

Long-term frequency shifts in the chromosomal polymorphisms of *Drosophila robusta* in the Great Smoky Mountains

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We resurveyed an elevational transect in the Great Smoky Mountains National Park first sampled in 1947 for chromosomal polymorphisms in populations of *Drosophila robusta*. Combining these results with those from previous surveys, unpublished data, and long-term meteorological data from this region up to 2003, we found that these chromosomal polymorphisms had continued to shift in frequency consistent with long-term temperature changes, yet had maintained elevational clines. Intensity of linkage disequilibrium for X-chromosome gene arrangements had shifted up and down the transect over the 56-year sampling period, suggesting shifting patterns of adaptation. Chromosomal frequency changes through the 1980s clearly demonstrated concerted directional evolution in response to cooler temperatures, but over the 20 years until 2003, frequency changes in most high-elevation populations reversed for many of the most temperature-sensitive gene arrangements. © 2006 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2006, 88, 131–141.

ADDITIONAL KEYWORDS: chromosomal polymorphism – climate change – cline – linkage disequilibrium – natural selection.

INTRODUCTION

Long-term changes in trait distributions have provided some of the clearest examples of directional natural selection in response to environmental change (Kettlewell, 1961; Antonovics, Bradshaw & Turner, 1971; reviewed in Endler, 1986; Grant, Owen & Clarke, 1996; Bradshaw & Holzapfel, 2001; Grant, 2002; Cook, 2003). However, the genetic consequences to global biotas, particularly those considered rare or endangered, of large-scale ecological change have been considered only recently (Holt, 1990; Rice & Emery, 2003). Consequences of climate change to genetic polymorphism have been suggested in *Drosophila* species (Orengo & Prevosti, 1996; Rodríguez-Trelles & Rodríguez, 1998; Levitan, 2001; Solé *et al.*, 2002; Levitan, 2003; Schilthuizen, 2003; Levitan & Etges, 2005; Umina *et al.*, 2005), yet we still know very little of the

phenotypic effects of chromosomal polymorphism. These cases underscore the value of assessing historical trends in *Drosophila* inversion polymorphisms, given their early prominence in studies of population genetics and evolution (Patterson & Stone, 1952; Lewontin *et al.*, 1981; Krimbas & Powell, 1992; Powell, 1997; Hoffmann, Sgrò & Weeks, 2004).

Sampling records of inversion frequencies in *D. robusta* Sturtevant now extend to almost 60 years (see Carson & Stalker, 1946) and in a number of cases have implicated temperature as a causal factor responsible for long-term genetic change (Levitan, 2001; Levitan, 2003; Levitan & Etges, 2005). Populations sampled along an elevational transect in the Great Smoky Mountains National Park revealed one of the early examples of a genetic cline associated with elevation (Stalker & Carson, 1948); the other was described for *D. pseudoobscura* Frowla in the western Sierra Nevada mountains (Dobzhansky, 1948). When the same gene arrangements, and later linkage com-

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binations, were found to show similar latitudinal and elevational clines (Carson & Stalker, 1947; Carson, 1958a; Levitan, 1978; reviewed in Levitan, 1992; Levitan & Scheffer, 1993) and it was found that the same gene arrangements showed similar clines on eight geographically isolated mountainsides (Levitan, 1978), it became clear that many of these inversion polymorphisms were responding to natural selection.

Early evidence from 'classic' population cage experiments suggested sensitivity to temperature by some of these inversion polymorphisms (Levitan, 1951). Variation among karyotypes was shown to influence response to selection (Carson, 1958b), population fitness (Carson, 1961), mating speed and fertility (Prakash, 1967; Prakash, 1968), temperature-dependent life-history variation (Etges, 1989), and viability selection and male mating success in a natural population (Etges, 1996). Furthermore, Levitan (1992) described replicate perturbation experiments in the wild, where he released thousands of adults with very different inversion frequencies into a local population in New Jersey. After carefully documenting interbreeding between local and introduced adults, he documented inversion frequencies returning to preperturbation levels in just a few months. Thus, *D. robusta* chromosomal polymorphisms influence components of fitness and are temperature-sensitive.

Unlike the relatively invariant, range-wide temporal changes in *D. pseudoobscura* inversion frequencies (Anderson *et al.*, 1991), Etges (1984) documented increased frequencies of 'high elevation, high latitude' gene arrangements in all populations along the Smoky Mountains transect, suggesting that these frequency shifts from 1947 to 1981 were very likely a response to environmental change. Thus, the main purpose of this study was to resample the Smoky Mountains elevational transect, and, by combining heretofore unpublished inversion frequency data from these populations, long-term meteorological data, and previous collection data from other workers, attempt to establish more carefully the causes for these micro-evolutionary changes.

