

Positive Selection Driving the Evolution of a Gene of Male Reproduction, *Acp26Aa*, of *Drosophila*: II. Divergence Versus Polymorphism

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The evolution of the gene for a male ejaculatory protein, *Acp26Aa*, has been shown to be driven by positive selection when nonsibling species in the *Drosophila melanogaster* subgroup are compared. To know if selection has been operating in the recent past and to understand the details of its dynamics, we obtained DNA sequences of *Acp26Aa* and the nearby *Acp26Ab* gene from 39 *D. melanogaster* chromosomes. Together with the 10 published sequences, we analyzed 49 sequences from five populations in four continents. The southern African population is somewhat differentiated from all other populations, but its nucleotide diversity is lower at these two loci. We find the following results for *Acp26Aa*: (1) The R : S (replacement : silent changes) ratio is significantly higher in the between-species comparisons than in the within-species data by the McDonald and Kreitman test. Positive selection is probably responsible for the excess of amino acid replacements between species. (2) However, within-species nucleotide diversity is high. Neither the Tajima test nor the Fu and Li test indicates a reduction in nucleotide diversity due to positive selection in the recent past. (3) The newly derived nucleotides in *D. melanogaster* are at high frequency significantly more often than predicted by the neutral equilibrium. Since the nearby *Acp26Ab* gene does not show these patterns, these observations cannot be attributed to the characteristics of this chromosomal region. We suggest that positive selection is active, but may be weak, for each amino acid change in the *Acp26Aa* gene.

Introduction

The role sexual selection may play in the evolution of hybrid male sterility has been suggested by a series of studies on the genetic basis of speciation (Wu and Davis 1993; Wu, Johnson, and Palopoli 1996; Hollocher et al. 1997). A logical question to ask is whether the signature of sexual selection can be observed at the molecular level. One might expect that genes specific for male reproduction are more likely to be driven by sexual selection and, hence, experience more rapid evolution than genes of female or nonsexual functions. This logic is best explained by Eberhard (1985) in his treatise on the evolution of male genitalia.

The collection of accessory gland protein genes (*Acp*) are excellent candidates for testing this hypothesis. This is a group of specialized proteins in the seminal fluid of *Drosophila* (Chen 1984; Monsma and Wolfner 1988; Monsma, Harada, and Wolfner 1990; Park and Wolfner 1995) that have been suggested to be variously involved in egg-laying stimulation (Herndon and Wolfner 1995), remating inhibition (Chen 1976), female longevity (Fowler and Partridge 1989), and sperm competition (Clark et al. 1995), although the last aspect is not conclusive (Herndon and Wolfner 1995). Among the *Acp* genes, *Acp26Aa* and *Acp26Ab* have been most extensively analyzed for their evolutionary dynamics. These two genes exist in tandem but show no sign of homology (Monsma and Wolfner 1988). They are different in size (about 264 and 90 amino acids, respectively), and only the larger one, *Acp26Aa*, shows deviation from the neutral expectation in its DNA sequence evolution (Aguade, Miyashita, and Langley 1992; Tsaur and Wu 1997). We do not expect all male reproductive

proteins to experience rapid evolution and, indeed, at least two other *Acp* genes have been shown to evolve at a moderate rate (Schmidt et al. 1993; Cirera and Aguade 1997). Our objective is to learn how and why some of them, such as *Acp26Aa*, have evolved faster than the neutral expectation (Kimura 1983).

The divergence of the *Acp26Aa* gene between distantly related species of *Drosophila* has been shown to have the characteristics of genes driven by positive selection (Tsaur and Wu 1997). The most salient feature is the higher rate (sometimes twice as high) of nonsynonymous than synonymous substitutions. In this study, we ask whether this selective pressure has been a constant force driving the evolution of *Acp26Aa* or whether it worked sporadically in the gene's evolutionary past. By examining the divergence of *Acp26Aa* between the sibling species of *Drosophila simulans* and *Drosophila melanogaster*, as well as the pattern of within-species polymorphisms, we may gain insight into how often and how strong positive selection has been at this locus. This was also the focus of Aguade, Miyashita, and Langley (1992), who sampled 10 sequences from a North American population. In this study, we add 39 sequences from four other populations and have been able to perform additional analyses.

