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Comparative population genomics of latitudinal variation in *Drosophila simulans* and *Drosophila melanogaster*

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Abstract

Examples of clinal variation in phenotypes and genotypes across latitudinal transects have served as important models for understanding how spatially varying selection and demographic forces shape variation within species. Here, we examine the selective and demographic contributions to latitudinal variation through the largest comparative genomic study to date of *Drosophila simulans* and *Drosophila melanogaster*, with genomic sequence data from 382 individual fruit flies, collected across a spatial transect of 19 degrees latitude and at multiple time points over 2 years. Consistent with phenotypic studies, we find less clinal variation in *D. simulans* than *D. melanogaster*, particularly for the autosomes. Moreover, we find that clinally varying loci in *D. simulans* are less stable over multiple years than comparable clines in *D. melanogaster*. *D. simulans* shows a significantly weaker pattern of isolation by distance than *D. melanogaster* and we find evidence for a stronger contribution of migration to *D. simulans* population genetic structure. While population bottlenecks and migration can plausibly explain the differences in stability of clinal variation between the two species, we also observe a significant enrichment of shared clinal genes, suggesting that the selective forces associated with climate are acting on the same genes and phenotypes in *D. simulans* and *D. melanogaster*.

Keywords: comparative genomics, *Drosophila*, latitudinal cline, latitudinal variation, parallelism, population genomics

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Introduction

Latitudinal transects have been studied across the tree of life, in a large number of bacteria, plant and animal species, revealing phenotypic and genetic clines (Feder & Bush 1989; Weber & Schmid 1998; Salgado & Pennings 2005; Fuhrman *et al.* 2008; Baumann & Conover 2011). A correlation between phenotypic variation and latitude is suggestive of local adaptation. For example, local adaptation to temperature is implicated in the correlation between decreased lifespan and latitude in ectotherms (Munch & Salinas 2009), and local adaptation to photoperiod is implicated in the correlation between flowering time and latitude in plants (Keller *et al.* 2011). However, neutral demographic processes also generate clinal variation. For example, 'isolation by distance', where gene flow is decreased between geographically distant populations, can produce patterns of variation similar to those resulting from local adaptation (Endler 1977). Strong patterns of clinal variation can also be generated by introgression between separate invading populations (Cruzan 2005) or range expansion of a single founding population (Excoffier *et al.* 2009). These demographic processes can be coincident with selective processes. Although disentangling selective and demographic scenarios is challenging, genomic data sets have the power to identify patterns associated

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either with selection or with demography. We perform a genomic study across two closely related *Drosophila* species, allowing us to elucidate general patterns that are shared between the species as well as refine our understanding of how the processes underlying clinal variation differ between these species.

The genus *Drosophila* represents a powerful system for the study of selection and demography. This group is composed of several species with broad distribution and represents Old and more recent New World colonizations. *Drosophila melanogaster* has been studied extensively in a latitudinal context. Several phenotypic traits and genetic loci vary with latitude in *D. melanogaster* (Vigue & Johnson 1973; Mettler *et al.* 1977; Voelker *et al.* 1977; Knibb *et al.* 1981; Oakeshott *et al.* 1982; Singh *et al.* 1982; David *et al.* 1985; Coyne & Beecham 1987; James *et al.* 1995; Munjal *et al.* 1997; Karan *et al.* 1998; Schmidt *et al.* 2000, 2005 2008; Gockel *et al.* 2001; Mitrovski & Hoffmann 2001; Hoffmann *et al.* 2002; Sezgin *et al.* 2004; Pool & Aquadro 2007; Emerson *et al.* 2009; Paaby *et al.* 2010), and *D. melanogaster* latitudinal variation has been studied in a genomic context and on multiple continents (North America, Australia, Europe, Asia and Africa) (Turner *et al.* 2008; Kolaczkowski *et al.* 2011; Fabian *et al.* 2012; Bergland *et al.* 2015; Reinhardt *et al.* 2014). One advantage to using *D. melanogaster* for the study of adaptation to latitude is that it is a relatively recent colonizer of temperate climates (10 000−20 000 years since expansion out of central Africa; Lachaise *et al.* 1988; Li & Stephan 2006). Temperate-adapted characters such as cold tolerance and starvation resistance are more pronounced at higher latitudes in *D. melanogaster*, suggesting that clinal variation in *D. melanogaster* is a result of local adaptation to temperate climates (Karan *et al.* 1998; Hoffmann *et al.* 2002; Schmidt *et al.* 2008). Additionally, there is some parallelism in clinal allele frequency patterns along the North American and Australian latitudinal clines, suggesting that there has been convergent adaptation to latitude (Turner *et al.* 2008; Fabian *et al.* 2012; Reinhardt *et al.* 2014). The *D. melanogaster* latitudinal clines are also subject to confounding demographic effects. Both North American and Australian populations seem to be a result of admixture (either pre- or postcolonization) between European and African populations (Duchen *et al.* 2013; Bergland *et al.* 2015; Kao *et al.* 2015). Although the *D. melanogaster* latitudinal clines are robust and some do seem to result from local adaptation, demography complicates the inference of selection.

Comparative studies can help us understand general patterns of latitudinal variation. The sister species *D. simulans* and *D. melanogaster* ($\sim 3 \times 10^6$ years diverged; Hey & Kliman 1993) represent a powerful system for comparative study. These species are similar in their range, ecology and evolutionary history (Cariou 1987; Hey & Kliman 1993). They have experienced parallel expansions out of Africa, adaptation to temperate climates and development of human commensalism (David & Capy 1988; Lachaise *et al.* 1988; Lachaise & Silvain 2004). Unfortunately, the limited amount of research on clinal variation in *D. simulans* has made a large comparative study of latitudinal variation impossible.

While *D. simulans* exhibits clinal variation in some of the same traits as *D. melanogaster* (pigmentation: David *et al.* 1985; body size: Arthur *et al.* 2008), *D. simulans* also seems less temperate adapted (McKenzie & Parsons 1974; Gibert *et al.* 2004; Arthur *et al.* 2008). For example, *D. simulans* has less physiological tolerance to cold and starvation (reviewed in Hoffmann & Harshman 1999). Another key clinal trait in *D. melanogaster* is a reproductive diapause, which is hypothesized to be important for survival through the high-latitude winters (Saunders *et al.* 1989; Schmidt & Conde 2006). Reproductive diapause has not been observed in *D. simulans*. Certain phenotypes that are clinal in both species vary less with latitude in *D. simulans* than in *D. melanogaster* (starvation: Arthur *et al.* 2008; desiccation: McKenzie & Parsons 1974), supporting the hypothesis of a more shallow cline in *D. simulans* (reviewed in Gibert *et al.* 2004).

While local adaptation could explain the above patterns, a shallow cline in *D. simulans* could also result from demographic patterns. Contemporary demographic patterns such as seasonal bottlenecks and migration may contribute to clinal variation. Although the true demographic patterns in *D. simulans* are not known, *D. simulans* has been hypothesized to experience strong bottlenecks and/or employ migratory behaviour in response to seasonal fluctuations. This is supported by the temporal abundance patterns found along latitudinal clines in Europe and North America (Boulétreau-Merle *et al.* 2003; Fleury *et al.* 2004; Schmidt 2011; Behrman *et al.* 2015). Specifically, *D. simulans* tends to be in greater relative abundance in the more equatorial populations and does not appear at the higher latitudes until later in the year than *D. melanogaster*. Additionally, in temperate North America, there are distinct differences between *D. melanogaster* and *D. simulans* in the population age structure across seasonal time that are also indicative of different overwintering strategies (Behrman *et al.* 2015). In *D. melanogaster*, the earliest observed spring populations have a uniformly young age distribution, shifting to a heterogeneous age distribution over time. This pattern is consistent with populations that overwinter locally. In *D. simulans* the earliest observed postwinter populations are already age heterogeneous, which is more con-

sistent with annual recolonization from a refugia (either local or more distant) than with in situ overwintering. The *D. simulans* relative abundance and age distribution patterns can be explained by either (i) annual extirpation and recolonization of high-latitude populations, (ii) in situ overwintering and maintenance of a small resident population or (iii) both a strong annual bottleneck and subsequent input of migrants *with* the maintenance of a small resident population. Each of these scenarios would also contribute to a shallow cline.