MATERIAL AND METHODS

Buckets of fermenting bananas were tied to trees at the same sites as described in Etges (1984), along the West Prong of the Little Pigeon River (WPLPR) and its tributaries near US 441 in the Great Smoky Mountains National Park. In addition, a site at an elevation of 1000 ft along Caney Creek just north of Pigeon Forge, an area thought to have been destroyed since the initial collections of Stalker & Carson (1948), was sampled in 1982 and 2003. All buckets were sweep-netted daily until at least 50 male and 50 female *D. robusta* were collected at each site in late

July to early August in 1982, 1983, and 2003. Cytological analyses of adult karyotypes were performed using standard methods (Levitan, 1955).

Frequency data from all populations along this transect were compared across years with contingency table *G*-tests (Sokal & Rohlf, 1995). Significance of frequency changes with elevation across years (1947, 1958–59, 1981, 1982, and 2003) was assessed by correlation and ANOVA (SAS Institute, 1989). Gene arrangements in *D. robusta* were labelled as in Carson (1958a) and Levitan (1958). Individual gene arrangements were labelled by chromosome, for example, 2L-1 is inversion one on the left arm of the second chromosome. As X-chromosome left and right arm gene arrangements are in linkage disequilibrium in these populations (Levitan, 1961), they were given shorthand labels. For example, X-chromosome combination XL-1.XL-2 was written as 12. The intensity of linkage disequilibrium among gene arrangements on the left and right arms of the X chromosome were assessed using the techniques of Lewontin & Kojima (1960) and compared across years.

Meteorological data from several stations in the Great Smoky Mountains National Park were assessed for possible temporal trends in temperature and precipitation. These data were obtained online from the National Park Service and the National Climatic Data Center (<http://www.ncdc.noaa.gov/oa/ncdc.html>) for stations near the elevational transect. Polynomial regression analysis of temperature and precipitation data with time was performed with PROC REG (SAS Institute, 1989).

RESULTS

Elements of the 2003 data (Tables 1, 2, 3), like those of previous collections, were clinal in nature. Frequencies of X-chromosome combination 1S tended to rise with elevation, particularly in 2003 ($r = 0.752$, $F = 11.69$, $P < 0.01$), whereas those of SS and S2 tended to decrease. Combination 12 exhibited little clinal variation, yet increased in frequency at most sites from 1981 to 2003, with a concomitant decrease in 1S (Tables 1, 3). Combination 22 showed no overall shifts in frequency. Gene arrangement 2L-1 decreased in frequency with elevation over all years, yet these clines differed among years (Table 2). The significant cline in 2L-2 frequencies observed in 1947 has diminished with time resulting in a significant elevation \times year interaction (Table 3). Arrangement 2L-3 showed significant clines in all years, but frequencies of 2L-3 had remained static in most populations since the 1980s collections, with some indication of decreases in the upper-elevation populations concurrent with increases in 2L-1 (Table 2). Furthermore, frequencies of 2R-1 and 3R-1 increased in this interval in many of

Table 1. Frequencies of X-chromosome gene arrangement combinations in natural populations of *Drosophila robusta* along an elevation transect in the Great Smoky Mountains National Park

Elevation (ft)	Year	N_s	X-chromosome combination					G
			SS	S2	1S	12	22	
1000	1947	53	47.2	37.7	0.0	7.5	7.5	58.27***
1000	1982	176	14.2	30.7	10.8	23.9	20.5	
1000	2003	122	7.4	32.8	4.1	29.5	26.2	
1200	1947	78†	33.3	25.6	6.4	7.7	26.9	69.04***
1400	1947	95†	11.6	34.8	10.5	18.9	24.2	
1400	1958–59	51	7.8	29.4	7.8	15.7	39.2	
1360	1981	147	7.5	32.0	15.6	25.2	19.7	17.20***
1360	1982	200	9.0	24.5	28.0	26.5	12.0	
1360	2003	121	2.5	22.3	7.4	38.8	28.9	
1560	1981	123	3.3	28.5	30.1	22.0	16.3	203.64***
1560	2003	142	3.5	16.9	16.9	32.4	30.3	
2000	1947	161	6.8	25.5	13.7	16.8	35.4	
2000	1958–59	798	6.6	21.8	25.4	22.1	24.1	12.86*
2000	1981	112	2.7	12.5	59.8	16.1	8.9	
2080	1981‡	149	3.4	18.8	43.0	24.2	10.7	
2000	1982	124	5.7	16.9	44.4	21.0	12.1	36.47***
2000	1983	366	8.2	16.9	44.5	21.9	8.5	
2000	2003	131	5.3	13.0	28.2	37.4	16.0	
2440	1981	127	2.4	12.6	53.5	22.8	8.7	29.33***
2440	2003	125	4.8	20.8	32.0	32.8	9.6	
3000	1947	40	0.0	15.0	27.5	17.5	40.0	
3000	1958–59	58	1.7	10.3	51.7	13.8	22.4	39.46***
3040	1981	33	0.0	9.0	55.0	27.0	9.0	
3040	2003	122	2.5	13.1	24.6	40.2	19.7	
3620	1981	87	1.2	10.3	48.3	32.2	8.0	30.72***
3620	1982	60	0.0	3.3	58.3	35	3.3	
3620	2003	111	0.0	13.5	32.4	30.6	23.4	
4000	1947	34	0.0	14.7	8.8	26.5	50.0	26.20***
3980	1981	97	4.1	12.4	47.4	23.7	12.4	
3980	2003	107	0.0	12.2	29.9	32.7	24.3	
4520	1981	99	1.0	14.1	36.4	42.4	6.1	8.95*
4520	1982	76	1.3	9.2	51.3	25.0	13.2	
4520	2003	138	0.0	13.8	23.2	40.6	22.5	
4680	1981	55	3.6	7.3	69.1	9.1	10.9	26.20***
4680	2003	78	0.0	11.5	30.8	29.5	28.2	
4840	1981	10	10.0	10.0	60.0	10.0	10.0	
4840	2003	91	0.0	7.7	35.2	30.8	26.4	