Materials and Methods

Samples

We surveyed 39 isofemale lines of *D. melanogaster* collected from four different continents: (1) 10 African (Af) lines from Zimbabwe that have been described in Begun and Aquadro (1993) and Hollocher et al. (1997); (2) 10 Australian (Au) lines collected in 1995; (3) 11 Asian lines from Taiwan (Tw) collected in 1993 and 1996; and (4) 8 North American lines collected in upstate New York (Ny) in the late 1980s. We also include the 10 published sequences (Aguade, Miyashita, and Langley 1992) from flies collected in North Carolina (Nc), bringing the total to 49 lines. To avoid heterozy-

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gosity, we used *Df(2L)PM101*, a deficiency that uncovers the region containing *Acp26A*, to extract single copies of the genes from each isofemale line. The DNA sequences of this study are available from GenBank under accession numbers AF053250–AF053276 and AF052470–AF052481.

PCR and DNA Sequencing

A 1.4-kb fragment encompassing both transcription units of *Acp26Aa* and *Acp26Ab* was PCR-amplified by the primers 110⁺ (5'-ATGAACCAGATTTTATTATGC-3') and 1549⁻ (5'-GCTTGAAGTAACTGCGG-3'). Two additional internal primers, 613⁻ (5'-TCCAAA-AGTCTGCGGTTTAC-3') and 705⁺ (5'-CCCA-CCTGCCGCCAATC-3'), were also used for sequencing. The + and - superscripts indicate the sense and antisense strands, respectively. The numbers refer to the 5' nucleotide positions of the *D. melanogaster* sequence as reported in Aguade, Miyashita, and Langley (1992). Amplification was performed in 50 μ l reaction volume. Reactions were carried out for 30 cycles of 30 s at 94°C for denaturation, 30 s at 56°C for annealing, 2 min at 72°C for extension, and the final extension at 72°C for 6 min. The desired fragment was then purified (Wizard PCR Preps, Promega Corp., Madison, Wis.). Sequencing was done on an ABI Prism 377 automated sequencer using AmpliTaq DNA Polymerase, FS. All sequences were determined from both strands.

Analysis

In the analysis of divergence between species, we used only coding regions for the McDonald and Kreitman (1991) test. In this test, fixed differences are sites where the nucleotide of the *D. simulans* sequence of Aguade, Miyashita, and Langley (1992) is absent from all 49 *D. melanogaster* sequences. These nucleotide changes are then classified as either R (for amino acid replacements) or S (for silent changes). In all other analyses (gene diversity, population differentiation, frequency spectrum, and the HKA test), whole genes excluding the primer sites were used. *Acp26Aa* corresponds to positions 47–898, and *Acp26Ab* corresponds to positions 1079–1413 of Aguade, Miyashita, and Langley (1992) and figure 1. The sequences thus include intron, replacement, and silent sites.

We use H to designate nucleotide diversity and present it as the average number of pairwise nucleotide differences per kilobase. Following Hudson, Slatkin, and Maddison (1992), H_w denotes the average nucleotide diversity within each population, and H_b denotes the average nucleotide diversity between populations. Population differentiation, F_{ST} , is defined as $1 - H_w/H_b$. Three statistical tests were performed on the entire *Acp26Aa* and *Acp26Ab* genes: the HKA test (Hudson, Kreitman, and Aguade 1987), the Tajima (1989) test, and the Fu and Li (1993) test. These tests are explained in the text. Calculations for nucleotide diversity, population differentiation, and the three tests were carried out by using DnaSp (Rozas and Rozas 1997, version 2.51). In the HKA test, we used the *D. simulans* sequence for

the divergence estimate and pool all *D. melanogaster* sequences for the polymorphism analysis.

Results

DNA sequences at polymorphic sites are presented in figure 1. In total, there are 60 segregating sites in the sample of 49 sequences from five populations.

Divergence Between Species

The first question we ask is whether the amino acid substitutions between the *Acp26A* genes of *D. melanogaster* and *D. simulans* can be accounted for by neutral evolution. Since the rate of nonsynonymous substitutions is not significantly different from that of synonymous substitutions in *Acp26Aa* (Aguade, Miyashita, and Langley 1992), we do not know whether the protein experiences no selection or whether positive selection, which accelerates substitutions, is roughly cancelled out by negative selection, which retards them. To address this issue, we compared the level of polymorphism with that of divergence for both synonymous changes (S) and amino acid replacements (R), a procedure pioneered by McDonald and Kreitman (1991). The results are presented in table 1.