Genomic analyses of latitudinal variation have been performed in *D. melanogaster*; however, no such studies exist for *D. simulans*. Genomic data sets are critical to understanding general patterns of clinal variation. With genomic data, we can statistically differentiate subtle patterns, such as the enrichment of functional genic classes and parallelism in clinal variants between species. Here, we present a multiyear, multiseason, genomewide analysis of population differentiation and latitudinal variation in *D. simulans* and *D. melanogaster*. We directly compare the amount of clinal variation in *D. simulans* and *D. melanogaster* using these genomic data and confirm that, in line with phenotypic observations, *D. simulans* has less clinal variation than *D. melanogaster*. We find evidence for a strong contribution of annual variation to *D. simulans* population genetic structure, which is not found in *D. melanogaster*. The strong, stable cline in *D. melanogaster* is a stark contrast to the weak cline seen across *D. simulans* populations, where we see greater evidence of processes that increase differentiation from year-to-year, such as migration and bottlenecks. We also observe signatures of spatially varying selection in *D. melanogaster* and to a lesser extent in *D. simulans*, and evidence for convergent evolution of clinal variation across genes.

Materials and methods

Sequence data

D. simulans. We sampled individuals from four *D. simulans* populations along the East Coast of North America, spanning 19 degrees latitude (Table S1, Supporting information). From north to south, the population and year of collection are as follows: Maine 2011 (ME), Pennsylvania 2011 (PA), Virginia 2010 (VA) and Florida 2011 (FL). Three separate samples of the PA population were taken, one each in August, September and November (named PA8.2011, PA9.2011 and PA11.2011, respectively). Populations were sampled by direct aspiration of flies from substrates and by collection with banana and yeast baited traps. We extracted DNA from a total of 267 female flies (an average of $\sim$50 files per sample) using Favorgen 96-well genomic DNA extraction kits and quantified the DNA with a Picogreen fluorescence assay. Moleculo (now Illumina TruSeq) performed the per-individual library preparation and sequenced paired-end 100-bp reads on an Illumina HiSeq 2000. Depth of sequencing coverage per individual varied from 0.01× to 5×. We aligned reads to the *D. simulans* v2 reference genome (Hu *et al.* 2013) with BWA version 0.6.2 *aln* and *sampe* functions (default parameters; Li *et al.* 2009). We performed PCR duplicate removal with SAMtools version 0.1.19 (Li *et al.* 2009) and indel realignment with GATK version 2.4 (McKenna *et al.* 2010). A total of $\sim$3.2 M single nucleotide polymorphisms (SNPs) were called with GATK Unified Genotyper version 2.4 using all reads combined (DePristo *et al.* 2011). Genotype calls were made per individual for each SNP (ploidy 2). For each individual and SNP, we randomly chose a single chromosome of the diploid genotype for use in the final analysis, to avoid bias from individuals with higher coverage. SNPs were then filtered for $\sim$2.5 M nucleotides of repetitive DNA identified using RepeatMasker (Smit *et al.* 2013). We also filtered out high coverage sites (upper 95th quantile) and low coverage sites (<5× per collection site). For consistency with the *D. melanogaster* data, we filtered out sites within 5 bp of an indel, with low minor allele frequency (MAF) sites (mean MAF <10% across the four collection sites) and that were non-bi-allelic. Allele frequencies for each population were calculated relative to the reference genome. The filtered data set had a mean coverage of 20× per population, with 2.2 M SNPs on autosomes and 0.3 M SNPs on the X chromosome. For functional analysis, we used a *D. simulans* cDNA-guided genome annotation (Rogers *et al.* 2014) and the SNP functional annotator snpEff v4.0 (Cingolani *et al.* 2012).

We also sequenced two 2010 temporal samples (July and September) from the Pennsylvania population (named PA7.2010 and PA9.2010, respectively). These population samples consisted of male files sequenced using pooled population sequencing (pool-seq), where individuals from each sample were pooled prior to DNA extraction and sequencing. We extracted DNA from these two samples using a lithium chloride precipitation. Sequencing library construction followed the protocol described in Bergland *et al.* (2014). The samples were sequenced with paired-end 100-bp reads on an Illumina HiSeq 2000. The effective number of chromosomes (N_C) represented in the pooled samples was calculated as

$$N_C(N,R) = \left(\frac{1}{N} + \frac{1}{R}\right)^{-1} \quad (1)$$

where N is the number of chromosomes in the pool, and R is the read depth at that site (Kolaczkowski *et al.*

2011; Feder *et al.* 2012; Bergland *et al.* 2014). This adjusts for the additional error introduced by sampling of the pool at the time of sequencing. Sequencing reads mapped to autosomes were down-sampled to match the N_C of the X chromosome.

D. melanogaster. We compared *D simulans* data to published *D. melanogaster* data from a study conducted by Bergland *et al.* (2014). Three of the four *D. melanogaster* collection sites (FL, PA, ME) were the same as the
D. simulans collection sites. The fourth *D. simulans* site (VA) was imperfectly matched to a *D. melanogaster* Georgia (GA) collection site (Table S1, Supporting information). We used two *D. melanogaster* temporal samples of the PA site (November 2009 and November 2010). The other sites were sampled once each; FL in 2010, GA in 2008 and ME in 2009. Bergland *et al.* (2014) produced sequence data by pooling males files within each population and sequencing on an Illumina HiSeq 2000. We mapped the raw reads to the *D. melanogaster* genome version 5.5 using BWA version 0.7.9 *aln* and *sampe* algorithms, with default parameters (Li & Durbin 2009). Reads mapping to autosomes were down-sampled to match the N_C of the X chromosome for each population. Allele frequency was calculated relative to the reference allele for each SNP used in Bergland *et al.* (2014) ($\sim$600 K SNPs). SNP calling in Bergland *et al.* (2014) differed from the *D. simulans* SNP calling. The data in Bergland *et al.* (2014) were exclusively pool-seq data, for which SNPs were called using the program CRISP (Bansal 2010). Additional filtering also took place, notably, the exclusion of SNPs not also identified in the *Drosophila* Genetic Reference Panel (DGRP). The differences in SNP calling and filtering, along with real differences in genetic diversity between the two species, account for the smaller number of SNPs in the *D. melanogaster* data set.

Pool-seq error model

In this study, we compare pooled *D. melanogaster* population samples with (primarily) nonpooled *D. simulans* population samples. Pool-seq is known to have inherent errors in allele frequency estimation; therefore, we must take care to model this variance appropriately (Kofler *et al.* 2011; Zhu *et al.* 2012; Lynch *et al.* 2014). This is particularly important for our analysis of the relative proportion of clinal variation in *D. simulans* and *D. melanogaster*. As all of the *D. melanogaster* samples are pooled, these samples inherently have an additional source of error that is not accounted for, resulting in an overestimate of the sample size. As clinal patterns are expected to be more pronounced in *D. melanogaster*, a perceived increase in clinal variation in *D. melanogaster* could be attributed to the pool-seq variance. To arrive at a conservative estimate of clinal variation in *D. melanogaster* a liberal estimate of pool-seq error should be used.

Two methods for accounting for extra variance in pool-seq data are (i) modifying the statistical tests used (e.g. modification of the null expectation, as in Bastide *et al.* 2013) and (ii) translating the additional variance into an effective sample size. We chose the latter, using our comparable barcoded data set to assess the additional pool-seq error. To model the pool-seq error, we compared the level of genetic differentiation among barcoded temporal samples (*D. simulans* PA8.2011, PA9.2011, PA11.2011) with differentiation between pooled temporal samples (*D. simulans* PA7.2010, PA9.2010), with the assumption that within-population samples should have similar amounts of month-to-month variation from one year to the next. This assumption is reasonable, as we observe this to be the case (see below) for populations within a few months of each other. Note that this may not be the case for certain months, particularly for those during or directly following a winter bottleneck. For this analysis, we use the proportion of SNPs found to be at significantly different allele frequencies as a measure of genetic differentiation (Fisher's exact test for each SNP). Only SNPs with a total of 40 chromosomes between the two samples being tested were used to ensure equal power between data sets. The range of chromosomes per population varied (PA8.2011: 8-35; PA9.2011: 8-36; PA11.2011: 4-32; PA7.2010: 18-22; PA9.2010: 22-18). The Fisher's exact test provides a test of the deviation from panmixia (i.e. variation above binomial sampling error) with a standard expectation of a uniform *P*-value distribution and is robust to small and unequally distributed sample sizes. Panmixia is rejected if there is enrichment of differentiated SNPs above the expectation. For pool-seq data, we do not expect a uniform *P*-value distribution under panmixia for two reasons: (i) to account for the two levels of sampling (chromosomes and reads), we use a single effective sample size (N_C), which is close to but not exactly the same as correctly using the convolution of two binomials, and (ii) the average error in allele frequency estimation for pool-seq data may be greater than binomial, even with the effective N_C calculation. We use Fisher's exact test on pooled and nonpooled data to estimate this second error component.