N_s is the number of X-chromosomes sampled.

Contingency table G -tests assessed heterogeneity at each elevation across years for all gene arrangement combinations with $(r - 1)(c - 1)$ d.f.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$.

†Observed numbers of 2S chromosomes were not included in the analyses.

‡A population sampled in 1981 from Elkmont at c. 2000 ft in an adjacent watershed was not included in the statistical analyses.

Table 2. Frequencies of second and third chromosome gene arrangements in natural populations of *Drosophila robusta* along an elevation transect in the Great Smoky Mountains National Park

Elevation (ft)	Year	Second chromosome arrangement									Third chromosome arrangement			
		2L					2R				3R			
		N_2	2L	2L-1	2L-2	2L-3	G_{2L}	2R	2R-1	G_{2R}	N_3^\dagger	3R	3R-1	G_{3R}
1000	1947	145	26.9	37.3	17.2	18.6	38.85***	86.9	13.0	10.29**		33.8	66.2	11.45**
1000	1982	237	21.1	28.7	7.6	42.6		95.4	4.6			30.4	69.6	
1000	2003	167	11.4	37.1	14.4	37.1		88.6	11.4		169	18.3	81.7	
1200	1947	127	24.4	33.9	15.7	26.0		89.8	10.2			28.3	71.7	
1400	1947	278	19.4	19.8	15.5	45.3	23.41*	91.7	8.3	6.33 ns		25.2	74.8	3.07 ns
1400	1958–59	58	25.9	25.9	6.9	41.4		91.4	8.6		57	24.6	75.4	
1360	1981	186	17.2	21.5	5.9	55.4		91.9	8.1			30.6	69.4	
1360	1982	249	19.7	17.7	8.0	54.6		95.2	4.8		245	29.8	70.2	
1360	2003	165	15.8	17.6	8.5	58.2		88.5	11.5		166	25.3	74.7	
1560	1981	158	12.6	18.5	7.5	61.4	2.50 ns	95.6	4.4	4.64*		31.1	68.9	11.66***
1560	2003	180	8.9	20.6	11.1	59.4		89.4	10.6		181	15.5	84.5	
2000	1947	301	18.6	23.6	7.0	50.8	45.31***	88.7	11	13.44*		19.9	80.1	13.1*
2000	1958–59	977	12.4	17.2	7.0	63.5		89.3	11		983	25.2	74.8	
2000	1981	168	11.3	17.9	5.3	65.5		93.4	6.6			25.6	74.4	
2080	1981‡	200	13.0	12.0	8.0	67.0		92.5	7.5			25.0	75.0	
2000	1982	180	7.8	16.7	4.5	71.0		94.5	5.5			28.7	71.3	
2000	1983	476	9.5	15.6	5.3	69.8		93.3	6.7			30.3	69.7	
2000	2003	176	6.3	15.9	6.8	71.0		92.1	7.9		173	23.1	76.9	
2440	1981	168	7.7	16.0	6.6	69.7	3.05 ns	94.6	5.4	3.98*		32.3	67.7	5.55*
2440	2003	168	6.6	22.6	8.3	62.5		88.7	11.3			20.8	79.2	
3000	1947	102	22.5	15.7	7.8	54.0	66.88***	85.3	15	1.12 ns		22.5	77.5	20.99***
3000	1958–59	95	16.8	16.8	8.4	57.9		88.4	12		93	31.2	68.8	
3040	1981	52	5.8	11.5	5.8	76.9		88.5	12			30.8	69.2	
3040	2003	164	9.2	12.8	12.2	65.9		89.6	10.4		163	10.4	89.6	
3620	1981	122	7.4	13.9	3.3	75.4	7.43 ns	94.3	5.7	1.73 ns		20.5	79.5	2.84 ns
3620	1982	95	11.6	11.6	11.6	65.4		89.5	11			24.2	75.8	
3620	2003	153	9.2	13.1	6.5	71.2		92.8	7.2			15.7	84.3	
4000	1947	74	20.3	8.1	5.4	66.2	11.72 ns	91.9	8.1	0.40 ns		21.6	78.4	10.15**
3980	1981	140	11.4	8.6	4.3	75.7		89.3	11.0			25.0	75.0	
3980	2003	146	9.6	11.6	11.6	67.1		89.7	10.0		145	11.0	89.0	
4520	1981	144	11.8	5.6	6.9	75.7	7.49 ns	95.1	4.9	7.21*		26.4	73.6	14.71***
4520	1982	129	7.0	7.0	7.0	79.1		93.0	7.0			31.8	68.2	
4520	2003	187	9.6	10.2	11.2	69.0		87.2	13.0			14.4	85.6	
4680	1981	82	1.2	8.5	4.9	85.4	8.43*	95.1	4.9	1.06 ns		26.4	73.6	1.96 ns
4680	2003	129	5.4	17.8	7.8	69.0		91.5	8.5			18.6	81.4	
4840	1981	14	7.0	21.0	0.0	72.0	4.39 ns	86.0	14.0	1.04 ns		36.0	64.0	1.48 ns
4840	2003	130	6.2	7.7	9.2	76.9		93.9	6.2			20.8	79.2	

