Biological Journal of the Linnean Society, 2008, **95**, 702–718. With 3 figures

Variable evolutionary response to regional climate change in a polymorphic species

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Received 21 April 2008; accepted for publication 6 May 2008

The evolutionary response to regional and global climate change may vary in widespread polymorphic species, so predicting future genetic responses will require careful tracking of genetic variability in local populations. We surveyed chromosomal inversion polymorphisms in 25 populations of *Drosophila robusta*, many of which have been sampled repeatedly starting in the 1940s, 50s, and 60s up until 2007, across its range in the USA. Frequencies of some northerly, or cold-adapted, gene arrangements have declined in the face of increasing temperatures, whereas frequencies of several southern, or warm-adapted, gene arrangements were positively correlated with increasing temperature changes. Over a finer geographic scale, populations from the west-central part of the species range from the Ozark Plateau, Ouachita mountains, and eastern Oklahoma showed genetic differentiation between south-central Ozark and western Ozark/Ouachita regions that has persisted in the face of recent shifts in gene arrangement frequencies. Overall, populations of *D. robusta* exhibited dynamic genetic changes over time, with some populations shifting chromosome frequencies in just 10–15 years. Some temporal genetic shifts were widespread and significantly correlated with temperature increases, but regions of the genome marked by different gene arrangements have responded in different sections of the species range. In some parts of the species range, chromosome frequencies shifted but were not associated with changing temperatures, showed little or no temporal change, or temporal shifts stopped for temperature sensitive gene arrangements near fixation. *Biological Journal of the Linnean Society*, 2008, **95**, 702–718.

ADDITIONAL KEYWORDS: chromosome polymorphism – *Drosophila robusta* – gene arrangement – global warming – inversion – North America.

INTRODUCTION

Only recently have long-term genetic data revealed the effects of temperature or seasonal shifts on the evolutionary potential of species associated with climate change (Rodríguez-Trelles & Rodríguez, 1998; Reale *et al.*, 2003; Levitan & Etges, 2005; Umina *et al.*, 2005; Balanya *et al.*, 2006; Bradshaw & Holzapfel, 2006; Franks, Sim & Weis, 2007). Although natural and sexual selection are pervasive in natural populations (Endler, 1986; Reznick *et al.*, 1997; Hendry & Kinnison, 1999; Hoekstra, *et al.*, 2001; Kingsolver *et al.*, 2001), there are fewer examples that have revealed the precise time course of selectively driven evolutionary change: industrial melanism (Cook, 2003), guppy life history evolution (Reznick, Bryga & Endler, 1990), morphological evolution in finches (Grant, 1986; Grant & Grant, 1989) range shifts in prairie grasses (Etterson & Shaw, 2001), reproductive isolation in introduced salmon populations (Hendry *et al.*, 2000), phenological shifts in mosquitoes (Bradshaw & Holzapfel, 2001), and concerted temperature-related shifts in *Drosophila* inversion polymorphisms (Etges, 1984; Prevosti *et al.*, 1988; Rodríguez-Trelles, Rodríguez & Scheiner, 1998; Levitan & Etges, 2005; Umina *et al.*, 2005; Balanya *et al.*, 2006; Van Heerwaarden & Hoffmann, 2007). These studies suggest that evolutionary responses to

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specific ecological factors, including climate related causes, can occur in time periods spanning a few decades or less, although climate change has not been implicated in all cases (Anderson *et al.*, 1991; Coyne *et al.*, 1992).

Drosophila chromosomal polymorphisms have been a model system for understanding evolutionary genetics for the last 75 years (Dobzhansky, 1951; Dobzhansky, 1970; Lewontin *et al.*, 1981; Krimbas & Powell, 1992; Hoffmann, Sgrò & Weeks, 2004), and have provided confirmation that natural and sexual selection maintains genetic polymorphism (Powell, 1997). Many of these features have been particularly well documented in populations of *Drosophila robusta* Sturtevant collected over more than 50 years from more than 140 natural populations, demonstrating north–south, east–west, and elevational clines in the frequencies of some gene arrangements and linked arrangement combinations, as well as a number of consistent, but geographically variable patterns of linkage disequilibrium (Levitan, 1992; Levitan & Etges, 1995).

Despite these observations, it remains unclear how long these observed geographical patterns have existed. Certainly, over the millions of years that *D. robusta* has inhabited North America subsequent to its invasion from southeast Asia, frequencies of these chromosome arrangements have waxed and waned as populations responded to repeated glaciations and climate change in eastern North America, leaving footprints of clinal variation and abundant inversion polymorphism (Etges & Levitan, 2004). Concerted temporal genetic changes in populations of *D. robusta* (Levitan, 2003; Levitan & Etges, 2005), as well as other *Drosophila* species (Rodríguez-Trelles & Rodríguez, 1998; Umina *et al.*, 2005; Balanya *et al.*, 2006; Stamenkovic-Radak *et al.*, 2008) strongly implicate large scale warming-related patterns (Barry *et al.*, 1995; Parmesan & Yohe, 2003; Root *et al.*, 2003; Parmesan & Galbraith, 2004) as a cause of recent inversion frequency shifts.

Early on, study of frequency shifts of *D. robusta* chromosome polymorphisms in local populations suggested genetic stability rather than temporal change (Carson, 1958; Levitan, 1992). Carson (1958) first characterized such genetic equilibrium over 10 years in a local Missouri population that he interpreted as a general characteristic of this widespread species, despite the extensive accumulated data showing significant geographic variation across eastern North America (Carson, 1959). However, collections from the early 1980s in the Smoky Mountains National Park revealed parallel frequency shifts in multiple populations that had been previously sampled in 1947 and 1958–59 (Etges, 1984). More historical changes in these frequencies have recently been uncovered in a number of *D. robusta* populations (Levitan, 2001; Levitan, 2003). The correlation of some of these frequency shifts with long-term temperature trends led to the hypothesis that these historical frequency shifts were symptomatic of regional or global warming (Levitan, 2003; Levitan & Etges, 2005; Etges, Arbuckle & Levitan, 2006).

However, how temperate zone warming has caused inversion frequency shifts in *Drosophila* species is not completely clear. Lessening of extreme winter temperatures, longer growing seasons, and higher summer temperatures can have related phenological effects on small, multivoltine insects that may influence chromosome frequency changes in ways not due to direct effects of long-term temperature changes *per se* (e.g. selection for increased heat tolerance; Bradshaw & Holzapfel, 2006; Bradshaw & Holzapfel, 2008). Earlier initiation of spring for drosophilids that over-winter as adults in diapause (Carson & Stalker, 1948; Lumme & Lakovaara, 1983; Schmidt *et al.*, 2005) implies an earlier start of warm season population growth, particularly in more southerly populations. This, in turn, has been hypothesized to have potentially increased migration rates from southern to northern populations, another explanation for long-term trends in gene arrangement frequencies (Balanya *et al.*, 2006; Santos, 2007). Longer growing seasons may also impose selection for shorter critical day lengths for entering diapause in fall (Bradshaw & Holzapfel, 2008). Therefore, using temperatures recorded when flies were sampled as predictors of inversion frequency change (Rodríguez-Trelles *et al.*, 1998; Levitan & Etges, 2005; Umina *et al.*, 2005; Balanya *et al.*, 2006) may not directly represent the causal factors responsible for microevolutionary change in these *Drosophila* species.