We first tested the differentiation among the three barcoded PA 2011 samples. We found that each of the three comparisons had similar levels of differentiation (between 0.99% and 1.05% of SNPs differentiated at $P < 0.01$, an average of 0.01% over expected; Fig. S1, Supporting information), representing near-uniform *P*-value distributions (the null distribution). In contrast,

the pooled temporal samples (PA7.2010 and PA9.2010) showed an enrichment of differentiated SNPs (1.26% of SNPs differentiated at $P < 0.01$). This is consistent with additional sampling error being introduced in the process of pooled DNA extraction, amplification, sequencing and mapping, resulting in an overestimate of the effective number of chromosomes sampled.

To determine how much additional variance is introduced by pool-seq, above what is accounted for by the $N_C(N,R)$ correction already implemented, we tested two models of pooled error. We used the data from all three barcoded PA temporal comparisons to perform a linear regression of differentiation with increasing N_C, providing the barcoded null model. We then found the additional variance component, which we call ϵ, that results in the best fit of the pooled PA comparison to the barcoded null (lowest sum of square deviations from the null). The first model tested fits an ϵ that is independent of R:

$$N_C(N,R,\epsilon) = \left(\frac{1}{N} + \frac{1}{R} + \epsilon\right)^{-1} \quad (2)$$

The second model tested fits an ϵ that is inversely proportional to R (greater error at lower read depth):

$$N_C(N,R,\epsilon) = \left(\frac{1}{N} + \frac{1}{R} + \frac{\epsilon}{R}\right)^{-1} \quad (3)$$

We found Model 2 (with the R dependence) to be a better fit to the data than Model 1, with a best-fit value of ϵ to be 0.1 (Fig. S5, Supporting information). Using this error model, we can calculate a more conservative N_C, which we use for the calculation of N_C for all pooled samples (*D. simulans* PA7.2010 and PA9.2010 samples; all *D. melanogaster* samples). Applying this $N_C(N,R,\epsilon)$ correction to the pooled *D. simulans* PA7.2010 and PA9.2010 samples, we find a slight depletion of significantly differentiated SNPs compared to the barcoded samples (0.84% at $P < 0.01$, compared with 1.01%). This indicates that our correction for pooled-error results in a conservative estimate of the effective number of chromosomes in a pooled sample.

Use of this correction for pooled error also decreases the average coverage per population. However, even with the use of our pool-seq error correction, our pool-seq libraries are still more efficient in estimating population allele frequency than our barcoded libraries (per raw sequencing read). For example, from 898 M raw barcoded reads, we retrieved a total of 115× coverage across all populations, which is an average of 8.1 M reads per 1× coverage. This is compared to 2.7 M and 4.3 M reads per 1× coverage for the *D. simulans* PA7.2010 and PA9.2010 pool-seq libraries, respectively. In summary, our two *D. simulans* pool-seq libraries were 39–53% more efficient in population allele frequency estimation per raw sequence read than our barcoded libraries. This increased pool-seq efficiency may be particularly pronounced in our study, as our barcoded libraries had high heterogeneity in coverage across individuals.

Measures of genetic variation, genetic differentiation and isolation by distance

We calculated two measures of within-population genetic variation – mean expected heterozygosity (H) and Watterson's theta (θ_S). For these analyses, we considered only sites covered by exactly 20 chromosomes in a given population, to avoid any biases resulting from differences in coverage among populations. Mean heterozygosity was calculated as

$$H = \frac{1}{N}\sum_{i=1}^{N} 2p_i(1-p_i) \quad (4)$$

where N is the number of sites (polymorphic and monomorphic) and p is the allele frequency of each site. θ_S was measured as the proportion of polymorphic SNPs, divided by the sample size correction:

$$\theta_S = \frac{S}{\sum_{i=1}^{n-1}\frac{1}{n}} \quad (5)$$

where S is the proportion of SNPs in the genome, and n is the number of chromosomes (i.e. 20).

We measured between-population genetic differentiation with the F_{ST} statistic (Weir & Cockerham 1984, equations 1:4). F_{ST} calculations were performed for each pairwise population comparison, for each SNP. As sample size affects the results of the F_{ST} statistic, we consider only SNPs with a total depth of coverage of 40–44 chromosomes between the two populations, with a minimum of 5 per population. In *D. simulans*, the maximum number of chromosomes per population ranged from 36–39 (FL: 39; VA: 39; PA8.2011: 37; PA9.2011: 39; PA11.2011: 36; ME: 37; PA7.2010: 39; PA9.2010: 39). In *D. melanogaster*, the maximum ranged from 31–39 (FL: 31; GA: 39; PA8.2011: 39; PA.2009: 39; PA.2010: 31; ME: 39). As the variance in the pool-seq allele frequency estimates is accounted for by the measure of effective number of chromosomes, $N_C(N,R,\epsilon)$, no additional pool-seq correction is necessary for F_{ST} or genetic variation calculations.

We assessed isolation by distance with a linear regression of F_{ST} with geographic distance between populations (degrees latitude). We incorporated into a multiple linear regression model the effect of comparison with the Maine population (vs. comparison between two non-Maine populations) and within-year

(vs. between-year) comparison. The final regression model is of the form:

$$y_i = d + m + y + d \times m + d \times y + m \times y + \epsilon_i \quad (6)$$

where y_i is the pairwise F_{ST}, d is the distance (degrees latitude) between two populations, m is whether or not one of the two populations of the comparison is Maine, y is between vs. within-year comparison, and ϵ_i is the gaussian error at the ith SNP.

Measures of clinal variation

To identify clinal SNPs, we used a generalized linear model (conducted in R version 3.1.0; R Core Team 2014) of allele frequency and population latitude, using a binomial error model and weights proportional to the effective number of chromosomes at each site (N_C):

$$y_i = \text{latitude} + \epsilon_i \quad (7)$$

where y_i is the allele frequencies at the ith SNP, and ϵ_i is the binomial error given the N_C at the ith SNP. This type of regression is particularly appropriate for the analysis of clinal variation of allele frequencies, as it takes into account precision (number of chromosomes sampled per population) and the curve-linear behaviour at low allele frequencies. For each species, we used five population measurements sampled from the four populations – one sample from each population, with an additional year's sample for PA (for *D. simulans*, we used PA7.2010 and PA8.2011). Each year of Pennsylvania samples was treated as a separate datapoint in the regression analysis, with a single timepoint for each year.

The average N_C across the populations used in the clinal regression varied little from chromosome to chromosome, ranging from 21.2−21.4 in *D. melanogaster* and 20.9−21.8 in *D. simulans*. There was no significant difference between the two species in mean N_C (t-test $P = 0.19$) or total N summed over all five populations (t-test $P = 0.13$) (Fig. S2, Supporting information). This equality of sample sizes is important because it allows us to compare the two data sets without confounding differences in power.

We identified two sets of clinal SNPs based on the results of the clinal regressions – SNPs that were statistically significant at $P < 0.01$, and SNPs that were statistically significant at false discovery rate (FDR) of $Q < 0.2$. FDR Q values represent the proportion of false positives in a set of tests and were calculated with the R package *qvalue* (Storey 2015). We use the $P < 0.01$ set to estimate the relative proportion of clinal loci in *D. melanogaster* and *D. simulans*, allowing us account for the number of false positives due to multiple testing (using the null expectation) in a way that does not skew the false-negative rates. The proportion of clinal loci (SNPs) is calculated as:

$$\frac{\text{obs}_{P<0.01} - \text{exp}_{P<0.01}}{L} \quad (8)$$

where L is the number of SNPs tested, $\text{obs}_{P<0.01}$ is the observed number of tests with $P < 0.01$, and $\text{exp}_{P<0.01}$ is the expected number of tests with $P < 0.01$ under the null expectation ($L \cdot 0.01$). For the remainder of the analyses (i.e. clinal consistency, functional genic classes, shared clinal genes), we use FDR Q-values, ensuring equal proportions of false positives in the *D. melanogaster* and *D. simulans* data sets.