Contingency table G -tests assessed heterogeneity at each elevation across years for all gene arrangement combinations with $(r - 1)(c - 1)$ d.f.

N is the numbers of chromosomes sampled in each population. N_3^\dagger is the number of third chromosomes sampled. This number is the same as the number of second chromosomes sampled unless indicated otherwise.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; ns, not significant.

‡A population sampled in 1981 from Elkmont at *c.* 2000 ft in an adjacent watershed was not included in the statistical analyses.

Table 3. ANOVA results for temporal and clinal changes in the frequencies of X-chromosome arrangement combinations and autosomal inversions sampled in 1947, 1958–59, 1981, 1982, and 2003 in the Great Smoky Mountains National Park

Source	d.f.	Type III SS	F-value	Pr > F	r ²
A. X chromosome combination SS					
Model	3	1827.93	15.08	< 0.0001	0.601
Elevation	1	602.72	14.91	0.0006	
Year	1	886.90	21.95	< 0.0001	
Elevation × year	1	591.25	14.63	0.0006	
B. X chromosome combination S2					
Model	3	1909.03	24.58	< 0.0001	0.711
Elevation	1	131.78	5.09	0.032	
Year	1	178.48	6.89	0.014	
Elevation × year	1	123.47	4.77	0.037	
C. X chromosome combination 1S					
Model	3	3948.97	5.44	0.0042	0.352
Elevation	1	409.79	1.69	ns	
Year	1	413.81	1.71	ns	
Elevation × year	1	388.32	1.60	ns	
D. X chromosome combination 12					
Model	3	1690.56	14.11	< 0.0001	0.585
Elevation	1	86.92	2.18	ns	
Year	1	598.08	14.98	0.0005	
Elevation × year	1	85.62	2.14	ns	
E. X chromosome combination 22					
Model	3	115.37	0.95	ns	0.087
Elevation	1	43.74	0.36	ns	
Year	1	2.39	0.02	ns	
Elevation × year	1	43.82	0.36	ns	
F. Gene arrangement 2L					
Model	3	963.25	21.11	< 0.0001	0.679
Elevation	1	15.69	1.03	ns	
Year	1	180.43	11.86	0.0017	
Elevation × year	1	43.82	14.60	ns	
G. Gene arrangement 2L-1					
Model	3	1501.20	24.08	< 0.0001	0.707
Elevation	1	126.95	6.11	0.0193	
Year	1	116.85	5.62	0.0243	
Elevation × year	1	119.42	5.75	0.0230	
H. Gene arrangement 2L-2					
Model	3	125.41	4.61	0.0090	0.3157
Elevation	1	83.59	9.23	0.0049	
Year	1	66.64	7.36	0.0110	
Elevation × year	1	82.49	9.10	0.0052	
I. Gene arrangement 2L-3					
Model	3	4809.14	18.59	< 0.0001	0.6502
Elevation	1	835.76	9.69	0.0040	
Year	1	1478.98	17.15	0.0003	
Elevation × year	1	810.06	9.39	0.0046	
J. Gene arrangement 2R-1					
Model	3	6.94	0.28	ns	0.0277
Elevation	1	0.28	0.03	ns	
Year	1	2.58	0.32	ns	
Elevation × year	1	0.28	0.03	ns	
K. Gene arrangement 3R-1					
Model	3	331.37	3.41	0.0302	0.2541
Elevation	1	13.79	0.43	ns	
Year	1	9.82	0.30	ns	
Elevation × year	1	14.23	0.44	ns	

r² is an estimate of the proportion of the total variance explained by the model used. Arcsin transformation of the data had no effect on the results.

ns, not significant.

the populations along the transect (Table 2), although across all years, these trends were not significant. Indeed, the overall structure of the chromosomal clines in 2003 was similar to that observed in the early 1980s, but the frequency changes observed then had not continued in all populations sampled.