We view local temperatures as a proxy for the influences of regional climatic shifts on *D. robusta* inversion polymorphism in the present study. In an attempt to control for seasonal temperature influences, we previously evaluated correlations with temperatures for the month prior to collection and average temperature for the 3 months prior to collection. Both were found to be nonsignificant (Levitan & Etges, 2005). In the present study, we explicitly assessed potential seasonal influences on long-term shifts in chromosomal polymorphism in *D. robusta* by including month of collection as a variable in our analyses.

Thus, one purpose of this report is to assess further frequency shifts up until the 2006 and 2007 collections of wild *D. robusta*. We also reanalysed previously published and unpublished data, compared local population structure and inversion frequency shifts in the Ozark and Ouachita mountain regions, and compared these local populations with others

across the species range to more precisely characterize rates of temporal frequency change. We show that inversion frequency changes are continuing in geographically disparate populations consistent with the hypothesis of regional or global warming in many cases, but some populations show evidence of: (1) temporal change not associated with changing temperatures; (2) little or no temporal change; or (3) in some cases, temporal shifts have stopped for temperature sensitive gene arrangements that are near fixation.

MATERIAL AND METHODS

Adult *D. robusta* were trapped in deciduous woods over fermenting bananas in mid-summer, with most collections made in June, July, or August at 26 long-term and recently collected sites (Fig. 1). We concentrated on populations that were sampled previously, some with records starting in the 1940s. Wild-caught adults were karyotyped using standard techniques (Levitan, 1955). 'Egg samples', or salivary gland smears from larvae derived from matings in the wild, were sometimes used to supplement the adult data.

Carson & Stalker (1947) designated certain band sequences of the salivary gland chromosomes as 'Standard' arrangements and named them for the respective arms of the three metacentric chromosomes (i.e. XL, XR, 2L, 3R, etc.). Most gene arrangements in natural populations are the result of one-step inversions from the Standards. Inverted arrangements were named and numbered in the order of their discovery (e.g., XL-1, XL-2, 2L-1, 2L-2, etc.) (Carson, 1958). X chromosome gene arrangements show widespread linkage disequilibrium (Levitan, 1992), and so whole X chromosome frequencies were analysed. Each is labelled by left and right arm arrangements (e.g. XL.XR is labelled SS, XL-1.XR-2 is labelled 12, etc.). Autosomal disequilibrium was of a more limited nature (Levitan & Etges, 1995).

Significance of year-to-year and among site differences for each chromosome or chromosome arm was determined by G-tests (Sokal & Rohlf, 1995). Analysis of covariance in PROC GLM and regression analysis in PROC REG (SAS Institute, 2004) were used to test for overall significance of temporal chromosome frequency changes across sites and years. Although more complex regression models were evaluated, particularly due to the apparent increases in temperatures starting in the 1970s and 1980s, linear regressions of chromosome frequencies on year were used as they

Figure 1. Locations of *Drosophila robusta* populations included in the present study. The dashed line indicates a division into eastern and western regions based on the historical biogeography of eastern deciduous tree species and previously documented longitudinal differences in chromosome arrangement frequencies.

best described patterns of change over time. All frequency data were arcsin-transformed to improve normality. Principal components analysis (PCA) was used to assess overall frequency shifts using PC scores in analysis of covariance (ANCOVA) to identify correlated genetic shifts over time. Spearman rank correlations between arcsin-transformed chromosome frequencies and average temperatures of the month for each collection were calculated with PROC CORR (SAS Institute, 2004). Temperature data were obtained from the National Climatic Data Center (http://www.ncdc.noaa.gov/oa/ncdc.html) for stations nearest to each site. We assessed inversion frequency correlations with average daily temperature, average high daily temperature, and average low daily temperature.

Over a finer geographical scale, a number of Ozarks and Ouachita populations were resampled to assess recent temporal change and population structure. Neighbour-joining (NJ) trees (Saitou & Nei, 1987) were constructed from 1000 bootstraps of the inversion frequency data and the resulting Nei's genetic distances between populations (Nei, 1972) in PHYLIP, version 3.67 (Felsenstein, 2007). Majority rule consensus trees were constructed to compare populations sampled 15–20 years apart. Associations between genetic distance matrices based on gene arrangement frequencies from these populations sampled in the 1980s and 1990s versus 2004–2007 and geographic distance matrices were evaluated with Mantel tests (Mantel, 1967; Smouse, Long & Sokal, 1986) to assess changes in the relationships between geographic distance and changing genetic distance between populations. Pairwise, great circle distances between sites were calculated using the 'Haversine' formula (Veness, 2006).

RESULTS

Historical frequency changes across the species range

Overall variation in chromosome frequencies across all 22 populations for which there were temporal sequences of data was subjected to PCA. The first five principal components accounted for 88% of the variation in the data (Table 1). We focused on the first three principal components that accounted for 73% of the variation, with PC I accounting for 37% of the overall variation in gene arrangement frequencies. We attempted to resample most populations that had been previously sampled, but this was not always possible. Some time series were more complete than others, such as data spanning over 60 years at Englewood, New Jersey, and Olivette, Missouri (see data summary in the Appendix, Table A1). Some populations were sampled only two times and others infrequently but, by combining all such cases, ANCOVA revealed the degree of heterogeneity in PC score slopes for temporal changes, as well as region X year interactions. We grouped populations from Iowa and those from central Indiana due to geographical proximity.

Changes in PC I over time revealed concerted directional evolution for most populations sampled (Fig. 2). PC I encompassed a latitudinal component of the variation in gene arrangement frequencies with high positive loadings for arrangements in the southern

Table 1. Loadings on the first five principal components for X chromosome and autosomal gene arrangement frequencies for 22 populations of *Drosophila robusta* in the present study, sampled over time

	PC I	PC II	PC III	PC IV	PC V
SS	–0.1415	–0.5332	–0.0113	–0.1630	0.3062
S1	0.0186	–0.0848	0.5734	0.1835	–0.3903
S2	0.2815	0.1479	–0.0960	–0.0978	0.6603
1S	–0.3097	0.1150	–0.3241	–0.0984	–0.0395
11	–0.1001	0.3089	0.3213	0.5909	0.2370
12	0.0223	0.3834	–0.2981	–0.2146	–0.4302
22	0.3770	0.1490	–0.1743	0.0245	–0.0609
2L	–0.1617	0.2369	0.4250	–0.5496	0.1068
2L-1	0.2003	–0.4845	–0.1916	0.2651	–0.1424
2L-2	0.4033	0.0999	0.0267	–0.0519	–0.0776
2L-3	–0.2838	0.3026	–0.2654	0.3813	0.1321
2R-1	0.3904	0.1184	0.1968	–0.0316	0.1170
3R-1	0.4358	0.0481	–0.0847	0.0567	–0.0513
Eigenvalue	4.843	2.451	2.170	1.015	0.859
Percent of the total variance	0.37	0.19	0.17	0.08	0.07