To test the consistency of clinal patterns of allele frequency across years, we measured how well the regression coefficient from one year predicts the directionality in a second year. Allele frequency measures from three *D. simulans* sites from 2011 and two from 2010 were available. We performed a logistic regression across the three 2011 sites (FL, PA2011, ME) and asked whether the same trend of either increasing or decreasing frequency with latitude was observed in 2010. Specifically, we asked if the sign of the regression coefficient agreed with the sign of the difference between the 2010 populations (VA, PA2010). If there was agreement, these SNPs were deemed to be 'consistently clinal'. If SNPs truly are clinal from year to year, it is expected that the proportion of SNPs found to be consistently clinal will increase with the stringency of the regression test (lower Q-value). We then performed a similar analysis in *D. melanogaster*, comparing the regression of the three 2008/2009 sites (GA2008, PA2009, ME2009) with two 2010 sites (FL2010, PA2010). As the inclusion of sites from two different years in the regression might bias towards identifying sites that truly are persistently clinal, thereby increasing the amount of clinal consistency detected, we compared this analysis with a mixed-year analysis of *D. simulans*. For this analysis, we performed a regression of *D. simulans* VA2010, PA2010 and ME2011, compared with the difference between the FL2011 and PA2011 sites. This provided a comparison that was liberal to finding clinal consistency in *D. simulans*. Results from the *D. simulans* mixed-year analysis were not significantly different from the single-year analysis (within two standard deviation), with the exception of chromosome 2L, for which the mixed-year analysis shows a decrease in clinal consistency (Fig. S3, Supporting information).

Enrichment tests

To test for enrichment of genic categories and of polymorphisms shared between *D. melanogaster* and *D. simulans* in sets of clinal SNPs ($Q < 0.2$), we compared our data sets with 100 bootstrap control data sets matched for mean allele frequency across the populations (by 20th quantile bin), inversion status (within the same

inversion or outside inversions, applicable to *D. melanogaster* only; by 7th quantile bin), chromosome and effective sample size N_C (by 10th quantile bin). The sizes of matching bins were chosen to result in the most well-matched controls that were also independent of one another. Genic categories for each species were identified with SNPeff (Cingolani *et al.* 2012), except for short introns. We used the set of *D. melanogaster* short introns identified in (Lawrie *et al.* 2013) and identified short introns in *D. simulans* as those <68 bp in the annotation by Rogers *et al.* (2014). We used the same *D. melanogaster* inversion breakpoints as in (Corbett-Detig & Hartl 2012).

We tested for an enrichment of genes identified as clinal in both *D. melanogaster* and *D. simulans*. We identified a gene as clinal if it had at least one clinal genic SNP (i.e. in the CDS, UTR or intronic regions). We measured the per cent of shared clinal genes as the overlap of *D. simulans* clinal genes with *D. melanogaster* clinal genes (contains at least one SNP with $Q < 0.2$). This was performed for five sets of *D. simulans* clinal genes, ranging in stringency from $Q < 0.5$ to $Q < 0.1$. For each set of *D. simulans* clinal genes, we produced 100 control sets of *D. simulans* genes matched for gene length (by 10th quantile bin) and SNP density (by 10th quantile bin) and measured the proportion of control genes shared with *D. melanogaster* clinal genes. Genes were omitted if <85 unique control genes could be identified. The distributions of gene length and SNP density for the clinal compared with the control gene sets overlapped well, and the majority (87%) of control genes were unique across permutations (Fig. S4, Supporting information).

Results

D. simulans SNPs across space and time

Here, we study *D. simulans* population genetic variation using genomic sequence data from 382 individual fruit flies (267 individually barcoded and 115 in pooled samples). Samples represented a spatial transect of four populations over 19 degrees latitude and a temporal transect of multiple time points over the course of two years (Table S1, Supporting information). We identified 2.5×10^6 bi-allelic *D. simulans* single nucleotide polymorphisms (SNPs) across the four major autosomal chromosome arms and the X chromosome (see Methods for filtering parameters). We utilized a matched *D. melanogaster* data set of pooled population sequence data ($\sim 6 \times 10^5$ SNPs; Bergland *et al.* 2014) to compare patterns of within, between, interannual and latitudinal population genetic variation. For all pool-seq samples, we applied a stringent pool-seq error correction that accounted for finite sampling and additional pool-seq variance (see Methods), allowing us to confidently compare the *D. melanogaster* data set with the *D. simulans* data set.

Larger proportion of clinal variants in D. melanogaster than D. simulans

We found a larger proportion of latitudinally clinal variants in *D. melanogaster* (3.7%) than in *D. simulans* (2.5%) ($P < 0.01$; Fig. 1D). The difference in the proportion of clinal variants was even greater when we considered only autosomal SNPs (4.3% in *D. melanogaster* compared with 2.1% in *D. simulans*; Fig. 2). As major chromosomal inversions in *D. melanogaster* show clinal patterns in frequency (Mettler *et al.* 1977), we asked whether inversions account for the difference between species. We found an elevated proportion of clinal SNPs in *D. melanogaster* inversions; however, *D. melanogaster* had a higher proportion of clinal SNPs than *D. simulans* in noninverted regions as well (Table S2, Supporting information). Similarly, although we did see an enrichment of clinal SNPs in low-recombination regions for *D. melanogaster*, the proportion of clinal SNPs outside low-recombination regions was still greater for *D. melanogaster* than *D. simulans* (Table S2, Supporting information).

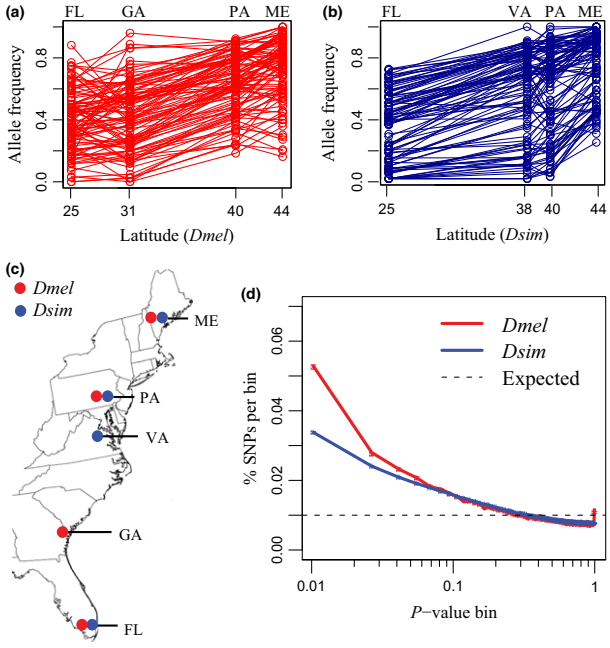

Fig. 1. Clinal genetic variation with latitude. (a, b): Allele frequency trajectories for clinal SNPs ($P < 0.01$, sample of 100). Allele frequencies are polarized such that FL < ME. (c) Distribution of populations used to assess clinal variation. (d) *P*-value distributions from logistic regressions of allele frequency with latitude (bins of 0.01). Error bars are two standard error (not visible).

We found substantial variation in clinality among chromosomes. The most striking pattern in *D. melanogaster* was the strong enrichment of clinal variants on chromosome 3R (9% clinal; Fig. 2). In *D. melanogaster*, much of the 3R chromosome is covered by three large cosmopolitan inversions. These inversions, particularly In(3R)P, have previously been found to be strongly clinal (Mettler *et al.* 1977; Kapun *et al.* 2014). On the X chromosome *D. melanogaster* and *D. simulans* had the opposite patterns of clinal variation. *D. melanogaster* had less clinal variation on the X chromosome (1% clinal) than any of the autosomes, whereas *D. simulans* had more clinal variation on the X chromosome (4% clinal) than any of the autosomes. Lower levels of clinal variation on the *D. melanogaster* X chromosome have been observed in previous studies (David & Capy 1988; Fabian *et al.* 2012; Kolaczkowski *et al.* 2011).

We asked whether the increased amount of clinal variation observed in *D. melanogaster* could be explained by greater *D. melanogaster* population structure. We looked at the effect of population structure by comparing genomewide mean pairwise F_{ST}. First, we noticed that on average (across all SNPs) *D. simulans* had a greater mean F_{ST} than *D. melanogaster*, indicating that a net increase in population structure was not driving the increased proportion of clinal variants in *D. melanogaster*. To look at the effect population structure had on the magnitude of clinal variation, we asked how mean F_{ST} scaled with the clinal effect size β (regression coefficient). We found that *D. melanogaster* had a stronger relationship between F_{ST} and β than *D. simulans* (Fig. S5, Supporting information), indicating that in *D. melanogaster* more of the observed population structure was due to clinal genetic differentiation.