Under the neutral theory, the R:S ratio for fixed differences between species should be the same as that for within-species polymorphisms. The ratios for *Acp26Aa*, 3.57 and 1.38, respectively, are significantly different at the 5% level. On the other hand, the ratios for *Acp26Ab* are comparable (although the level of divergence is too small to be truly informative). Since the ratio of the number of replacement sites over that of silent sites is about 3:1, the low R:S value for the *Acp26Aa* polymorphism suggests that negative selection against amino acid changes has been operating. The near parity in the rates of synonymous and replacement substitutions between species thus cannot be explained by neutral evolution over the entire gene. The number of amino acid replacements between species must have been enhanced by positive selection.

The higher-than-expected level of divergence in the *Acp26Aa* gene can also be seen in the comparison with the *Acp26Ab* gene by means of the HKA test. The test compares the level of within-species polymorphism with that of between-species divergence for two different loci (see *Analysis in Materials and Methods*). It yields a significant χ^2 value (7.27, df = 1, $P < 0.01$), indicating that the divergence/polymorphism ratio is higher in the *Acp26Aa* gene than in *Acp26Ab*. Much of the difference is due to the high level of divergence in the former.

Variation Within Species

To explain the excess in the number of amino acid substitutions between *D. simulans* and *D. melanogaster*, the most obvious hypothesis is directional selection, under which a favorable mutation increases its frequency until fixation. These selection-driven events themselves contribute very little to the observed level of within-species variation, because they introduce only transient polymorphism into the population. An important consequence of positive selection is that, if it has happened

Table 1
Nucleotide Polymorphism Within *Drosophila melanogaster* and Divergence Between *D. melanogaster* and *Drosophila simulans* for *Acp26Aa* and *Acp26Ab*

	FIXED DIFFERENCES (between species)		POLYMORPHIC DIFFERENCES (within species)		G VALUE
	R	S	R	S	
<i>Acp26Aa</i> ...	75	21	22	16	5.33*
<i>Acp26Ab</i> ...	1	2	4	6	0.044

NOTE.—R and S stand for amino acid replacements and silent changes, respectively.

* $P < 0.05$.

ferentiated from populations of other continents at the molecular level (Begun and Aquadro 1993). However, part of the differentiation is due to the reduced nucleotide diversity of the Zimbabwe population, an observation that is contrary to the pattern reported for X-linked genes (Begun and Aquadro 1993). In the rest of this study, we shall concentrate on the *Acp26Aa* gene, which appears to be influenced by positive selection (Aguade, Miyashita, and Langley 1992; Tsaur and Wu 1997). Since *Acp26Aa* shows little differentiation among the four non-African populations and only moderate differentiation between the African and non-African populations, we shall combine the data from all populations.

A normal level of nucleotide diversity does not necessarily mean the absence of a recent selective sweep. If the region evolves faster than other regions due to a greater mutation rate, for example, the population can recover quickly from the loss of diversity swept away by positive selection. (The synonymous substitution rate in this region is indeed higher than are those in other regions [Aguade, Miyashita, and Langley 1992; Tsaur and Wu 1997].) One characteristic of populations in the process of recovering nucleotide diversity lost through selective sweep or population bottleneck is the excess of rare or recent mutations relative to the total amount of variation observed (Nei, Maruyama, and Chakraborty 1977). In Tajima's (1989) test and Fu and Li's (1993) test, a negative statistic indicates an excess of rare or recent mutations. Both tests for both genes yield negative values, but none is significant at even the 10% level (table 3). Therefore, the overall nucleotide diversity and the test statistics related to it fail to yield evidence of a recent selective sweep.

In general, the tests above utilize only a portion of the information on the mutant frequency. For example, Fu and Li's (1993) test examines whether the relative abundance of singletons in the sample (variants that occur only once) agrees with the neutral prediction. In figure 2, the entire frequency spectrum of *Acp26Aa* is displayed. On the abscissa is the number of occurrences of the new nucleotide, which ranges from 1 to 48 in the sample of 49. On the ordinate is the number of sites having a particular occurrence. It is possible to determine the new nucleotide in *D. melanogaster* because the sequences from its three sibling species are available (Aguade, Miyashita, and Langley 1992). We only con-

Table 2
Between- and Within-Population Nucleotide Diversity and the F_{ST} Measure of Population Differentiation