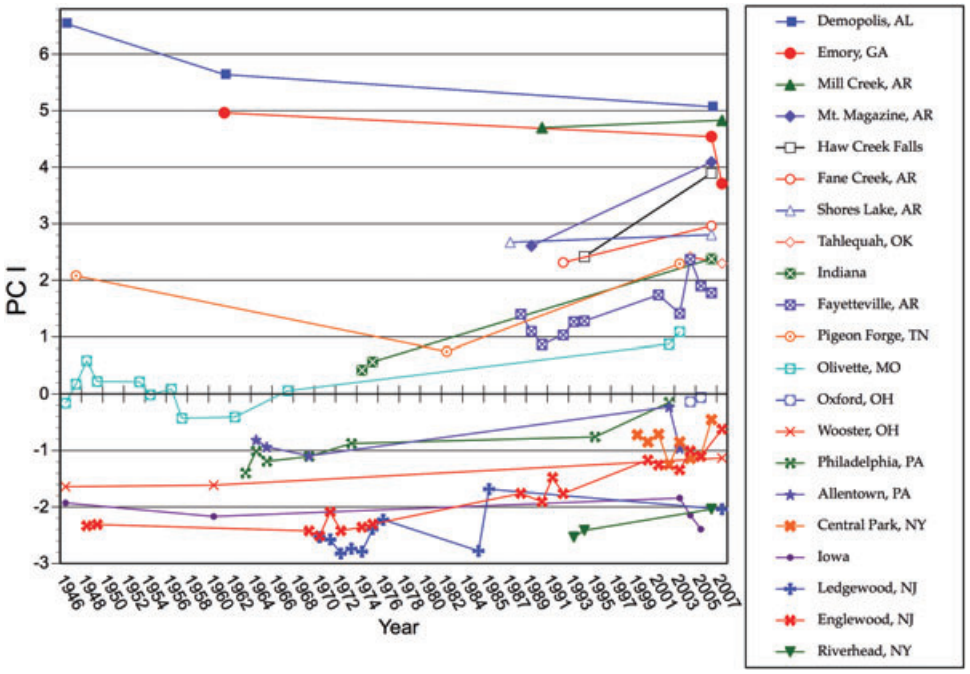

Figure 2. Historical genetic changes in *Drosophila robusta* populations illustrated by variation in principal component I (PC I) with time.

parts of the species range, including S2, 22, 2L-1, 2L-2, 2R-1, and 3R-1, and negative loadings for more northerly chromosomes, 1S and 2L-3. Thus, increases in PC I scores over time in many of the populations sampled (Fig. 2) reflected widespread increases in the frequencies of these 'southern' arrangements (i.e. S2, 22, 2L-1, 2R-1, and 3R-1) and decreases in 'northern' 1S and 2L-3 (Table 1). PC II and PC III encompassed 19% and 17% of the total variation in these data, respectively (Table 1). PC II contained a component of geographic variation due to high negative correlations with SS and gene arrangement 2L-1, and positive correlations with X chromosomes 11 and 12 and gene arrangements 2L and 2L-3. Because overall frequencies of SS and 2L-1 were positively correlated (Spearman's $r = 0.446$, $P < 0.0001$, $N = 113$), and both were negatively correlated with PC II, this component of variation represents both geographical variation from west to east, as well as temporal changes in populations in the western part of the range (see below). S1, 11 and 2L were positively loaded on PC III (Table 1). S1 and 11 have smaller west–east distributions across the northern part of the species range, and 2L is distributed along a southwest to northeast axis (Etges & Levitan, 2004), indicating PC III encompassed aspects of this east–west variation.

We further inspected the temporal shifts in PC scores by grouping populations into eastern and western groups (Fig. 1) because of the significant longitudinal frequency differences for some arrangements across the species range (Etges & Levitan, 2004). This geographical division was also based on the historical influences of the Mississippi River on the biogeography of the eastern deciduous forest tree species (Delcourt & Delcourt, 1987) used as breeding sites by *D. robusta*, as well as the Appalachian Mountains, hypothesized to have influenced biogeographical patterns of chromosomal polymorphisms in *D. robusta* (Etges & Levitan, 2004). Focusing on the first three PCs, temporal shifts were significant for PC I and PC III, and strong regional differences were apparent for PC II and PC III consistent with PC loadings described above. Significant year X region interactions were observed for all three PCs (Table 2) signifying region-specific temporal shifts in these chromosomal arrangements. Thus, the long-term genetic changes in populations described by Levitan (2003) and Levitan & Etges (2005) were not uniform across the species range.

We then investigated whether smaller-scale heterogeneity in chromosome frequency shifts was significant within eastern and western regions by performing nested ANCOVAs, with populations nested within regions (Table 2). Using sequential sums of squares (Type I) for the first three PCs, the effect of year was significant in all cases ($P < 0.0001$; results not shown), and using Type III sums of squares, the effects of population nested within region and population X year were significant, but the main effect of year was not (Table 2). Because Type III

Table 2. Analysis of covariance results for principal component (PC) I, PC II, and PC II based on all X chromosomes and autosomal gene arrangement frequencies for temporal genetic changes of populations grouped into eastern and western regions and populations nested with geographical regions

		PC I			PC II			PC III		
A. Source	d.f.	Type III SS	*F*	$P_r > F$	Type III SS	*F*	$P_r > F$	Type III SS	*F*	$P_r > F$
Populations grouped into eastern and western regions										
Model	3	129.873	11.56	< 0.0001	67.146	11.90	< 0.0001	137.038	49.26	< 0.0001
Region	1	14.402	7.14	NS	40.950	21.77	< 0.0001	17.744	19.14	< 0.0001
Year	1	20.549	2.24	0.021	7.032	3.74	0.055	26.671	28.76	< 0.0001
Year × Region	1	15.166	6.77	0.047	41.495	22.06	< 0.0001	16.891	18.22	< 0.0001
Error	105	393.184			197.544			97.360		
Populations nested with geographical regions										
Model	41	514.782	101.65	< 0.0001	249.688	27.20	< 0.0001	223.478	33.44	< 0.0001
Region	1	0.017	0.14	NS	0.785	3.51	NS	0.009	0.06	NS
Year	1	0.114	0.92	NS	0.251	1.12	NS	0.082	0.50	NS
Population*	19	10.022	4.27	< 0.0001	15.388	3.62	< 0.0001	7.734	2.50	0.003
Year × Region	1	0.019	0.15	NS	0.777	3.47	NS	0.010	0.06	NS
Year × Pop*	19	9.393	4.00	< 0.0001	15.805	3.71	< 0.0001	7.552	2.44	0.004
Error	67	8.275			15.002			10.920		

*Populations nested within region.
NS, not significant.

sums of squares for each level take into account all other factors in the model, these results show that temporal chromosome frequency changes have occurred across the species range in *D. robusta*, but were specific to particular populations and years for PC I, II, and III. This was anticipated given the temporal changes in all populations shown in Figure 2. For example, the two most southeasterly populations, Demopolis, AL, and Emory, GA, showed decreases in PC I scores through 2007. High frequencies of 2R-1 and 3R-1, both positively associated with PC I (Table 1), have not increased appreciably through 2007 in these populations, although SS and S2 have. Gene arrangement 3R-1 has either reached fixation or is close to it (see Appendix, Table A1). Several other populations were characterized by irregular temporal shifts, adding to the population by year interaction.

We included year and month of collection in linear regression models to detect temporal frequency changes because within-year, seasonal frequency shifts could have biased estimates of long-term chromosome frequency shifts for populations collected in different months. In addition to the effect of year, month of collection was significant for X chromosome combinations SS ($t = -2.78$, $P = 0.008$) and 11 ($t = 2.15$, $P = 0.037$) in western populations only. Because 88% of all collections were made in June, July, and August (see Appendix, Table A1), the possible influences of the few sites collected in spring and fall on yearly genetic trends were thus likely to be small.