Consistency of clinal variants from year to year

To assess the stability of clinal variation over time, we measured how well the clinal regression coefficient in 1 year predicted the allele frequency directionality in a second year. To ensure equal power and noise for the *D. melanogaster* and *D. simulans* analyses, we used false discovery rate (FDR) corrected *Q*-value significance thresholds for the clinal regressions and down-sampled the number of SNPs to the same number in each species and chromosome. We found evidence for clinal consistency from year to year in both species, with the proportion of clinal consistency increasing with *Q*-value stringency to 67% and 54% for *D. melanogaster* and *D. simulans*, respectively (at clinal $Q < 0.3$; Fig. 3; all chromosomes). Note that the *Q*-values are generally higher in this analysis than in the full clinal regression, as we use three populations instead of five. We found that *D. melanogaster* had significantly greater clinal consistency from year to year than *D. simulans* for each chromosome (Fisher's exact test $P < 10^{-14}$) except the X chromosome ($P = 0.3$).

Selection and parallelism in clinal variants

If clinal SNPs have phenotypic effects that are under spatially varying selection, we expect functional sites to be over-represented in the sets of clinal SNPs. Our expectation is that intergenic regions, short introns and synonymous sites are less likely to be functional than UTR's, nonsynonymous sites and long introns. We used a constant FDR ($Q < 0.2$) and number of SNPs per species (25 134 autosomal and 805 X chromosome SNPs) to ensure equal noise and power for the *D. melanogaster* and *D. simulans* analyses. For the set of SNPs clinal in *D. melanogaster* autosomes, we found a significant

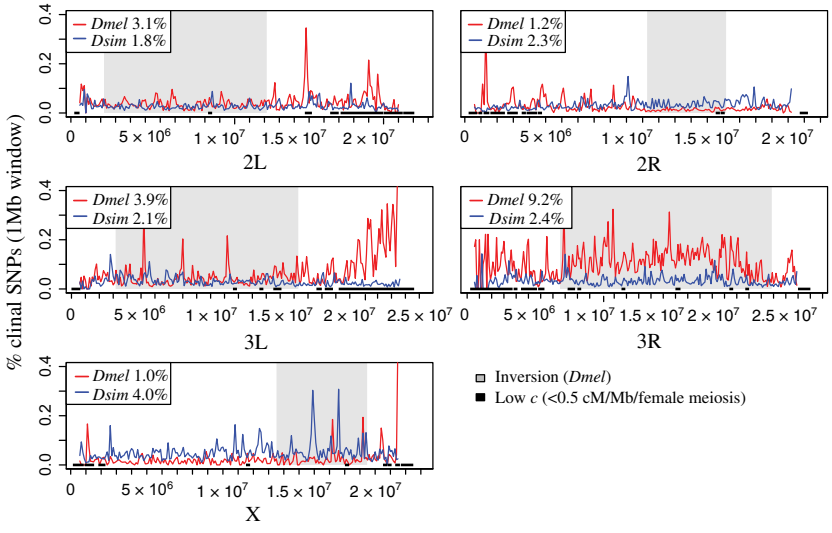

Fig. 2. The distribution of clinal SNPs across the genome. The mean proportion of clinal SNPs ($P < 0.01$) per 1Mb window is plotted across the *Drosophila melanogaster* genome. Shaded areas represent the *D. melanogaster* major inversions. Black along the *x*-axis represents low-recombination rate regions (<0.5 cM/Mb/female meiosis, 100-kb bins). The proportion of SNPs clinal on each chromosome is listed in the legends.

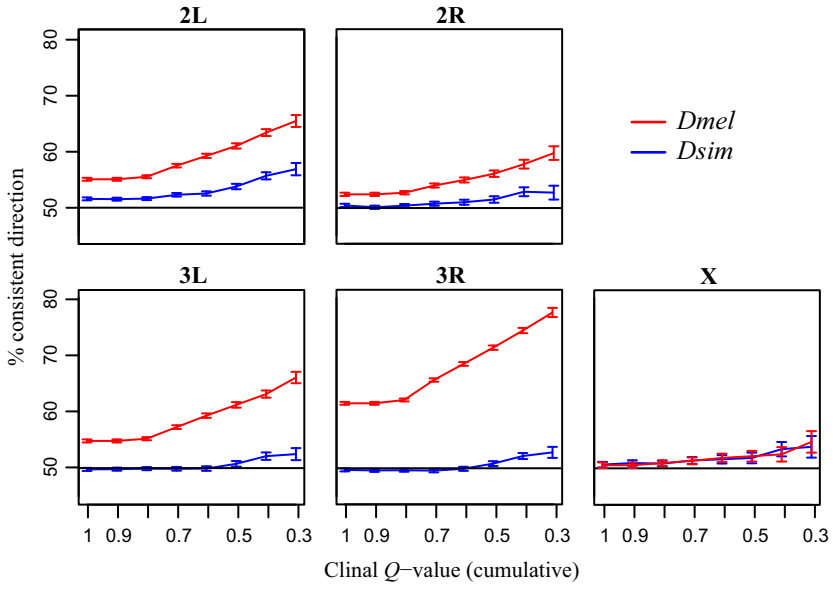

Fig. 3. Consistency of clinal variation across years. The proportion of SNPs for which the clinal regression coefficient from 1 year predicts the directionality in a second year is plotted for sets of clinal SNPs of increasing clinal stringency (decreasing *Q*-value). Error bars are two standard error.

enrichment of all genic classes (UTR's, long intron, synonymous coding and nonsynonymous coding) except short introns and found a depletion of intergenic regions, compared with 100 bootstrap control data sets matched for chromosome, mean minor allele frequency, sample size, recombination rate and inversion status (see Methods; Fig. 4A). Additionally, we found a marginal increase in the proportion of nonsynonymous SNPs compared with synonymous SNPs ($P = 0.1$). Conversely, the *D. melanogaster* X chromosome was enriched for intergenic SNPs and depleted for long introns and nonsynonymous SNPs (Fig. S6, Supporting information). The set of *D. simulans* clinal SNPs showed a marginal enrichment ($P < 0.1$) of 5′UTR SNPs and a marginal depletion of intergenic SNPs (autosomes; Fig. 4B).

If selection is acting similarly on both species, we might find evidence of convergent evolution of clinal variants. We asked whether there was an enrichment for SNPs or genes that are clinal in both *D. simulans* and *D. melanogaster*. We found no significant enrichment for shared clinal SNPs (61 shared clinal polymorphisms of 32 136 shared polymorphisms total). However, we did observe an enrichment of shared clinal genes (Fig. 5). We compared the proportion of shared clinal genes with the proportion for 100 bootstrap control sets of genes, matched for *D. simulans* gene length and SNP density (see Methods). Of the genes with at least one clinal SNP ($Q < 0.2$; 5559 *D. simulans* genes and 5556 *D. melanogaster* genes), 56% were clinal in both species, compared to a mean of 45% across the bootstrap replicates ($P = 0.01$). This enrichment became even more pronounced at more stringent *D. simulans* clinal regression thresholds (for *D. simulans* clinal regression $Q < 0.1$, observed: 65%, control: 46%; Fig. 5). We did not find the shared clinal genes to be enriched in SNPs that were also clinally consistent (Fisher's exact test; $P > 0.3$ for both species).

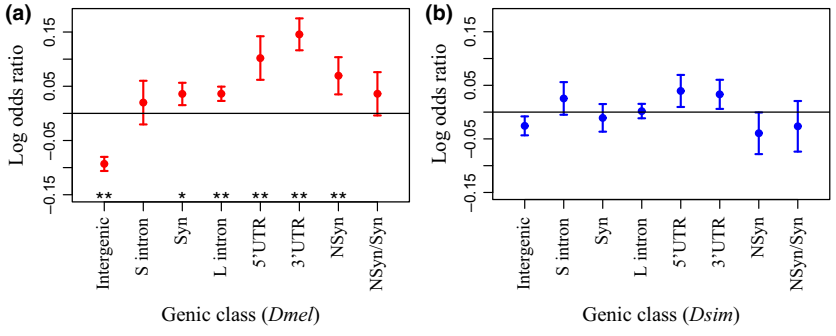

Fig. 4. Enrichment of clinal autosomal SNPs ($Q < 0.2$, down sampled to 25134 SNPs) in each functional genic class. Plotted is the log of the odds ratio of the proportion of each genic class in the set of clinal SNPs compared with 100 matched controls. Error bars are one standard deviation. Bootstrap *P*-value $*P \leq 0.05$; $**P \leq 0.01$.