The most striking observation in 1981 was that frequencies of 'high-elevation' gene arrangements and X-chromosome combinations, for example 2L-3 and 1S, had increased in all populations since 1947 at the expense of 'low-elevation' gene arrangements 2L-1 and X-chromosome combinations SS and S2 (Etges, 1984). Although they were not complete surveys of all populations, the 1982 and 1983 collections suggest that year-to-year fluctuations were rather small in comparison with the long-term changes (Tables 1, 2). Only X-chromosome combination frequencies at 1360 ft shifted significantly ($P < 0.05$) between 1981 and 1982 because of the near doubling of 1S and the decreases in S2 and 22 (Table 1). There were no significant shifts at the 2000-ft site from 1981 to 1983. All data are presented in a way comparable with those of Etges (1984).

FREQUENCY SHIFTS FROM 1981 TO 2003

The dynamics of individual X-chromosome combinations changed between the early 1980s and 2003. This could be seen most notably in the reversal at *all elevations* of the systematic increases of 1S frequencies that had occurred between 1947 and 1982. At the same time, the frequencies of 22, which had been decreasing at many localities, and those of 12, which had been relatively stable earlier, had increased, often more than doubling, between 1981/82 and 2003 (Table 1). Frequencies of X-chromosome combination 22 along the transect are of particular interest as this X-chromosome showed a significant, positive association with elevation in 1947, but this had reversed to a negative correlation with elevation by 1981 (Etges, 1984). There was no relationship between 22 and elevation in 2003, and over all years, no trends were evident (Table 3). Such systematic changes along this transect are unlikely to be results of random shifts in different populations, but their cause is unknown.

Overall, the steepness of the X-chromosome clines was evident from the lowest elevation sampled, 1000 ft, to 2440 ft, above which the remaining high-elevation populations were dominated by high frequencies of 1S and 12 (Table 1). Temporal shifts in these upper-elevation populations in X-chromosome combinations were all statistically significant as a result of increases in frequency of 22 and decreases in 1S (Table 1). At elevations up to 2000 ft, clines were steeper in 2003 for 2L-1 and 2L-2, with most upper-elevation populations showing little temporal heterogeneity since 1947 (Table 2).

Frequencies of 2R-1, a 'southern' gene arrangement (Etges & Levitan, 2004), showed no elevational clines in any year from 1947 to 2003, but temporal heterogeneities were apparent at a number of sites. In four of five cases where significant temporal changes occurred, 2R-1 increased in frequency from the 1980s to 2003 (Table 2).

In 1947, frequencies of 3R-1 were positively correlated with elevation, although frequencies leveled off above 2000 ft (Stalker & Carson, 1948). None of the subsequent collections (Table 3) revealed clines for third chromosome gene arrangements (Etges, 1984). By 2003, however, nine of 11 populations with comparable data showed significant increases in 3R-1 (Table 2). Taken together, the decreases in 1S and increases in 2L-1, 2R-1, and 3R-1 at the upper elevations suggest these chromosomes may have been responding to common factors, possibly higher temperatures, in the 20 years before 2003 in this region of the Great Smoky Mountains National Park.

Significance of the elevational clines for the most frequent gene arrangements and arrangement combinations was assessed by polynomial regression (results not shown) because there was no a priori reason to assume that these clines should be linear. In only one case (2L) was any higher order effect detected. In fact, an ordination analysis suggested that populations above 2000 ft were genetically more similar to each other than a linear cline would suggest, but this analysis did not include populations from lower than 1360 ft (Etges, 1984). It is clear that including these low-elevation populations increased the statistical significance of many of the clines, particularly those involving SS, S2, and 1S. Gene arrangements 2L-1 and 2L-3 exhibited significant association with elevation in all years (Table 3), emphasizing their sensitivity to environmental variation along this transect.

A noteworthy aspect of these clines is the increase in linkage disequilibrium (D) among X-chromosome gene arrangements with elevation (Table 4). Levitan (1958) arbitrarily classified X-chromosomes SS and 12 as 'coupling' and S2 and 1S as 'repulsion' combinations. In many southern regions, *D. robusta* populations contain excess frequencies of these repulsion combinations (Levitan, 1978), and in the Smokies such linkage disequilibria are adaptive (Levitan, 1961). The significance of linkage disequilibrium was estimated by summing the square roots of the χ^2 values at each elevation, yielding a standard normal deviate (Simpson, Roe & Lewontin, 1960). Across years, the intensity and significance of linkage disequilibrium varied with elevation, and in most cases it peaked at 2000–3500 ft and then declined again at higher elevations (Table 4). Analysis of covariance (Freund & Littell, 1991) revealed that D varied significantly with elevation