Linear regressions of gene arrangement frequencies on year often revealed contrasting temporal changes in eastern and western populations (Table 3). Frequencies of arrangement 2L-1 increased and 2L decreased significantly with time across the range (Table 3), but all other arrangements either showed evidence of change in the west or the east, but not both, or in some cases, slopes of different sign in different regions. In the western populations sampled, frequencies of SS and S1 have decreased whereas S2, 12, and 22 have significantly increased over time; however, SS frequencies also declined later in the year of collection. In the eastern group, SS and S1 have significantly increased, but 1S has decreased in frequency with time, suggesting temperature-associated causes. Two 'southern' arrangements, 2R-1 and 3R-1 have significantly increased in western populations along with 2L-2, but this latter arrangement has significantly declined over time in the east (Table 3). Thus, a majority of these gene arrangements has shifted in frequency over time, but many of these evolutionary changes have occurred in a region-specific fashion consistent with the PCA results (Fig. 2).

All three temperature indicators, average daily temperature, average daily high temperature, and average daily low temperature for the month in which

Table 3. Linear regression slopes, their standard errors, and significance values for yearly changes of X chromosome arrangement combination and autosomal gene arrangement frequencies in populations of *Drosophila robusta* grouped into eastern and western groups

	Eastern populations				Western populations			
Arrangement	Slope	SE	*t*	*P*	Slope	SE	*t*	*P*
SS	0.00502	0.00154	3.27	0.0018	–0.00299	0.00103	–2.91	0.0056
1S	–0.00340	0.00127	–2.67	0.0097	0.00018	0.00020	0.90	NS
S1	0.00197	0.00053	3.73	0.0004	–0.00575	0.00123	–4.66	< 0.0001
11	–0.00033	0.00023	–1.44	NS	–0.00027	0.00085	–0.32	NS
S2	–0.00101	0.00064	–1.57	NS	0.00185	0.00053	3.47	0.0011
12	–0.00047	0.00072	–0.66	NS	0.00253	0.00093	2.71	0.0094
22	–0.00160	0.00094	–1.70	NS	0.00411	0.00130	3.17	0.0028
2L	–0.00472	0.00103	–4.59	< 0.0001	–0.00405	0.00087	–4.66	< 0.0001
2L-1	0.00645	0.00120	5.38	< 0.0001	0.00238	0.00097	2.46	0.0178
2L-2	–0.00117	0.00042	–2.78	0.0072	0.00088	0.00041	2.13	0.0388
2L-3	–0.00022	0.00078	–0.28	NS	0.00057	0.00084	0.69	NS
2R-1	–0.00008	0.00059	–0.14	NS	0.00077	0.00028	2.77	0.0081
3R-1	0.00126	0.00244	0.52	NS	0.01015	0.00201	5.04	< 0.0001

All frequency data were arcsin-transformed.
NS, not significant.

Table 4. Spearman correlations between X arrangement combination frequencies and average daily temperature, average daily maximum temperature, and average daily minimum temperature for the month of capture for each population listed in the Appendix

	SS	S1	S2	1S	11	12	22
Average daily temperature							
All	0.176	0.168	0.154	**–0.432******	–0.149	–0.208*	0.190*
East	**0.463******	0.073	0.002	–0.354**	–0.258*	–0.419***	0.082
West	0.149	–0.121	0.137	–0.332*	–0.408**	0.035	0.125
Average daily maximum temperature							
All	0.035	0.100	0.288**	**–0.416******	–0.108	–0.134	0.246**
East	0.247*	–0.165	0.215	–0.309*	–0.099	–0.291*	0.214
West	0.110	–0.134	0.214	–0.218	–0.343*	0.070	0.095
Average daily minimum temperature							
All	0.277**	0.164	0.026	**–0.377******	–0.221*	–0.245**	0.140
East	**0.524******	0.176	–0.105	–0.388**	–0.177	–0.406***	0.045
West	0.188	–0.083	0.035	–0.411**	–0.459**	–0.035	0.135

Values in bold were significant after strict Bonferroni correction. For all populations, $N = 113$; east populations, $N = 65$; west populations, $N = 48$.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

each population was collected were highly correlated, as expected (all $P < 0.0001$). Of the more 'southern' chromosomes (i.e. SS, S2, 22, 2L-1, 2R-1, and 3R-1) that increased over time in eastern or western populations (Tables 4, 5), only SS, 2L-1, and 2R-1 were positively correlated with one or more indices of temperature after Bonferroni correction. Frequencies of widespread 'northern' 1S and 2L-3 were negatively correlated with temperature, with the latter mostly in the east. However, 2L-3 showed no consistent decreases over time (Table 3). Frequencies of 'southern' arrangements 2R-1 and 3R-1 have increased with temperature overall, particularly with average maximum temperatures, although correlations for 3R-1 and temperature were not significant after Bonferroni correction.

Frequencies of X chromosome arrangement combination 11 and gene arrangement 2L-3 were negatively

Table 5. Spearman correlations between autosomal gene arrangement frequencies and average daily temperature, average daily maximum temperature, and average daily minimum temperature for the month of capture for each population listed in the Appendix

	2L	2L-1	2L-2	2L-3	2R-1	3R-1
Average daily temperature						
All	0.043	0.255**	0.204*	**–0.465****	0.299**	0.274**
East	0.010	0.395**	–0.126	**–0.524****	0.005	0.056
West	–0.087	0.146	0.155	–0.212	0.106	0.179
Average daily maximum temperature						
All	0.120	0.097	0.309***	**–0.420****	**0.353****	0.284**
East	0.104	0.157	0.143	–0.401***	0.124	0.047
West	–0.061	0.181	0.087	–0.129	0.086	0.144
Average daily minimum temperature						
All	–0.094	**0.393****	0.095	**–0.426****	0.192*	0.257**
East	–0.195	**0.538****	–0.267*	**–0.494****	–0.057	0.130
West	–0.130	0.222	0.198	–0.283	0.121	0.213

Values in bold were significant after strict Bonferroni correction. For all populations, $N = 113$; east populations, $N = 65$; west populations, $N = 48$.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

correlated with temperature despite the absence of widespread temporal changes (Table 3). The absence of detectable changes with time, but significant correlations with increasing temperatures, was likely due to the smaller number of populations with appreciable frequencies of these arrangements that have been sampled repeatedly (e.g. the large number of populations in which 11 and 2L-3 were not present; see Appendix, Table A1) or perhaps subtle seasonal influences (11 frequencies increased seasonally). Also, frequencies of X chromosome arrangement combination S1 have increased in the east and decreased in the west (Table 3), and these shifts were also unrelated to ambient temperature changes (Tables 4, 5). Thus, factors unrelated to temperature were associated with long-term changes for some *D. robusta* chromosomes.

Genetic structure and change in the Ozark and Ouachita Interior Highlands

Across a smaller spatial scale, short-term temporal trends were assessed in *D. robusta* populations distributed in the western Ozarks of southwestern Missouri and western Arkansas, the south-central Ozarks and Mt Magazine, and the west-central Ouachitas (Fig. 1). Because these populations comprised much of the western group (above) and were located in forested sites away from large urban areas, we hypothesized that they should share more similar responses to environmental change over the approximately 13–20-year period they have been studied. Western Ozarks populations (i.e. Fayetteville, Tahlequah, and Washburn, Missouri) are genetically differentiated from both the south-central Ozarks (i.e. Shores Lake, Fane Creek, Haw Creek Falls, and Mt. Magazine) and the Ouachita populations (i.e. Mill Creek) (Levitan & Etges, 1995).