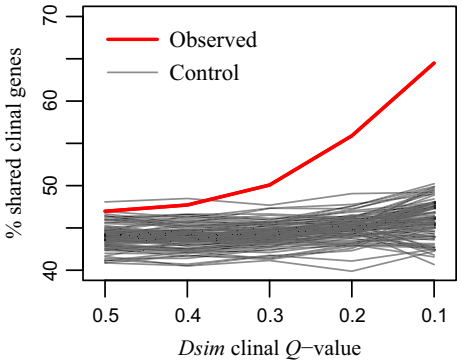

Fig. 5. Per cent overlap of *Drosophila simulans* clinal genes with *Drosophila melanogaster* clinal genes ($Q < 0.2$), over increasing stringency of *D. simulans* clinal regression.

We next queried the list of 3342 shared clinal genes for its overlap with a set of 13 genes previously found to be clinal in *D. melanogaster*. To arrive at a set of putatively clinal genes, we gathered genes from targeted studies of clinal variation (rather than genomic scans). The result was 13 genes with strong support in the literature and was comprised of the seven metabolism genes *Pgm* (Verrelli & Eanes 2001; Sezgin *et al.* 2004), *G6pd* (Oakeshott *et al.* 1983), *Gpdh* (Oakeshott *et al.* 1982), *UGP* (Sezgin *et al.* 2004), *Treh* (Sezgin *et al.* 2004), *Pgd* (Oakeshott *et al.* 1983), and *Hex-C* (Duvernell & Eanes 2000) and the six nonmetabolism genes *sgg* (Rand *et al.* 2010), *mth* (Schmidt *et al.* 2000; Duvernell *et al.* 2003), *cpo* (Schmidt *et al.* 2008), *per* (Costa *et al.* 1992), *Adh* (Vigue & Johnson 1973; Berry & Kreitman 1993) and *InR* (Paaby *et al.* 2010). All except one of these genes (*mth*) were analysed in both species, leaving a final set of 12 genes. Of these 12 genes, 10 were clinal in both species. The two genes that were not found to be clinal in both species were *Pgd* and *Hex-C*.

We also compared our results to a recent study of gene expression in *D. melanogaster* and *D. simulans* low- (Panama) and high- (Maine) latitude populations (Zhao *et al.* 2015). For each population, gene expression was measured at 21 ° C and 29 °C. Zhao and colleagues identified sets of 76 and 106 genes with latitude-specific expression in both species, at 21 °C and 29 °C, respectively (Zhao *et al.* 2015, Table S8, Supporting information). We compared the intersection of these data sets and our shared clinal genes data set with the intersection for 100 bootstrap control data sets matched for *D. simulans* gene length and SNP density (see Methods). We found only a marginal ($P = 0.1$) enrichment of latitude-specific genes at 29 °C, and no enrichment of latitude-specific genes at 21 °C, in our set of shared clinal genes. Zhao and colleagues also identified sets of genes with differential expression between temperatures (21 °C and 29 °C) in both species- 375 genes in the Maine populations and 861 in the Panama populations (Table S10, Supporting information, Zhao *et al.*). Also controlling for gene length and SNP density, we did find an enrichment of temperature-responsive genes in our set of shared clinal genes; however, this was only true for the Panama populations ($P = 0.02$) and not the Maine populations ($P = 0.18$).

Population genetic patterns in space

Visual inspection of frequency trajectories along the cline showed a more monotonic increase in allele frequency with latitude in *D. melanogaster* than *D. simulans* (Fig. 1). To further investigate this, we asked whether genetic differentiation between populations increased monotonically with physical distance between populations, a pattern know as 'isolation by distance'. We found that *D. simulans* had a weaker pattern of isolation by distance than *D. melanogaster* (Fig. 6). While in *D. melanogaster* the regression of genetic differentiation (F_{ST}) and physical distance between populations (degrees latitude) was significant ($P < 10^{-5}$, $R^2 = 0.94$), in *D. simulans* this was only significant ($P = 0.001$) in a regression model that included Maine (ME) as an explanatory variable (Table S3, Supporting information). In *D. melanogaster*, there was no effect of ME comparison. The significant effect of ME comparison in *D. simulans* was due to the disproportionate amount of divergence of ME from the other populations. Interestingly, we also found less genetic diversity in the *D. simulans* ME population than the other *D. simulans* populations (Fig. S7, Supporting information). In addition, the level of differentiation among the three southern *D. simulans* populations was considerably lower than for the three southern *D. melanogaster* populations (Fig. 6).

Population genetic patterns in time

The analysis of isolation by distance incorporated data from different years. We used this to determine whether there was a difference in the amount of interannual variation between *D. melanogaster* and *D. simulans*. As *D. simulans* has low clinal consistency, we might expect to also find a greater amount of interannual variation in *D. simulans*. We can test this with the isolation by distance regression model and ask whether there is a significant effect of between- vs. within-year comparison. Specifically, between-year comparisons should have greater F_{ST} than predicted by a regression of within-year comparisons. In *D. simulans*, we did indeed find that the effect of within- vs. between-year comparison was significant in the regression model ($P = 0.002$), with between-year comparisons showing greater genetic differentiation (Fig. 6; Table S3, Supporting information).

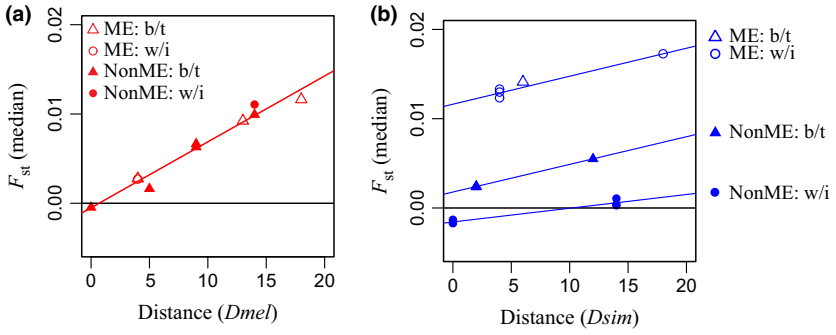

Fig. 6. Isolation by distance. Between-population genetic differentiation (median F_{ST}) is plotted against geographic distance (degrees latitude). (a) *Drosophila melanogaster*. (b) *Drosophila simulans*. For *D. simulans*, regression lines are plotted separately for population comparisons without ME within a year, without ME between years and with ME within a year, reflecting the significant effect of distance, ME vs. non-ME comparison, and within- vs. between-year comparison in the regression model. ME: F_{ST} between one non-ME population and ME; Non-ME: F_{ST} between two non-ME populations; b/t: F_{ST} between two samples taken between years; w/i: F_{ST} between two samples taken within a year.

The significant effect of between-year sampling implies that there was a detectable level of interannual variation in *D. simulans*. In contrast, in *D. melanogaster*, there was no effect of between-year comparison.

Although much of the clinal variation in *D. simulans* is not maintained from year to year (low clinal consistency) and there is interannual variation, can we still find evidence of genetic continuity in a population from year to year? We assessed the level of genetic continuity across years by comparing the level of differentiation (F_{ST}) among populations within a year to the level of differentiation within a population across years. We asked whether the PA.2010 samples were most similar to the PA.2011 samples (genetic continuity between years) or to the VA.2010 sample (genetic similarity between sites, within a year). We found significantly lower within-population differentiation (PA.2010/PA.2011) than between-site within-year differentiation (PA.2010/VA.2010) (chi-squared $P < 0.0001$; Fig. S4, Supporting information), indicating that a given *D. simulans* population does maintain some degree of genetic similarity from year to year.

Increased X chromosome differentiation and clinal variation in D. simulans

The X chromosome in *D. simulans* showed two patterns not observed in *D. melanogaster*- an increased proportion of clinal variants and increased population genetic differentiation compared with the autosomes. The increased level of X chromosome differentiation was particularly pronounced in any comparisons with ME (Fig. 7). We asked whether the increased differentiation on the X chromosome was consistent with its reduced effective population size resulting from hemizygosity in males. We used the formula proposed by Ramachandran *et al.* (2004) that predicts the relationship between autosomal F_{ST} and X chromosome F_{ST}, given a particular sex ratio. To perform this analysis, we calculated pairwise F_{ST} for autosomal loci and the corresponding expected X chromosome F_{ST} values, assuming equal proportions of breeding males and females. Only in the ME comparisons were the X chromosome F_{ST} values significantly greater than expected when accounting for decreased effective population size (Fig. 7). With regard to the proportion of clinal variants, it is impossible to say whether the increased level of clinal variation on the X chromosome was due to the general pattern of increased X chromosome differentiation because the two signals are both strongly affected by increased ME differentiation.