Table 4. Measures of linkage disequilibrium (D) and its significance in all populations sampled along the Great Smoky Mountains cline of *Drosophila robusta*

Year	Elevation (ft)	D	χ^2	χ
1947	1000	0.0416	9.76	3.1240
	1200	0.0172	0.59	0.7695
	1400	-0.0255	2.63	-1.6212
	2000	-0.0559	8.35	-2.8894
	3000	-0.1146	15.18	-3.8957
	4000	-0.0519	1.67	-1.2919
				$\Sigma\chi = -5.8047$ $t = -2.3697$ ns
1958–59	1400	-0.0291	0.58	-0.7611
	2000	-0.0715	24.63	-4.9624
	3000	-0.0849	10.09	-3.1765
				$\Sigma\chi = -8.9001$ $t = -5.1385^*$
1981	1360	-0.0484	5.39	-2.3224
	1560	-0.1119	22.87	-4.7825
	2000	-0.0850	24.63	-4.9624
	2080	-0.0911	23.72	-4.8699
	2440	-0.0744	19.74	-4.4429
	3040	-0.0600	5.00	-2.2361
	3620	-0.0547	8.80	-2.9664
	3980	-0.0637	9.31	-3.0512
	4520	-0.0546	8.51	-2.9173
	4680 + 4840	-0.0600	11.44	-3.3829
				$\Sigma\chi = -35.9340$ $t = -11.3633^{***}$
1982	1000	0.0012	0.004	0.0629
	1360	-0.0578	10.23	-3.1985
	2000	-0.0819	15.61	-3.9515
	3620	-0.0208	3.15	-1.7754
	4520	-0.0583	8.82	-2.9705
				$\Sigma\chi = -11.8330$ $t = -5.2919^{**}$
1983	2000	-0.0687	32.47	-5.6981
2003	1000	0.0153	0.65	0.8046
	1360	0.0138	0.60	-0.7744
	1560	-0.0353	2.88	-1.6959
	2000	-0.0236	1.50	-1.2252
	2440	-0.0622	8.92	-2.9862
	3040	-0.0347	3.38	-1.8372
	3620	-0.0747	13.38	-3.6581
	3980	-0.0634	10.16	-3.1875
	4520	-0.0531	9.86	-3.1396
	4680	-0.0689	8.04	-2.8359
4840	-0.0499	7.15	-2.6733	
				$\Sigma\chi = -23.2087$ $t = -6.9977^{***}$

The sum of the square roots of χ^2 in each year was distributed as a standard normal deviate. t -values and their significance are indicated.

over all years ($F = 7.78$, $P = 0.01$), but the regression slopes varied among years (elevation \times year interaction, $F = 4.14$, $P = 0.02$). In 2003, changes in disequilibrium with elevation seemed to be returning to levels recorded in 1947, although fewer populations were sampled in the latter study, particularly at the upper elevations. Thus, the intensity of linkage disequilibrium at different elevations is also dynamic, having shifted along this transect over the 56-year sampling period.

ANALYSIS OF METEOROLOGICAL VARIATION

Long-term temperature and precipitation data were available starting in 1921 for Gatlinburg (park headquarters at Sugarlands, 1460 ft, c. 2 km from the Boundary collecting site at 1360 ft and 1.5 km from the Sugarlands collecting site at 1560 ft). Annual observed temperatures for Gatlinburg, recorded each day at 06.00 h, were missing for some years. However, rather complete records of the average yearly maximum and minimum temperatures provided an in-depth record of temperature fluctuations going back for 80 years, at least at lower elevations. The average monthly precipitation showed evidence of small increases with time (total annual precipitation could not be calculated because of missing data), but none of the long-term trends in precipitation were statistically significant (Fig. 1). All three temperature indicators showed evidence of decrease from the early 1920s until the early 1980s and then evidence of increase to 2003. The linear regression coefficient for average maximum temperature (perhaps the most relevant temperature indicator for *D. robusta* during the summer months) was positive, but the squared coefficient was negative, indicating that the annual maximum temperature decreases from the 1950s to the late 1980s were significantly nonlinear (Fig. 1). Thus, there is direct corroborative evidence of a temperature decrease of c. 2 °C in average maximum temperatures, as well as minimum temperatures (Fig. 1), coincident with the decreases in frequencies of 'low-elevation' gene arrangements in these populations during the approximately 35-year period after Stalker & Carson (1948) made their initial collections in this valley.

The increases in frequency in 2L-1, 2R-1, and 3R-1, and decreases in 1S at the upper elevations were consistent with increases in all three temperature indicators from the mid-1970s until 2003, particularly minimum temperature (Fig. 1). Linear regression analyses of these temperature increases revealed that maximum temperatures increased from 1976 to 2003 ($T_{\max} = -140.77 + 0.073 \times \text{year}$, $F = 7.32$, 1/12 d.f., $P = 0.019$, $R^2 = 0.379$).