	Af	Au	Nc	Ny	Tw
<i>Acp26Aa</i>					
Af	5.7	9.3	7.7	8.7	6.9
Au	0.219	9.0	8.4	8.4	8.9
Nc	0.133	0.023	7.7	7.9	7.6
Ny	0.183	-0.030	-0.014	8.6	7.9
Tw	0.081	0.113	0.037	0.023	7.0
<i>Acp26Ab</i>					
Af	4.9	12.2	12.0	11.4	12.6
Au	0.398	9.9	9.9	11.8	13.5
Nc	0.392	0.013	9.9	10.8	12.0
Ny	0.270	0.081	0.001	11.9	13.0
Tw	0.207	0.071	-0.038	-0.035	15.1

NOTE.—For population designation and statistical definition, see *Materials and Methods*. Above diagonals, H_b (between-population nucleotide diversity); diagonals, H_w (within-population nucleotide diversity); below diagonals, $F_{ST} = 1 - H_w/H_b$ (Hudson, Slatkin, and Maddison 1992).

sider polymorphic sites that are unambiguous with respect to the ancestral versus derived nucleotide. There are 31 such sites. (The ancestral state is determined to be the particular nucleotide of the polymorphism which is also present in all three sibling species.)

At the neutral equilibrium, the expected number of sites at which the new nucleotide is present i times in the sample is given by $4Nv/i$ (Fu 1995), where N and v are the effective population size and mutation rate, respectively. To compute the expected spectrum, the estimated $4Nv$ according to Watterson's (1975) formula is the number of observed sites (31) divided by $(1 + \frac{1}{2} + \frac{1}{3} + \dots + \frac{1}{48})$. The expected and observed frequency spectra are both given in figure 2. There appear to be too many sites with the new nucleotide in high frequency (>0.5): 10 of the 31 are such sites. The expected values for the high- and low-frequency mutations are 5.04 and 25.96, respectively. A χ^2 test between the observed and expected numbers yields a value of 5.83, which is significant at the 5% level ($df = 1$).

The presence of the many sites with the derived nucleotide at a high frequency also explains the discrepancy in the test statistics between Tajima's and Fu and Li's tests shown in table 3 for *Acp26Aa* (-0.875 vs. -0.118). This is because whether a new mutation is at a frequency of x or $1 - x$ would not have an effect on Tajima's test, whereas the two situations may yield very different results in Fu and Li's test. Tajima's test examines the relative frequency of the rare alleles, while

Table 3
Summary Statistics of the Polymorphic Data

	n^a	s^b	H^c	Tajima's D	Fu and Li's D
<i>Acp26Aa</i> ..	49	37	8.01 ± 0.57	-0.875 ^d	-0.118 ^d
<i>Acp26Ab</i> ..	49	18	11.67 ± 0.79	-0.109 ^d	-1.294 ^d

^a Number of sequences.

^b Number of polymorphic sites.

^c Average nucleotide diversity (bp differences per kb).

^d No D value is significant.