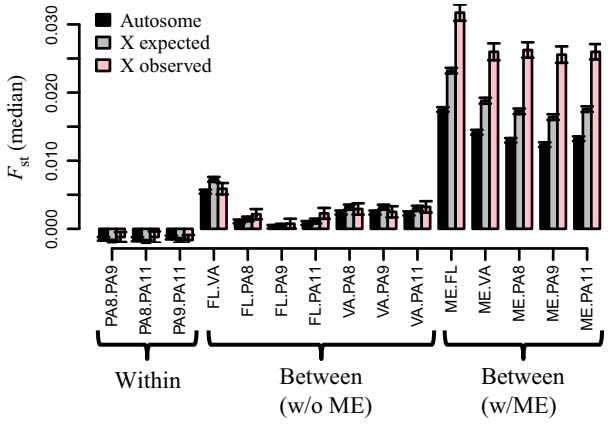

Fig. 7. Expected vs. observed X chromosome F_{ST} in *Drosophila simulans*. An expectation of X chromosome median F_{ST} is calculated from the autosomal F_{ST} values. Within-population F_{ST} measures are from the three PA samples taken over the course of 2011. Between-population F_{ST} measures are divided up into comparisons that include ME and those that do not include ME. Error bars are 2 standard error.

Discussion

Our study is the first to conduct a comparative genomic analysis of *D. simulans* and *D. melanogaster* latitudinal variation. We expect *D. melanogaster* to have a larger proportion of clinal genetic variants than *D. simulans*, as *D. melanogaster* has been documented to have more strongly clinal phenotypes (Gibert *et al.* 2004; Arthur *et al.* 2008). The absence of *D. simulans* at high latitudes early in the year (Boulétreau-Merle *et al.* 2003; Fleury *et al.* 2004; Behrman *et al.* 2015) can be explained by either a stronger *D. simulans* winter bottleneck or population extinction and recolonization, both of which would result in a less stable cline from year to year. Our experimental design focuses on testing these predictions of less clinal variation and less clinal stability in *D. simulans*, as compared with *D. melanogaster*.

Less clinal variation in D. simulans than D. melanogaster

We find strong support for a larger proportion of clinal variants in *D. melanogaster* than in *D. simulans*, particularly for *D. melanogaster* autosomes, which harbour twice as much clinal variation as *D. simulans* autosomes (4.3% and 2.1%, respectively). We have ensured that this result is not confounded by differences in power or the additional sampling error of pool-seq. With a greater sample size (i.e. additional populations), it is possible that we would find an even greater proportion of clinal variants. For example, using deeper coverage and additional populations, Bergland *et al.* (2014) identified approximately one-third of common *D. melanogaster* SNPs as clinal. Our study design of four populations along a latitudinal transect makes our measurements of clinal variation sensitive to outlier allele frequencies at the Florida and Maine populations. In *D. simulans*, we do find that Maine is a genetic outlier, which could be contributing to the lower proportion of clinal variation identified. However, multiple lines of evidence from this study do support the conclusion of a more robust cline in *D. melanogaster* than in *D. simulans*, including increased clinal consistency, stronger isolation by distance, and more clear signatures of selection in *D. melanogaster* than *D. simulans*.

The strong pattern of clinal consistency in *D. melanogaster*, where clinal SNPs tend to show the same allele frequency pattern from year to year, indicates that the *D. melanogaster* cline is stable, rather than transient and re-established on an annual basis. Not only does *D. simulans* have a smaller proportion of clinal variants, the variants that are clinal are much less likely to be clinal from year to year than *D. melanogaster*. This indicates that the *D. simulans* cline is less stable, with a greater proportion of clinal variants due to processes operating on annual timescales.

The strong pattern of isolation by distance in *D. melanogaster* is also indicative of a robust cline. The pattern of isolation by distance in *D. melanogaster* is independent of whether or not the population pair was sampled in the same or different years. In contrast, in the *D. simulans* isolation by distance regression model, there is a significant effect of within- vs. between-year sampling of population pairs, indicating that interannual variation drives a detectable amount of population genetic variation. One important note is that in *D. simulans*, the genetic continuity at a collection site (i.e. across years) is still greater that the genetic similarity between collection sites (within a year), indicating that there is a balance between the processes resulting in these two patterns. For *D. melanogaster*, the pattern of isolation by distance is unperturbed by interannual variation, possibly indicating low effective migration rate between populations or a balance between selection and migration not seen in *D. simulans*.

Although a demography-driven pattern of isolation by distance can result in stable clinal variation, stability can also result from local adaptation to variable conditions along a transect. We find that *D. melanogaster* clinal SNPs are significantly enriched for functional genic classes, including UTR's, coding regions and long introns and have a marginally elevated proportion of nonsynonymous to synonymous sites. This suggests that *D. melanogaster* clinal variants are under selection. We see weak evidence for selection in *D. simulans*, which shows a marginal enrichment for 5'UTR's and no enrichment for other genic classes, suggesting that neutral processes play a stronger role.

Our comparisons of clinal variation in these two species reveal robust patterns of allele frequency with latitude in *D. melanogaster*, and weaker patterns in *D. simulans*. *D. melanogaster* not only harbours a larger proportion of clinal SNPs, but allele frequency patterns of clinal variants persist more from year to year, and there is evidence that clinal variants are under increased spatially varying selection. These results are consistent with previous studies that suggest less clinality in *D. simulans*. Specifically, some characters show no clinality in *D. simulans* (weight, wing length: Gibert *et al.* 2004; hexokinases: Duvernell & Eanes 2000; absence of diapause: Schmidt *et al.* 2005), while others show a decreased amplitude of clinality (wing length, thorax length, ovariole number: Gibert *et al.* 2004, cold tolerance, starvation tolerance: Hoffmann & Harshman 1999).

Shared clinal genes

A given selection pressure may act on the same genes in closely related species. As selection pressures along the latitudinal cline are expected to vary in the same manner for *D. melanogaster* as for *D. simulans*, the two species may exhibit similar genetic responses. We find a significant enrichment for genes that are clinal in both species. Fifty-six per cent of the 5559 *D. simulans* clinal genes were also clinal in *D. melanogaster*, compared to 45% in the matched controls. The enrichment of shared clinal genes increases with increasing stringency of the clinal regression. This supports the hypothesis of convergent evolution in these species due to the action of similar selection pressures on similar genetic backgrounds. This result is also consistent with the finding of parallel latitudinal gene expression in *D. melanogaster* and *D. simulans* (Zhao *et al.* 2015).

Although there is a significant enrichment of shared clinal genes ($\sim$20% more shared clinal genes than expected), we still cannot say which of the $\sim$3000 shared clinal genes are true positives. However, we can ask whether genes previously identified as clinal tend to be shared clinal genes in our data set. When we look at a set of 12 genes with substantial literature support for latitudinal variation in *D. melanogaster*, 10 are clinal in both *D. melanogaster* and *D. simulans*. These genes include *Pgm* (Verrelli & Eanes 2001; Sezgin *et al.* 2004), *G6pd* (Oakeshott *et al.* 1983), *Gpdh* (Oakeshott *et al.* 1982), *UGP* (Sezgin *et al.* 2004), *Treh* (Sezgin *et al.* 2004), *sgg* (Rand *et al.* 2010), *mth* (Schmidt *et al.* 2000; Duvernell *et al.* 2003), *cpo* (Schmidt *et al.* 2008), *per* (Costa *et al.* 1992), *Adh* (Vigue & Johnson 1973; Berry & Kreitman 1993) and *InR* (Paaby *et al.* 2010).

We also find that our set of shared clinal genes is enriched for genes recently identified by Zhao *et al.* (2015) to have temperature-dependent expression in both *D. melanogaster* and *D. simulans* (Panama populations). Interestingly, we find only a marginal enrichment ($P = 0.1$) for genes with latitude-specific expression (Panama vs. Maine) in both species. One explanation for the lack of enrichment is the difference in sampling schemes. We sampled four populations along a continuous transect and identified loci that vary consistently with latitude. Zhao *et al.* (2015) sampled two populations from separate continents and identified gene expression differences between these two diverged groups.

Demographic implications of Drosophila clinal patterns

It is possible that *D. simulans* and *D. melanogaster* differ in both the initial establishment of clinal variation and the potential for that variation to be maintained. There is evidence that some of the latitudinal variation that we see in *D. melanogaster* is due to introgression between founding European and African populations (Duchen *et al.* 2013; Bergland *et al.* 2015; Kao *et al.* 2015). There is currently no evidence that this occurred in *D. simulans*. Additionally, the potential for maintenance of clinal variation might be diminished in *D. simulans*. As we discuss below, *D. simulans* population structure may be disproportionately affected by processes such as bottlenecks and migration.