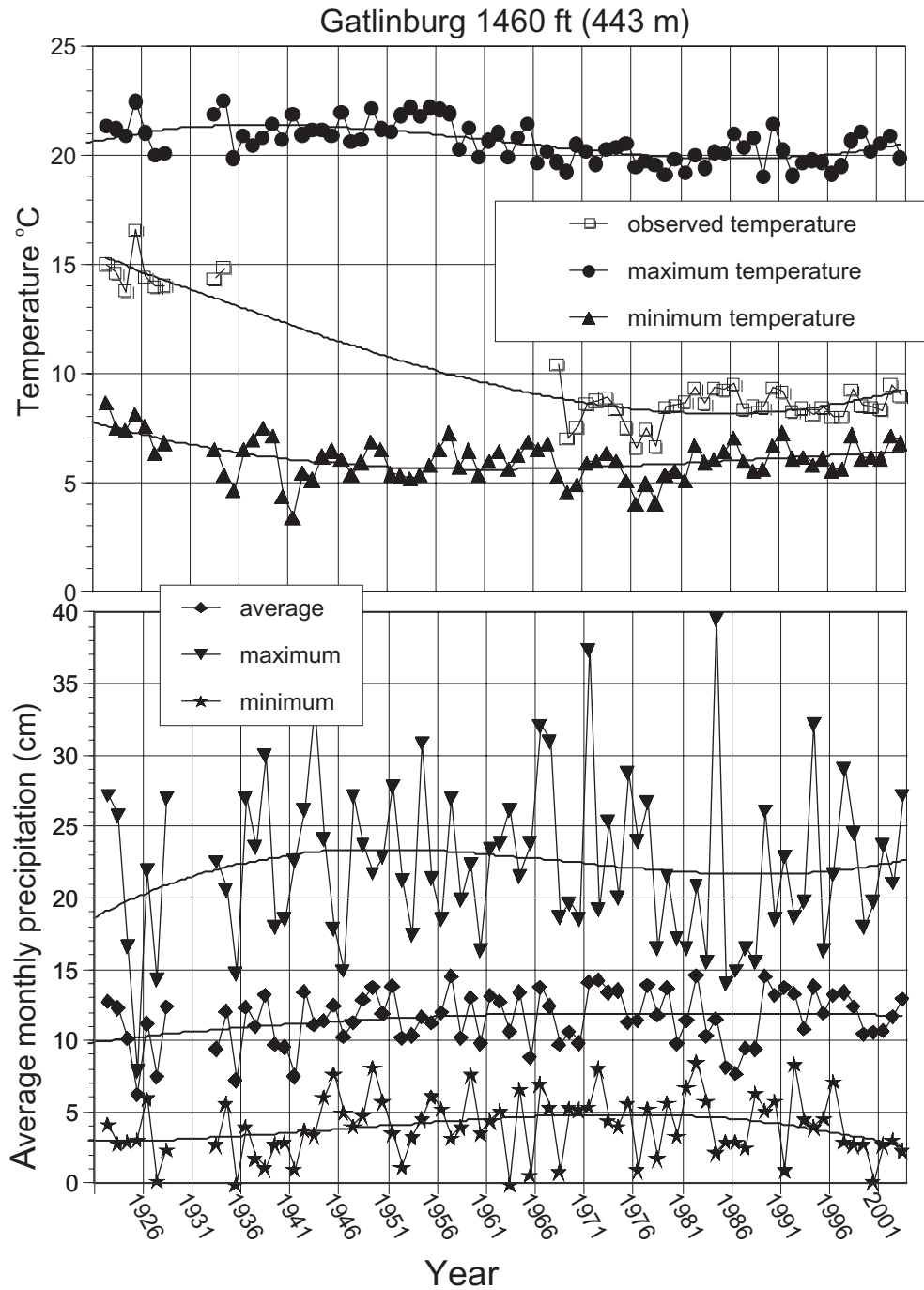


Figure 1. Annual temperature and daily precipitation variation from 1922 to 2003 for Gatlinburg. Annual temperature was calculated from daily observed temperatures (recorded at 06.00 h), daily maximum temperatures, and daily minimum temperatures from the National Climatic Data Center, Asheville, North Carolina. This station (Gatlinburg 2 SW) is located near the elevational transect at Park Headquarters near the Sugarlands Visitor's Center. Plotted lines are the results of third-degree polynomial regression analysis. For each temperature variable, these equations were: $T_{max} = 17.005 + 0.273^{**} \text{ year} - 0.005^{***} \text{ year}^2 + 0.000^{***} \text{ year}^3$, $F = 15.94$, 3/74 d.f., $P < 0.0001$, $R^2 = 0.368$. $T_{min} = 11.459 - 0.231^{*} \text{ year} + 0.003 \text{ year}^2 - 0.000 \text{ year}^3$, $F = 8.32$, 3/74 d.f., $P < 0.0001$, $R^2 = 0.252$. $T_{obs} = 18.750 - 0.136 \text{ year} - 0.002 \text{ year}^2 + 0.000 \text{ year}^3$, $F = 109.86$, 3/41 d.f., $P < 0.0001$, $R^2 = 0.889$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. None of the precipitation data showed any significant long-term trends.