Resampling the four south-central Ozark populations in 2006 and the Mill Creek Creek site in 2007 in the Ouachitas permitted closer inspection of temporal genetic changes subsequent to the late 1980s to 1990s. Frequencies of SS, 1S, 11, 12, 22, 2L-2, and 3R-1 changed significantly during this period (all $P < 0.05$). The Ozarks populations have become more genetically differentiated for X chromosome arrangement combinations over time, where the early samples did not differ in X chromosome arrangement (= XCH) frequencies, but were heterogeneous in second chromosome, left arm (= TWL) frequencies ($G = 19.17$, $P = 0.024$) and third chromosome frequencies ($G = 8.703$, $P = 0.034$). In particular, Haw Creek Falls and Mt Magazine contained lower frequencies of 3R-1 than the other populations. By 2006, increases in 22 and decreases in 12 caused significant heterogeneity in XCH ($G = 56.66$, $P < 0.0001$), TWL remained heterogeneous ($G = 53.79$, $P < 0.0001$), and 3R-1 increased to more homogeneous frequencies ($P = 0.08$) in all populations. Interestingly, frequencies at the Mill Creek site were unchanged, except for a small increase in 2R-1 (see Appendix, Table A1).

These patterns of genetic similarity in the Ozarks and Ouachita populations were further scrutinized by constructing NJ trees based on pairwise Nei's genetic distances from the early and more recent frequency data (Fig. 3). Branch support is shown at each node

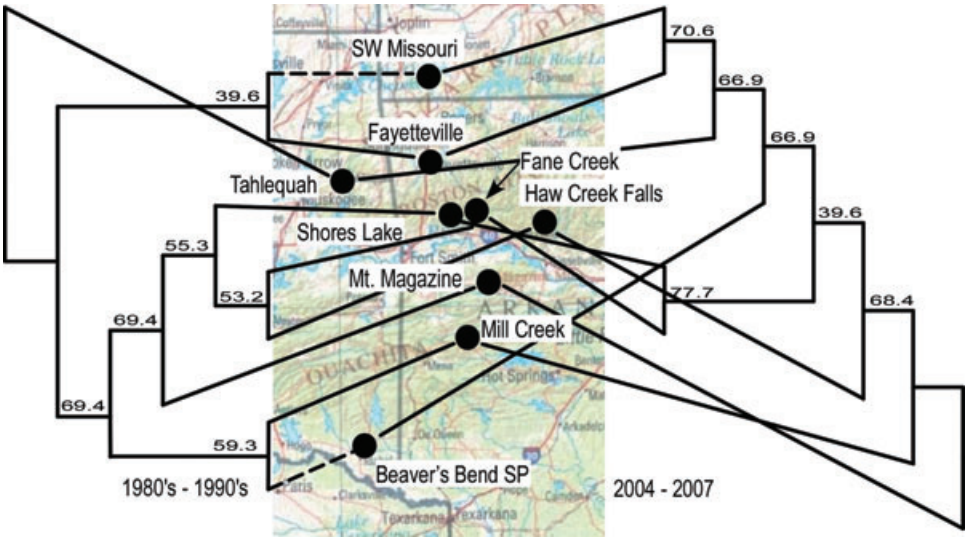

Figure 3. Temporal shifts in the topology of Neighbour-joining trees based on gene arrangement frequencies for nine populations in the Ozarks/Ouachita region. Numbers adjacent to nodes correspond to the percentage of times that node was recovered after 1000 bootstraps. Dashed lines indicate the two populations that were sampled once in 2004–5. For details, see text.

based on 1000 bootstraps of the frequency data. Populations sampled only once were included in both NJ trees for comparison. Tree topologies were unaffected by randomizing the input order of populations. Despite temporal frequency shifts, the central Ozarks populations remain clustered, except Mt Magazine (south of the Arkansas River) has evolved higher similarity to more southern populations. A 'western Ozarks' group (southwestern Missouri, Fayetteville, Tahlequah, and Beaver's Bend SP) has become better resolved, but branch support in some cases was weak. Overall, the genetic structure of these populations has persisted despite microevolutionary temporal change from the 1980s and early 1990s up until 2006 and 2007.

Associations between Nei's genetic distance and geographic distance were not significant in either early or recent data (Mantel test, early: $\rho = -0.192$, $P = 0.814$; late: $\rho = -0.254$, $P = 0.917$), allowing rejection of isolation by distance as an explanation for genetic differentiation in these populations. Thus, the geographic proximity of these populations was unrelated to overall genetic differentiation even though chromosome frequencies have shifted significantly in some populations associated with temperature change.

DISCUSSION

Populations of *D. robusta* continue to evolve in response to temperature-associated climatic changes across eastern North America (Fig. 2). However, overall temporal changes varied depending on geographic location and local chromosome frequencies. Some gene arrangements have increased in frequency associated with increasing temperatures throughout the range (e.g. 2L-1) but others have shifted regionally (Table 3). Those that have long been associated with latitudinal and elevational gradients (e.g. 1S, SS, 2L-1, 2L-3, and 3R-1; Carson, 1959; Levitan, 1992), showed consistent associations with temperature changes (Tables 4, 5) but not always consistent long-term temporal trends (Table 3). This may not be surprising in a species with such a large amount of structured geographic polymorphism (Levitan, 1992; Etges & Levitan, 2004), but is required information for understanding the course of future climatically induced evolutionary change.

Across the range of *D. robusta*, populations have evolved in response to shifting temperatures in as little as 15–20 years (Levitan & Etges, 2005; Etges *et al.*, 2006), which corresponds to recent regional warming trends that began in the 1980s (Karl & Trenberth, 2003). Regional shifts in temperature and precipitation in the USA have varied substantially subsequent to the 1930s. Average annual temperatures have increased in the northeastern and western states, but have decreased in the southeast, and as far west as Missouri and Oklahoma. This may explain the temporal decreases in PC I for the Alabama and Georgia populations, but not the increases observed for the Ozarks/Ouachita groups (Fig. 2). Most of these temperature increases have occurred in winter and spring (Karl *et al.*, 1996), consistent with hypotheses

that increasingly mild winters and longer growing seasons may be driving these climatically driven evolutionary changes (Bradshaw & Holzapfel, 2006). At present, we hypothesize that these regional differences in historical temperature changes are indicative of how regional warming has shaped geographical differences in gene arrangement frequency shifts in eastern versus western populations of *D. robusta*, but more data on the phenotypic effects of these chromosomes are needed.

Away from urban areas and their 'heat island' influences on air temperatures (Hudischewskyj, Douglas & Lundgren, 2001), most of the Ozarks and Iowa populations (Fig. 2) showed evidence of concerted chromosomal frequency shifts. In the western Ozarks, only the Fayetteville population has been repeatedly collected preventing comparisons with other populations within this area, and only 3R-1 frequencies showed evidence of increasing in this population from 1988 to 2006 (slope = 0.0113, $t = 4.53$, $P = 0.0014$; see Appendix, Table A1). The genetically differentiated south-central Ozarks populations have continued to evolve, all reaching high frequencies of 3R-1 ($P = 0.813$–0.913), but overall levels of genetic differentiation within the Ozarks–Ouachita region have shifted only slightly (Fig. 3). Further north, the Iowa populations were characterized by high relative frequencies of S1 and 11, X chromosomes not widely distributed across the range, and not strongly associated with PC I (Table 1). Thus, these Iowa populations exhibited different historical trajectories than most of the other populations studied (e.g. Demopolis, AL and Emory, GA; Fig. 2). In addition, frequencies of 2L-3, a 'northern' and 'high elevation' gene arrangement (Carson, 1958; Levitan, 1992), have increased in the Iowa samples (see Appendix, Table A1), but were negatively correlated with temperature (Tables 4, 5). The overall geographic diversity in response to temperature associated shifts in *D. robusta* is reminiscent of cases of uniform selection, where different populations or species have evolved in response to common environmental factors by a variety of genetic means (Bishop & Cook, 1981; Cohan & Hoffman, 1989).