Drosophila simulans overwintering. Drosophila populations experience a contraction as a result of temperate winters (Ives 1970). The decreased genetic diversity observed in high- relative to low-latitude populations of both *D. melanogaster* (Reinhardt *et al.* 2014) and *D. simulans* (Fig. S3, Supporting information) is consistent with stronger bottlenecks at high latitudes. *D. simulans* seems to be physiologically less winter-adapted than *D. melanogaster* (Hoffmann & Harshman 1999) and *D. simulans* is not observed at high latitudes until later in the year (Boulétreau-Merle *et al.* 2003; Fleury *et al.* 2004; Schmidt 2011; Behrman *et al.* 2015), suggesting a stronger bottleneck for *D. simulans* high-latitude populations than for *D. melanogaster* high-latitude populations. In addition to the decreased genetic variation we observe in the high-latitude *D. simulans* Maine population, we find that this population is much more genetically differentiated from the other three populations, a result that could be explained by strong bottlenecks or by complete extirpation and recolonization. Alternatively, these genetic patterns could be explained by selective sweeps in the Maine population or by effects due to the Maine population existing at the edge of the *D. simulans* range. Although we find evidence of year-to-year genetic continuity of the lower-latitude Pennsylvania population, indicating that there is not complete annual extirpation at the Pennsylvania site, additional sampling is needed to determine whether *D. simulans* is able to overwinter at latitudes as high as Maine (45° latitude).

Migration. While *D. melanogaster* has a strong, clear pattern of genetic isolation by distance, this is not true of *D. simulans*. A weak pattern of isolation by distance can be indicative of substantial gene flow among populations (Endler 1977). Genetic differentiation is particularly low among the three southern *D. simulans* populations (median F_{ST} 0.001–0.006, compared with 0.003–0.012 in *D. melanogaster*). The low level of differentiation indicates that there is a stronger effect of migration among these populations. Such a contribution of migration to *D. simulans* population genetic patterns is consistent with the reduced amount of clinal variation in *D. simulans*, as migration can disrupt clinal pat-

terns resulting from demographic processes or local adaptation. A strong effect of migration in *D. simulans* and not in *D. melanogaster* could also contribute to the increased interannual variation observed in *D. simulans*, as evidenced by the significant effect of between-year comparison in the isolation by distance regressions (between-year comparisons show increased differentiation) and by the reduced level of clinal consistency (the same variants are not clinal from year to year). The effect of annual migration would be more acute in *D. simulans* than in *D. melanogaster* if *D. simulans* does indeed experience stronger annual bottlenecks, such that migrants overwhelm the local population. An additional contributor to weaker population structure in *D. simulans* than *D. melanogaster* could be the lack of large cosmopolitan inversions, which could act as a barrier to gene flow among *D. melanogaster* populations (Mettler *et al.* 1977; Knibb *et al.* 1981; Noor *et al.* 2001; Hoffmann & Weeks 2007).

One caveat to each of the analyses that utilize interannual data is the reliance of the conclusions on few between-year comparisons. For example, if the Virginia sample from 2010 was aberrant in its genetic composition, such as might occur with human-mediated migration from a distant population, our conclusions of low clinal consistency and the interaction of sampling year with isolation by distance in *D. simulans* might change. Further temporal sampling could bolster these findings.

Increased differentiation on the D. simulans X chromosome

We find more population genetic differentiation on the X chromosome than on autosomes in *D. simulans*. This pattern is opposite of what we find in *D. melanogaster* and is particularly pronounced for any comparisons with Maine. Additionally, only in the Maine comparisons are the X chromosome F_{ST} values significantly greater than expected when accounting for decreased effective population size (Fig. 7). In contrast, we see a lack of differentiation on the *D. melanogaster* X chromosome, consistent with previous findings of a drop in X chromosome diversity relative to autosomal diversity in non-African populations (Andolfatto 2001). There are multiple evolutionary processes that can affect the relative rates of divergence of the X and the autosomal chromosomes. Examples of a 'faster-X' effect are found across various taxa, including in *D. simulans*, and to a lesser extent in *D. melanogaster* (Begun *et al.* 2007). Certain classes of genes, such as those with greater expression in males than females (Baines *et al.* 2008), have shown faster-X patterns in *Drosophila*, as have certain classes of genomic sites, such as nonsynonymous sites, UTR and long introns (in *D. melanogaster* and *D. simulans*; Hu *et al.* 2013). In addition, gene expression differences have accumulated faster between *Drosophila* species on the X than on autosomes (Meisel *et al.* 2012). Further evidence for the contribution of selection to faster-X evolution in *Drosophila* includes the increased selection on tandem duplication on the X chromosome (in *D. simulans*; Rogers *et al.* 2015) and faster-X evolution in nonsynonymous sites, UTR and long introns, but not found in synonymous sites and short introns (Hu *et al.* 2013). The latter study again finds the effect present in both *D. simulans* and *D. melanogaster,* but is more marked in *D. simulans*.

The increased divergence of the *D. simulans* Maine X chromosome could be due to Maine suffering more extreme winter population bottlenecks. This is consistent with our findings of decreased genetic diversity and high levels of divergence on the autosomes as well as the X chromosome. Strong drift and divergence of the Maine population could also be driving clinal variation. A demographic explanation for the observed clinal variation is consistent with the weak evidence for selection on clinal variants in *D. simulans*. Another process that could contribute to X chromosome divergence is that of unequal sex ratios. Although we do not have sex ratio data for our populations, multiple sex-distorter systems have been found in other *D. simulans* populations (Bastide *et al.* 2013).

Conclusions

We have presented genomic evidence that *D. melanogaster* has a greater proportion of latitudinally varying loci than *D. simulans*. In *D. simulans*, we observe a weak pattern of isolation by distance, with a significant effect of between-year differentiation, low consistency of clinal SNPs from year to year and less evidence for selection on clinal variants than in *D. melanogaster*. In *D. melanogaster*, we observe the opposite patterns-strong isolation by distance, strong clinal consistency, low interannual variation and clear evidence for selection acting on clinal variants. We argue that one contributing factor to these differences is the ability of the two species to overwinter in temperate climates, causing differences in bottlenecks and migration. However, despite differences in demography, we do see an enrichment of shared clinal genes between the two species, suggesting that climate-associated selection might act on similar genes and phenotypes in the two taxa.

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P.S., D.A.P., A.O.B. and H.E.M. designed the research. P.S., K.R.O. and E.L.B. contributed samples. H.E.M. performed experiments. H.E.M. analysed the data. P.S., D.A.P., A.O.B., K.R.O., E.L.B. and H.E.M. discussed conclusions. H.E.M. wrote the manuscript.

Data accessibility

Drosophila simulans sequence fastq files and alignment bam files: NCBI SRA: SRP063680 *D. melanogaster* sequence fastq files (Bergland *et al.* 2014): NCBI SRA: PRJNA256231 Allele frequency data: Dryad doi: 10.5061/dryad.3hf2q. F_{ST} and latitude matrices for isolation by distance analyses: Dryad doi: 10.5061/dryad.3hf2q. GLM results for clinal regressions: Dryad doi: 10.5061/dryad.3hf2q. *D. melanogaster* and *D. simulans* shared clinal genes: Dryad doi: 10.5061/dryad.3hf2q.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Populations sampled along the east coast of the North America.

Table S2 Proportion of clinal SNPs.

Table S3 Isolation by distance linear model.

Table S4 Annotation of *D. simulans* genome and SNPs by genic category.

Fig. S1 Levels of differentiation between barcoded samples compared with differentiation between pooled samples.

Fig. S2 Sample size (N_C) for clinal regressions. (a) Average N_C by chromosome. Error bars are 2 standard error (all <0.03). (b) Distribution of total N_C (sum over populations).

Fig. S3 Clinal consistency across years.

Fig. S4 Distribution of *D. simulans* (a) coding sequence (CDS) length and (b) SNP density (number of SNPs divided by the gene length), for the observed clinal genes ($Q < 0.2$; pink) and the matched control genes (blue).

Fig. S5 Relationship between population structure (mean F_{ST}) and clinal effect size (β).

Fig. S6 Enrichment of clinal X chromosome SNPs in each functional genic class.

Fig. S7 *D. simulans* autosomal diversity across populations.

Fig. S8 F_{ST} between the two PA 2010 samples and each of the other population samples in *D. simulans*.