DISCUSSION

Long-term temperature changes in this region of the Smoky Mountains mirror many of the temporal shifts in chromosomal frequencies documented since 1947 and provide some insight into the evolutionary history of these Appalachian populations. While it is not clear what factors are responsible for these trends from the early 1980s to 2003, the regional scale of this study, as well as the known history of this region, provide a useful background for interpreting these long-term evolutionary changes.

From 1947 to 1981, directional selection had influenced all of the populations from 1360 ft to the uppermost populations sampled, because the same inversions and X-chromosome combinations (2L-3 and 1S) had increased in frequency in all populations. Etges (1984) suggested that such systematic frequency shifts since 1947 in chromosomes known to be associated with high latitudes and elevations indicated temperature decreases in the region of the elevational transect, the watershed encompassing the WPLPR. He suggested that the most likely mechanism influencing all of the populations was a consequence of the park's history: much of the lower watershed was inhabited during the 1930s until the establishment of the park in 1934. Canopy regrowth once the parklands were protected probably influenced resource availability for these small, sap-breeding, forest insects. However, it is less certain whether regional climatic shifts accompanying forest regrowth (Fig. 1), or other factors associated with changes in forest composition, influenced *D. robusta* populations.

From the park's northern boundary at Gatlinburg to c. 3000 ft, humans lived and farmed the watershed, and there is no evidence for widespread fire in recent history. Above 3000 ft, the region is considered a historically uninhabited and undisturbed forest (Kunze, 2003), except for the paved highway that was constructed in 1934. Logging operations in the region were curtailed in 1939, but did not involve the WPLPR watershed (Stupka, 1964). Chestnut blight arrived in the area around 1926, so many of the dominant American chestnuts *Castanea dentata* (Marshall) Borkhausen had already been eliminated by the time the park was established. Thus, the most significant impact on *D. robusta* populations in terms of forest canopy-caused microclimate shifts should have been evident principally below 3000 ft during the early 1980s, consistent with the long-term frequency increases in 1S and 2L-3 (Tables 1, 2).

Stalker & Carson (1948) made their collections in 1947, just 13 years after the park was established. Carpenter & Giordano (1955) described these same sites 3 years later and clearly indicated the extent of forest regrowth. By the early 1980s, the entire WPLPR

watershed was reforested, and at 2000 ft, the upper canopy was dense, extending to at least 75 ft (W.J. Etges, unpubl. data) and consisting of mature tulip poplars, red maples, and several species of oak and hickory with little forest floor undergrowth. Additionally, Carpenter & Giordano (1955) reported that they considered *D. robusta* to be rare (1–4% of all species collected) along the transect in 1950, while the most abundant species collected were the cosmopolitan species *D. immigrans* Sturtevant, *D. melanogaster* Meigen, and *D. hydei* Sturtevant. The 1980s and 2003 collections revealed that *D. robusta* and *D. immigrans* were among the most abundant species at the lower elevations, along with *D. affinis* Sturtevant at higher sites (W.J. Etges, unpubl. data). *D. melanogaster* and *D. hydei* have apparently disappeared from these forest sites. Therefore, forest regrowth was also accompanied by increases in population densities of *D. robusta*, and changes in drosophilid community composition.

Changes in chromosomal frequencies revealed in the 2003 data suggest a new dynamic in which 'southern' chromosomes had shifted in concert towards higher frequencies in the upper-elevation populations since the early 1980s. High-elevation communities are expected to be more impacted by the effects of global warming (Beniston, Diaz & Bradley, 1997; Pounds, Fogden & Campbell, 1999; Root *et al.*, 2003), so equilibrium frequencies of chromosomal arrangements in these Smokies populations may have been shifting again in response to further changes in the environment associated with regionally increasing temperatures, as they are in other parts of North America (Levitan & Etges, 2005). Thus, the record of change in chromosomal frequencies has revealed insight into evolutionary change at local and regional levels consistent with recent trends in ambient climatic conditions. Systematic frequency shifts consistent with adaptation to warmer climatic conditions have also been revealed independently in European populations of *D. subobscura* (Rodríguez-Trelles, Rodríguez & Scheiner, 1998; Solé *et al.*, 2002; Balanya *et al.*, 2004) and Australian *D. melanogaster* (Umina *et al.*, 2005) on continent-wide scales. Not only do these revelations call for renewed focus on understanding *Drosophila* inversion polymorphism, but they suggest that further study is warranted of the genetic basis of such adaptive responses and the genetic interactions preserved by such chromosomal rearrangements.

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