Together with *D. subobscura* populations on three continents (Balanya *et al.*, 2004; Balanya *et al.*, 2006) and *D. melanogaster* in Australia (Umina *et al.*, 2005), the geographical range-wide frequency shifts in *D. robusta* have revealed a degree of evolutionary flexibility in widespread species in response to aspects of climatic change. Many of these chromosomal arrangements have responded to climate associated causes in natural populations, helping to put in perspective the causes for complex east–west, north–south, and elevational clines, as well as the more limited regional distributions of some chromosomes subsequent to *D. robusta* occupying North America (Etges & Levitan, 2004). These geographical patterns have shifted over decadal time periods (Fig. 2) but, over shorter intervals, can remain at stable equilibrium frequencies (Carson, 1958). Mechanisms thought to influence *D. robusta* chromosome frequencies in nature include chromosome dependent influences on fitness components (Etges, 1989), sex specific viability selection, and sexual selection (Etges, 1996). Those gene arrangements that have shifted along with increases in temperature such as 1S and 2L-1 should help to further resolve specific regions of the genome and, ultimately, the genes that are responsible for these microevolutionary patterns. The consequences of future climatic shifts to these and other species must therefore include the course of future genetic changes in widespread species, as well as phenotypic (plastic), demographic, and behavioral alterations when the long-term fates of species distributions and their persistence are considered.

ACKNOWLEDGEMENTS

We thank C. Marshall, K. Fields, T. Gregg, B. McAllister, A. Miles, and H. Wistrand for help in collecting wild flies and M. Castanata, S. Gmuca, A. Hafeez, E. Hoque, J. Katz, B. Li, P. N. Moussazadeh, and N. Khanna for their assistance in the laboratory. This work was partially supported grants from the M. M. Kaplan Family Foundation to M.L. and W.J.E. and an REU supplement to NSF DEB-02111025 to W.J.E. supporting A. Miles.

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APPENDIX

Table A1. X chromosome arrangement association and autosomal gene arrangement frequencies for populations of *Drosophila robusta* sampled by year in the present study

Population	Year	Month	N_X	SS	S1	S2	1S	11	12	22	N_2	2L	2L-1	2L-2	2L-3	2R-1	N_3	3R-1
Allentown, PA	1964	August	45	46.7	4.4	20	2.2	0	22.2	4.4	70	45.7	15.7	5.7	32.9	5.7	67	22.4
Allentown, PA	1965	July	75	26.7	2.7	12	9.3	0	44	5.3	94	48.9	18.1	7.4	25.5	3.2	88	14.8
Allentown, PA	1969	August	103	32	10.7	13.6	18.4	0	21.4	3.9	145	43.4	26.2	5.5	24.8	4.1	142	9.9
Allentown, PA	2002	August	66	36.4	18.2	10.6	15.2	0	18.2	1.5	86	17.4	60.5	2.3	19.8	7	87	34.5
Allentown, PA	2003	June	219	41.6	18.3	4.6	21.5	0	13.7	0.5	289	30.1	48.1	1.4	20.4	7.6	289	28
Beaver's Bend SP, OK	2004	June	158	55.7	1.9	10.1	0	0	0	31.6	241	15.4	63.5	21.2	0	12.4	241	98.3
Bloomington, IN	1974	July	94	40.4	13.8	16	2.1	4.3	8.5	14.9	114	68.4	22.8	5.3	3.5	16.7	119	43.7
Bloomington, IN	1975	June/July	969	35.3	14.6	20.4	5.3	1.6	11.5	11.2	1447	65.3	24.8	7	2.6	17.6	1439	31.9
Central Park, NY	1999	June/July	109	48.6	22	1.8	19.3	0	3.7	4.6	161	14.3	72	0	13.7	4.4	158	17.2
Central Park, NY	2000	June	52	53.8	19.2	0	19.2	0	7.7	0	72	13.9	75	1.4	9.7	0	72	20
Central Park, NY	2001	July	203	50.2	28.1	2	9.9	0	8.9	1	306	16	69.6	0.7	13.7	1.6	306	21.9
Central Park, NY	2002	July	164	61	26.2	3	6.7	0	2.4	0.6	214	29.4	52.8	0	17.8	0.5	213	21.6
Central Park, NY	2003	August	39	35.9	17.9	5.1	10.3	0	28.2	2.6	62	37.1	46.8	0	16.1	3.2	62	33.9
Central Park, NY	2004	June	126	48.4	26.2	3.2	8.7	0	11.9	1.6	183	31.7	53	0	15.3	1.1	183	17.5
Central Park, NY	2005	July	205	54.6	24.4	0	12.2	0	8.8	0	290	18.6	65.5	0	15.9	0.7	290	20
Central Park, NY	2006	June/July	196	57.7	34.7	0.5	3.6	0	3.1	0.5	250	9.6	77.2	0	13.2	3.2	249	26.5
Cossatot River, AR	2007	June	117	27.4	2.6	6.8	0	0	2.6	59	149	15.4	62.4	22.1	0	10.1	149	95.3
Demopolis, AL	1946	April	18	5.6	0	27.8	0	0	0	66.7	30	13.3	56.7	30	0	30.2	30	100
Demopolis, AL	1961	July	13	30.8	0	15.4	0	0	0	53.8	18	11.1	55.6	33.3	0	33.3	18	94.4
Demopolis, AL	2006	May	32	28.1	0	21.9	0	0	0	50	34	2.9	79.4	17.6	0	20.6	34	100
Emory, GA	1961	August	391	4.9	0.5	38.1	0.8	0	2	53.7	529	16.4	62.9	16.3	4.3	24.2	526	91.3
Emory, GA	2006	September	225	48.9	1.8	28.9	0	0	1.3	19.1	227	8.8	74.4	15.4	1.3	37.9	224	92.4
Emory, GA	2007	August/ September	74	64.9	1.4	24.4	0	0	0	9.5	98	13.3	70.4	13.3	3.1	33.7	97	94.9

