the S marker). We also used a chill-coma recovery measure of cold tolerance to see if the hybrid-derived L lines were more cold tolerant than the S lines in this different measure of cold sensitivity (Figure 3d). Again, the L lines were more resistant, recovering faster from the chill coma in both females ($F_{1,121} = 24.73$, $P < 0.01$) and males ($F_{1,116} = 5.70$, $P < 0.05$). The chromosome 3 element marked by L therefore provided elevated cold resistance over S-marked elements for two different types of cold stress. The data suggest that the alternate genetic elements fixed in the S and L lines each provide a positive boost to fitness under one type of thermal stress at the cost of conferring a negative fitness effect at the opposite temperature extreme.

We asked if the *hsr-omega* $^{L/S}$ variation marked a region of chromosome 3 that varied clinally as did the previously reported *hsr-omega*$^{a/b}$ variation. Frequency estimates of the L allele along a latitudinal transect of Australian east-coast sites (Figure 4a), all taken at low altitudes of <200 m, are plotted in Figure 4b and indicate a positive association with latitude. This geographical L/S variation is spatially autocorrelated, since a Mantel difference-matrix comparison between all sites considered pairwise for L allele frequency and linear distance indicated a highly significant association ($P < 0.0001$). Almost exactly the same level of association occurred if latitudinal differences are substituted for linear distance in the matrix comparison. Further, when population differences in L frequency are aligned with geographic separation distances in a partial Mantel test (Fortin and Gurevitch, 1993) that controls for latitudinal differences,

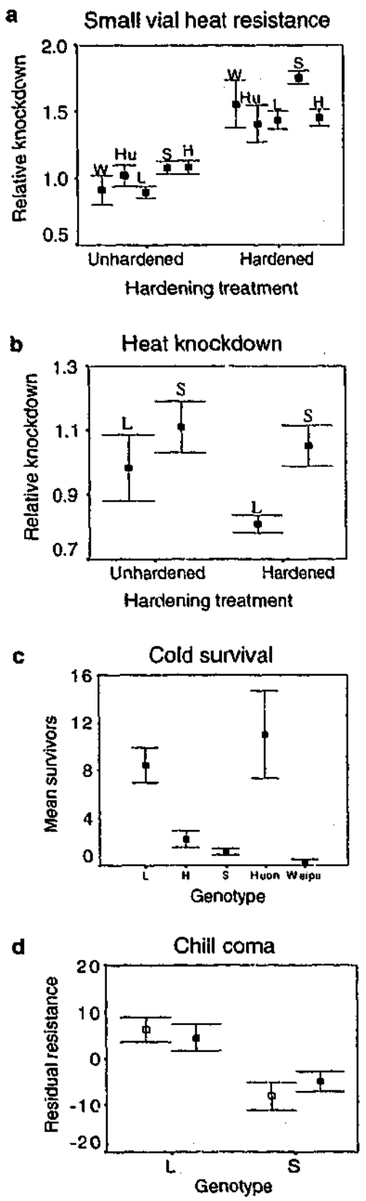

Figure 3 Thermal tolerance measurements. (a) Small-vial heat resistance of hardened and unhardened parental (W: Weipa, Hu: Huon) and hybrid lines (L: fixed for *hsr-omega*^{L}, H: 50% each allele, S: fixed for *hsr-omega*^{S}). ■: means based on 10 replicates for parental lines, 10 replicates for each of five lines for hybrid lines. Error bars represent standard error of the mean. (b) Large tube hardened and unhardened knockdown of two L lines and two S lines. ■: mean relative knockdown when compared to a control line, based on three replicates. Error bars represent standard error of the mean. (c) Mean survival after exposure to −2°C for 2 h of parental (Huon and Weipa) and hybrid (L, H, S) lines. ■: mean number of flies surviving based on four replicates for each of five lines in the hybrid lines, and four replicates for parental lines. Error bars represent standard error of the mean. (d) Estimates of chill coma recovery after a treatment of 16 h at 0°C. □: mean female residual recovery time based on measurements of 15 flies for each of four lines (S) and five lines (L). ■: mean male residual recovery time based on measurements of 15 flies for each of four lines (S) and five lines (L).