Englewood, NJ	1948	September	260	36.92	0.38	2.31	51.15	0	8.85	0.38	359	39.83	27.86	5.57	26.74	0.84	356	3.93
Englewood, NJ	1949	May	120	40	0	1.67	48.33	0	8.33	1.67	172	44.19	30.23	2.91	22.67	2.91	172	1.74
Englewood, NJ	1969	August	216	42.13	0.93	1.39	52.78	0	2.78	0	298	42.28	38.59	1.34	17.79	0.67	282	2.13
Englewood, NJ	1970	July	248	41.94	0	0.81	53.23	0	3.23	0.81	364	50.82	34.07	0.55	14.56	0.82	359	4.74
Englewood, NJ	1971	July	117	47.86	0	1.71	48.72	0	0.85	0.85	142	46.48	39.44	2.11	11.97	2.82	142	6.34
Englewood, NJ	1972	July	353	45.89	0	1.7	47.88	0	4.53	0	496	54.84	30.85	1.21	13.1	1.01	459	5.23
Englewood, NJ	1974	July/August	240	44.58	1.25	1.67	42.5	0	9.17	0.83	374	47.59	29.95	1.6	20.86	0.8	342	6.73
Englewood, NJ	1975	July	528	43.18	0.38	2.65	43.18	0.19	9.85	0.57	713	45.86	32.4	0.98	20.48	1.12	683	6.88
Englewood, NJ	1988	August	91	62.64	5.49	1.1	26.37	0	4.4	0	76	35.3	50.9	0	13.8	1.7	113	8.8
Englewood, NJ	1990	August	79	59.49	2.53	0	29.11	0	8.86	0	111	26.13	47.75	0	25.23	3.6	111	9.91
Englewood, NJ	1991	August	158	50.63	4.43	1.9	29.11	0	13.92	0	232	28.45	54.74	0.86	15.95	2.15	224	14.73
Englewood, NJ	1992	July	95	54.74	2.11	0	33.68	1.05	6.32	1.05	169	25.44	50.89	1.18	22.48	0.59	168	22.62
Englewood, NJ	2000	July	218	63.76	12.39	2.29	15.6	0	4.59	1.38	318	18.24	64.47	0	17.3	0.95	317	19.56
Englewood, NJ	2001	July	111	71.17	9.01	0	15.32	0	4.5	0	223	22.87	60.09	0	17.04	2.69	218	27.06
Englewood, NJ	2003	June	119	68.07	5.88	0	19.33	0	6.72	0	150	32.7	53.3	0	14	3.33	150	30.7
Englewood, NJ	2004	June	186	56.45	13.98	1.08	19.89	0	5.91	2.69	260	16.2	65.4	0.4	18.1	1.5	260	30.4
Englewood, NJ	2005	June	244	63.52	11.89	0.82	18.85	0	4.51	0.41	334	16.8	65.9	0	17.4	1.2	335	31.6
Fane Creek, AR	1992	July	116	0.9	3.4	12.1	0.9	0	52.6	30.2	182	46.2	37.4	6	10.4	25.3	179	85
Fane Creek, AR	2006	July	97	8.2	4.1	10.3	0	0	35.1	42.3	110	34.5	37.3	17.3	10.9	19.1	109	86.2
Fayetteville, AR	1988	June	334	22.5	39.5	9.3	3.3	2.4	17.1	6	458	32.5	52.6	11.4	3.5	15.1	458	60.7
Fayetteville, AR	1989	June/July	647	26	48.8	5.3	2.8	1.3	7.4	8.5	884	44.6	40.7	13	1.7	14.8	884	55

Table A1. *Continued*

Population	Year	Month	N_X	SS	S1	S2	1S	11	12	22	N_2	2L	2L-1	2L-2	2L-3	2R-1	N_3	3R-1
Fayetteville, AR	1990	June/July	118	19.5	45.8	4.2	2.5	2.5	14.4	11	146	49.3	36.3	11.7	2.7	14.4	146	49.3
Fayetteville, AR	1992	June/July	112	16.1	29.5	8	2.7	1.8	30.4	11.6	139	41	46.8	5.8	6.5	16.6	148	57.4
Fayetteville, AR	1993	July	225	19.1	30.7	9.8	2.7	2.7	24	10.7	297	38.1	49.5	8.4	4	13.8	295	64.4
Fayetteville, AR	1994	June	118	21.2	38.1	7.6	3.4	1.7	18.6	8.5	155	41.3	47.1	11.6	0	12.9	153	62.8
Fayetteville, AR	2001	June	162	35.2	35.2	8	0	3.1	13.6	4.3	230	30	52.6	16.1	1.3	15.2	229	68.6
Fayetteville, AR	2003	July	138	20.3	65.2	5.8	0.7	0	5.8	2.2	154	44.2	40.9	14.3	0.6	16.9	224	64.7
Fayetteville, AR	2004	August	181	25.4	36.5	11	0.6	0	7.7	18.8	250	30.4	57.6	12	0	20.8	247	72.5
Fayetteville, AR	2005	September	53	28.3	20.8	15.1	1.9	0	11.3	22.6	77	44.2	46.8	7.8	1.3	20.8	74	66.2
Fayetteville, AR	2006	June	84	26.2	41.7	9.5	1.2	0	13.1	8.3	120	36.7	45	16.7	1.7	12.5	118	73.7
Haw Creek Falls, AR	1994	July	41	2.4	2.4	12.2	0	0	51.2	31.7	53	35.8	41.5	11.3	11.3	24.5	50	66
Haw Creek Falls, AR	2006	July	58	6.9	3.4	10.3	0	0	20.7	58.6	74	28.4	37.8	25.7	8.1	20.3	75	81.3
Iowa	1946	August	124	0.8	50.8	0.8	2.4	44.4	0.8	0	130	56.2	9.2	3.8	30.8	10	130	8.5
Iowa	1960	September	105	1.9	55.2	1.9	5.7	34.3	1	0	138	56.5	7.2	0.7	35.5	9.4	135	7.4
Iowa	2003	August	137	3.6	35	4.4	4.4	45.3	7.3	0	179	51.4	12.3	0.6	35.8	15.1	179	10.6
Iowa	2004	July	203	3.9	38.9	3.4	6.4	39.4	6.9	1	275	43.6	8.4	0	48	13.8	274	10.2
Iowa	2005	June	155	7.1	18.1	8.4	16.8	39.4	9.7	0.6	222	39.6	10.4	0.9	49.1	7.2	221	11.3
Ledgewood, NJ	1970	July/Aug	159	21.4	3.1	3.1	50.9	0	19.5	1.9	238	39.9	18.5	3.8	37.8	0.8	238	10.1
Ledgewood, NJ	1971	July	121	9.1	2.5	4.1	55.4	0	26.4	2.5	181	48.1	15.5	1.7	34.8	2.8	181	9.9
Ledgewood, NJ	1972	May/June	261	11.9	2.3	3.1	59.8	0.4	20.7	1.9	192	42.7	18.8	1	37.5	1.6	190	7.4
Ledgewood, NJ	1973	June	210	16.2	1	3.3	56.2	0.5	21.9	1	275	44	16.4	1.5	38.2	2.5	275	10.9
Ledgewood, NJ	1974	July/Aug	79	16.5	0	3.8	63.3	0	13.9	2.5	117	41	20.5	2.6	35.9	0	111	9
Ledgewood, NJ	1975	June	329	11.8	0.9	4	48.6	0	33.4	0.9	451	45	16.9	1.8	36.4	3.3	451	14.9