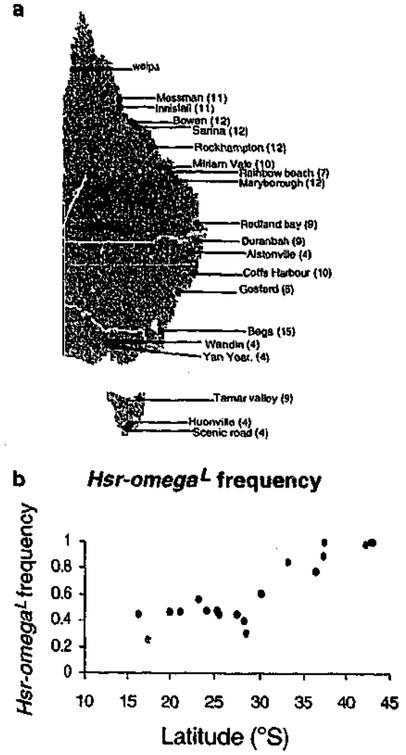

Figure 4 Clines. (a) Map of collection sites from the 3000 km transect on the eastern coast of Australia. Numbers in brackets represent number of isofemale lines genotyped for *hsr-omega*$^{L/S}$ variation (Weipa excluded from latitudinal analyses). Latitudes of sites: Weipa 12.40°S, Mossman 16.27°S, Innisfail 17.32°S, Bowen 20.01°S, Sarina 21.25°S, Rockhampton 23.22°S, Miriam Vale 24.19°S, Rainbow Beach 25.54°S, Maryborough 25.31°S, Redland Bay 27.39°S, Duranbah 28.18°S, Alstonville 28.5°S, Coffs Harbour 30.19°S, Gosford 33.25°S, Bega 36.41°S, Yan Yean 37.34°S, Wandin 37.45°S, Tamar Valley 42.24°S, Huon 43.01°S, Scenic Rd 43.03°S. (**b**) Estimates of *hsr-omega*^{L} frequency of 19 collection sites.

or aligned with latitudinal differences in a partial Mantel test that controls for separation distance, the significant associations are completely lost. Since the sample sites were chosen to lie along a near linear, north–south transect, these latitude and linear distance comparisons are behaving as one-and-the-same variable in the Mantel tests. The significant associations confirm the presence of a latitudinal/spatial cline in frequency of that region of chromosome 3 marked by the L allele among Australian east-coast populations, as previously indicated for the *hsr-omega*^{n1} variation (McColl and McKechnie, 1999). If strong population disequilibrium occurs between the L/S variation and *In(3R)P*, clinal variation of *In(3R)P* could also underlie the clinal variation in this gene. Ongoing investigations should resolve these issues.

Using these latitudinal-transect data we examine L frequency associations with six climatic variables (temperature, rainfall and humidity statistics) in simple difference-matrix Mantel tests. Three of these, average maximum temperature for the hottest month, average minimum temperature for the coldest month and monthly rainfall for the wettest month, show significant associations (all $P<0.0001$). Since population differences for each of these three climatic variables are significantly associated with latitude differences (all $P<0.01$), partial Mantel tests that controlled for latitude were undertaken. Only one of these, the partial Mantel test for maximum temperature differences, retained a significant association with L differences ($P<0.01$). The association between L and latitude differences is completely removed ($P=0.12$) in the partial Mantel test that controls for maximum temperature. Therefore, the L frequency differences between populations are robustly associated with population differences in maximum temperature in the hottest month, but not with latitude or with the other climatic variables. These data support the hypothesis that environmental temperature directly or indirectly acts as a proximate selective factor for the region of chromosome 3 marked by the L/S variation.

Discussion

Our field collection suggests that we have identified in *D. melanogaster* a major genetic element that occurs at high frequency in tropical populations from coastal eastern Australia and decreases in frequency at higher latitudes, being replaced clinally by the alternate element. The tolerance data support the idea that the element can have substantial effects on both heat and cold tolerance. These results are consistent with earlier data indicating that allelic variation of *hsr-omega* can underlie heat-tolerance variation (McKechnie *et al*, 1998). They are also consistent with an earlier study indicating that cold-tolerance variation in both adult and larval *D. melanogaster* is largely attributable to a chromosome 3 effect (Tucic, 1979).

What is the nature of the element marked by *hsr-omega* L/S? It seems likely that for our hybrid line data this element is the common cosmopolitan inversion *In(3R)P*, a large inversion covering about six major cytological units and therefore about 6% of the genome (perhaps 1000 genes). In an earlier study, the frequency of *In(3R)P* varied clinally in Australia, along almost the same east coast transect, being present at frequencies up to 60% in the north and almost absent in temperate populations (Knibb, 1982). The frequency of *In(3R)P* has been associated with latitude in several other continents, along with the other common cosmopolitan inversions (Knibb, 1982). What factors other than thermal tolerance may influence the *In(3R)P* cline? *In(3R)P* has also been postulated to be at a disadvantage at high latitudes because it is associated with reduced flight ability under cooler conditions, possibly related to decreased body size (Stalker, 1980). Body size increases with latitude (Partridge *et al*, 1999) and two microsatellite markers that associate with latitude and map within *In(3R)*P (Gockel *et al*, 2001) have also been related to body size in a QTL study (Gockel *et al*, 2002). Clearly, some fine disecction of variable clinal markers and trait associations in this chromosomal region is required to tease apart the chromosomal regions and selective processes at work. However, if the *In(3R)P* cline is still present in Australia, which seems likely, it may be this element that contributes to the opposing clines in tolerance to both temperature extremes (Hoffmann *et al*, 2002).

While an effect of inversions is to maintain coadapted blocks of genes that are inherited as a group (Lewontin, 1974; Krimbas and Powell, 1992), recombination at reduced frequency occurs for the common inversions and linkage disequilibrium is not always strong, especially for polymorphic sites centrally placed in the inversion (Hasson and Eanes, 1996; Andolfatto and Kreitman, 2000; Verrelli and Eanes, 2000). A number of single gene polymorphisms in *Drosophila*, that lie within or in close proximity to inversions, have shown various levels of population disequilibrium with the inversion, yet display independent clinal variation or climatic associations (Knibb, 1983; Van't Land *et al*, 2000). Thus, the genetic element responsible for these temperature tolerance effects might be smaller than that covered by *In(3R)P*. A group of tightly linked genes surrounding the *hsr-omega* alleles that were isolated in these lines could be the causal elements. If a genetic element smaller than the inversion were to underlie the L/S tolerance associations, we still need to be aware that these fitness associations might not be typical of all *hsr-omega* L and S alleles, given that our crosses started with two isofemale lines. Likewise, our data does not exclude the possibility that other *In(3R)P* chromosomes fail to show these thermal tolerance effects. Nonetheless, our data indicate that large thermal tolerance fitness effects, with potential to be ecologically relevant, are caused by a genetic element marked by *hsr-omega* L/S variation.

What are the candidate genes on the right arm of chromosome 3 that causally affect thermal tolerance and may be part of a coadapted genetic element? Four obvious candidates are *hsr-omega*, *hsp68*, *dca* and *frost*. *Frost* and *Dca* are two genes that are upregulated by cold treatment and map to chromosome 3R at 85E2 and 88D, respectively (Goto, 2000, 2001) (Figure 1). Of these four genes, the expression patterns and potential phenotype affects for *hsr-omega* are the best understood. The evidence is now substantial that allelic variation in *hsr-omega* underlies a significant fraction of the heat sensitivity variation in *D. melanogaster* (McColl *et al*, 1996; McKechnie *et al*, 1998). *Hsr-omega* is both constitutively expressed in most tissues and upregulated by environmental factors. The relative and total levels of two major *hsr-omega* RNA transcripts are labile and fast to change in response to many environmental signals. The gene is thought to play a role in the dynamic coordination of nuclear and cytoplasmic transcript processing under various stress and growth conditions (Garbe *et al*, 1989; Pardue *et al*, 1990; Lakhotia *et al*, 1999; Lakhotia *et al*, 2001). However, we need to be cautious about assigning any genotype effect on thermal sensitivity directly to the differential function of *hsr-omega* alleles. If thermal selection in the field is responsible for the *hsr-omega*^{L} cline, the target of such selection may be groups of genes in linkage disequilibrium with *hsr-omega* variation. Transgenic studies with molecular constructs of *hsr-omega* that vary only at the relevant polymorphic sites are required to provide unambiguous evidence of effects of any single gene on these thermal fitness components.

In summary, our data suggest that polymorphic variation of *hsr-omega* is a marker for a genetic element on the right arm of chromosome 3, quite possibly *In(3R)P*, that provides insight into the genetics of adaptation to thermal extremes in a species that is widespread across diverse climatic regions. Variation of this element, at least in part, explains the known opposing thermal tolerance clines that occur along the eastern coast of Australia. The element found at high frequency in the tropics provides high adult heat tolerance at this end of the cline but incurs a trade-off in also causing cold sensitivity. The element is clinally replaced in cool-temperate southern populations by an alternate element with reciprocal effects. Such clinal polymorphic variation may help the species have a broad climatic range and be a thermal specialist at each end of the range where perhaps only one thermal extreme is regularly encountered; each element, in the right climate, may provide the appropriate thermal resistance benefits with little cost. Limited gene flow along the transect could serve to smooth any 'bumps' in the cline if thermal selection-intensity thresholds happen to change abruptly over space. Also, the polymorphism at any particular latitude might provide seasonal population flexibility where temperature extremes in one generation are interspersed with the opposite extreme several generations later. In this simplistic model, the fitness trade-offs would facilitate selection that leads to seasonal changes in frequency, as has been reported for *In(3R)P* (Stalker, 1980). These data represent one of the few cases where opposing clinal selection on alternative genetic elements has been demonstrated and linked to geographic patterns and adaptive phenotypes.

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