Ledgewood, NJ	1976	May	41	17.1	0	2.4	43.9	0	34.1	2.4	59	32.2	25.4	3.4	39	0.9	59	13.6
Ledgewood, NJ	1985	May	94	9.6	0	4.3	63.8	1.1	21.3	0	150	37.3	20	1.3	41.3	1.3	149	20.1
Ledgewood, NJ	1985	October/ November	67	17.9	1.5	6	32.8	1.5	38.8	1.5	88	42	28.4	0	29.5	4.5	88	27.3
Ledgewood, NJ	2007	June	107	19.6	3.7	2.8	36.4	0.9	34.6	1.9	134	32.8	23.9	1.5	41.8	3.7	134	26.1
Mill Creek, AR	1990	July	293	11.9	3.1	11.6	0	0	5.5	67.9	399	25.8	49.9	23.6	0.8	18	399	98.2
Mill Creek, AR	2007	June	261	12.6	1.2	9.2	0.4	0	12.3	64	349	24.6	51.2	23.7	0.6	24.8	344	97.7
Mt. Magazine, AR	1989	June	22	4.5	0	4.5	0	0	40.9	45.5	44	50	34.1	11.4	4.6	20.5	44	88.6
Mt. Magazine, AR	2006	June	217	9.7	0.9	11.1	0	0	12	66.4	299	22.4	62.2	13.4	2	20.4	299	91.3
Olivette, MO	1946	June	803	43.1	55.2	0.5	0.1	0.5	0	0.6	474	52.3	38.6	8.6	0.4	11.5	474	20.6
Olivette, MO	1947	June	314	44.6	51	2.2	0	0.6	0	1.6	320	57.5	30.3	11.3	0.9	15.9	320	28.4
Olivette, MO	1948	June	358	45	53.1	0.8	0	0.3	0	0.8	214	45.8	40.2	14.1	0	16.4	214	31.3
Olivette, MO	1949	June	217	43.3	53	1.8	0	0.5	0.5	0.9	309	47.9	44	7.8	0.3	16.5	309	25.2
Olivette, MO	1953	June	340	39.7	56.2	2.9	0	0.6	0	0.6	354	58.2	34.5	7.3	0	18.6	354	32.8
Olivette, MO	1954	June	375	47.7	49.9	1.9	0	0	0	0.5	192	57.3	31.3	10.4	1	12.5	192	32.3
Olivette, MO	1956	June	334	40.1	57.8	0.9	0	0.6	0	0.6	479	47.6	42.6	9	0.2	11.9	460	27
Olivette, MO	1957	July	216	40.3	56	3.2	0	0	0	0.5	216	70.8	21.3	6.9	0.9	14.2	216	22.2
Olivette, MO	1962	June	277	46.2	49.8	2.2	0	0.4	0	1.4	302	62.6	32.5	4.3	0.7	11.9	302	31.1
Olivette, MO	1967	June	217	43.8	43.2	6	0.5	1.8	1.4	3.2	276	60.5	35.9	3.6	0	17.4	283	35
Olivette, MO	2002	July	158	53.8	24.7	14.6	0	0	4.4	2.5	227	39.2	52.4	5.7	2.6	15.9	227	51.5
Olivette, MO	2003	June	108	48.1	25.9	21.3	0.9	0	2.8	0.9	144	41.7	47.9	9	1.4	11.1	145	58.6
Oxford, OH	2004	July	148	56.1	12.8	8.8	3.4	8.1	8.8	2	203	53.2	37.9	3.4	5.4	16.7	200	36
Oxford, OH	2005	July	139	47.5	4.3	15.1	3.6	7.9	15.1	6.5	190	62.1	22.6	3.7	11.1	17.9	189	39.7
Philadelphia, PA	1963	July	179	62.01	3.35	5.59	11.17	0	15.64	2.23	298	60.1	29.9	2	8.1	3	294	19
Philadelphia, PA	1964	July	298	65.44	1.68	9.4	11.74	0	9.4	2.35	429	52.2	37.1	4.4	6.3	3.7	428	14.3
Philadelphia, PA	1965	July	139	64.75	3.6	8.63	12.95	0	7.91	2.16	181	52.5	38.7	1.7	7.2	5.5	178	9
Philadelphia, PA	1969	August	231	77.49	1.3	7.79	9.09	0	3.46	0.87	318	44.7	47.5	1.9	6	2.8	314	16.9
Philadelphia, PA	1973	August	185	57.3	0	11.89	9.73	0	14.59	6.49	236	52.1	38.6	1.3	8.1	4.2	254	18.5
Philadelphia, PA	1995	July	95	63.16	0	7.37	16.84	0	10.53	2.11	128	23.4	60.9	0	15.6	6.2	125	27.2
Philadelphia, PA	2002	June	97	75.26	2.06	6.19	2.06	0	11.34	3.09	148	29.7	55.4	4.7	10.1	6.8	147	44.2

Table A1. *Continued*

Population	Year	Month	N_X	SS	S1	S2	1S	11	12	22	N_2	2L	2L-1	2L-2	2L-3	2R-1	N_3	3R-1
Riverhead, NY	1993	August	51	70.6	0	0	29.4	0	0	0	79	54.4	30.4	0	15.2	0	79	1.3
Pigeon Forge, TN	1947	July	53	47.2	0	37.7	0	0	7.5	7.5	145	26.9	37.3	17.2	18.6	13	145	66.2
Pigeon Forge, TN	1982	July	176	14.2	0	30.7	10.8	10.8	23.9	20.5	237	21.1	28.7	7.6	42.6	4.6	237	69.6
Pigeon Forge, TN	2003	August	122	7.4	0	32.8	4.1	4.1	29.5	26.5	167	11.4	37.1	14.4	37.1	11.4	169	81.7
Riverhead, NY	1994	August	15	93.3	0	0	6.7	0	0	0	22	45.5	31.8	0	22.7	0	21	4.8
Riverhead, NY	2006	August	143	83.2	0.7	0	14	0	0	2.1	196	27.6	45.4	1	26	0.5	195	3.6
Robert Allerton Pk, IL	2002	June	264	12.5	36.4	23.9	0.8	10.2	11	5.3	354	64.7	21.4	2	11.9	17.5	354	34.5
Seymour, IN	2006	May	62	19.4	6.5	45.2	3.2	3.2	9.7	12.9	77	57.1	29.9	9.1	3.9	26	78	52.6
Shores Lake, AR	1987	October	121	4.1	3.3	8.3	0	1.7	46.3	36.4	150	31.3	45.3	16.7	6.7	17.3	150	74.7
Shores Lake, AR	2006	June	149	8.7	2.7	10.7	0	0	40.9	36.9	168	33.9	36.9	17.9	11.3	17.3	168	86.1
Tahlequah, OK	2004	June	198	19.2	46	5.6	0.5	0	7.1	21.7	268	32.5	54.1	12.7	0.7	17.9	268	86.9
Tahlequah, OK	2007	July	167	34.1	34.7	7.8	0	0	7.2	16.2	211	36	49.8	13.3	0.9	19.9	208	86.5
Unionville, IN	1974	July	86	23.3	7	30.2	3.5	1.2	17.4	17.4	123	67.5	26	4.9	1.6	15.4	123	43.1
Washburn, MO	2005	July	97	26.8	28.9	14.4	0	0	14.4	15.5	133	47.4	37.6	15	0	18	133	69.2
Wooster, OH	1946	August	67	9	17.9	7.5	32.8	18.4	14.9	1.5	102	80.4	2.9	5.9	10.8	10.8	122	17.2
Wooster, OH	1960	July	385	16.9	16.4	6.8	25.7	18.7	14.5	1	504	70.2	15.9	2	11.9	9.9	504	16.7
Wooster, OH	2007	June	52	13.5	9.6	11.5	34.6	1.9	23.1	5.8	65	40	27.7	1.5	30.8	12.3	65	20

Month refers to the month of collection. Numbers of each kind of X chromosome, N_X, second chromosome, N_2, and third chromosome, N_3, sampled are indicated. For locations, see Fig. 1.