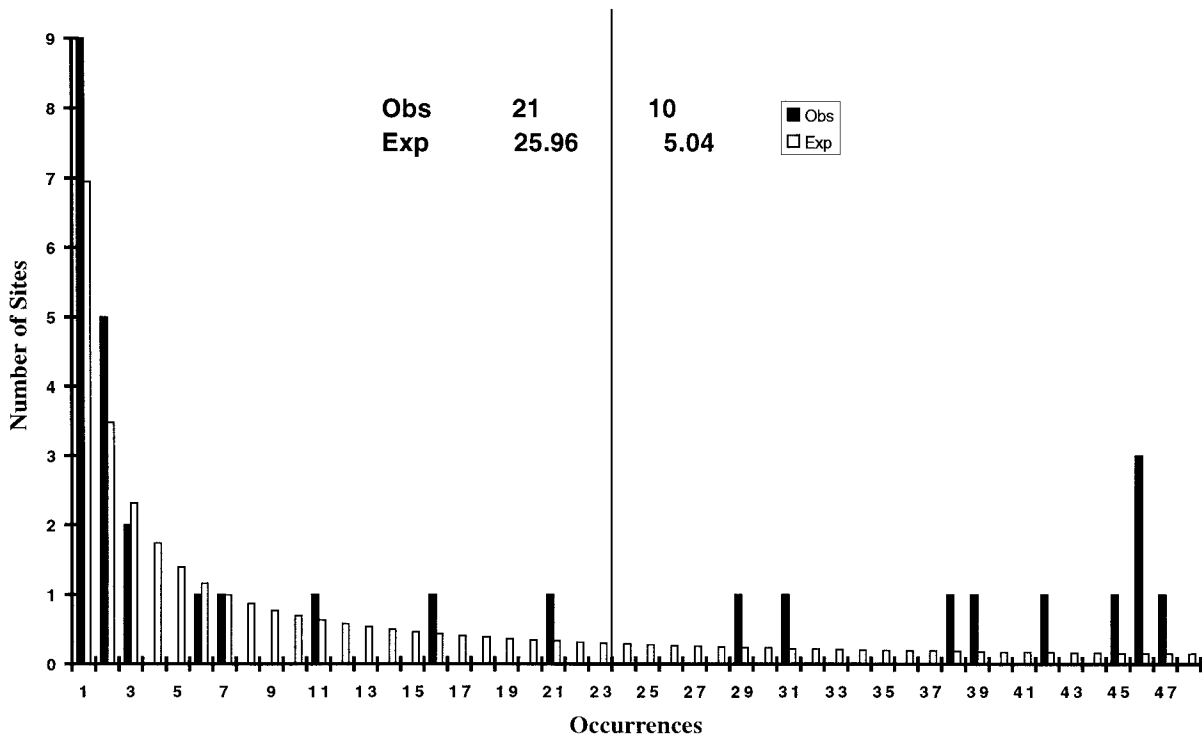


FIG. 2.—The observed and expected frequency spectra of the newly derived nucleotide in *Acp26Aa* of *D. melanogaster*. The abscissa is the number of occurrences (i) of the new nucleotide, ranging from 1 to 48 in a sample of 49 sequences. The ordinate is the number of sites with i occurrences. There are 31 sites where the derived nucleotide can be unambiguously inferred with reference to the three outgroup species. The expected frequency spectrum is given by Watterson (1975; see text). We divide the occurrences into high (≥ 24) and low (< 24) frequency classes for the goodness-of-fit test.

Fu and Li's test studies the frequency of the young alleles. The two tests should be comparable except in dealing with unusual frequency spectra like that of figure 2. The difference in these two tests for *Acp26Ab* may also be due to an unusual frequency spectrum, although the number of polymorphic sites is too small to be statistically trackable.

A more appropriate statistic than that provided by the goodness-of-fit test presented above may be difficult. Fu (1996) gives perhaps the most rigorous test for deviation of an observed frequency spectrum from the neutral expectation but his test generally requires the absence of recombination. As shown in figure 3, there is no indication of extensive linkage disequilibrium, and in a separate analysis, recombinations were often detectable even between sites less than 50 bp apart.

Discussion

The most parsimonious interpretation of the results presented in table 1, in conjunction with the observations of Tsauro and Wu (1997), is that positive selection drives the divergence between species at the *Acp26Aa* locus. This selection has been operating in both the distant and recent evolutionary past of the *D. melanogaster* species subgroup. However, if the excess of amino acid substitutions between *D. melanogaster* and *D. simulans* is due to positive selection, there is no sign of a recent selective sweep resulting in the loss of variation within the species of *D. melanogaster*. Because our estimate of

the number of selectively driven events between the two species is necessarily imprecise, it is possible that the actual number is much smaller and the last sweep occurred too long ago to leave a trace.

Alternatively, positive selection may have been constantly at work but too weak to depress the nucleotide diversity, thus leaving no trace detectable by either Tajima's (1989) or Fu and Li's (1993) test. Weak selection would take a long time to drive a favorable mutation to fixation, and therefore even very closely linked regions may escape the sweep through recombination. On the other hand, this selection should still be orders of magnitude stronger than genetic drift, resulting in the acceleration of between-species divergence. Is there a range of values for the selective coefficient that satisfy both conditions? Based on the results of Kaplan, Hudson, and Langley (1989) and the parameters from *Drosophila*, even sites as close as 50 bp may not share an identical history if $2Ns$ is less than 10^3 (s being the selective advantage at one of the two sites). Since $2Ns > 10$ is stronger than genetic drift, there exist at least two orders of magnitude for which selection is strong enough to overpower genetic drift but still weak enough to have only a restricted footprint (< 50 bp) on nucleotide diversity (see Braverman et al. 1995 for a detailed study). Such weak hitchhiking can be more effectively detected by examining the frequency spectrum of new mutations, as shown in figure 2. Diversity reduction may be viewed as an extreme example of strong selection

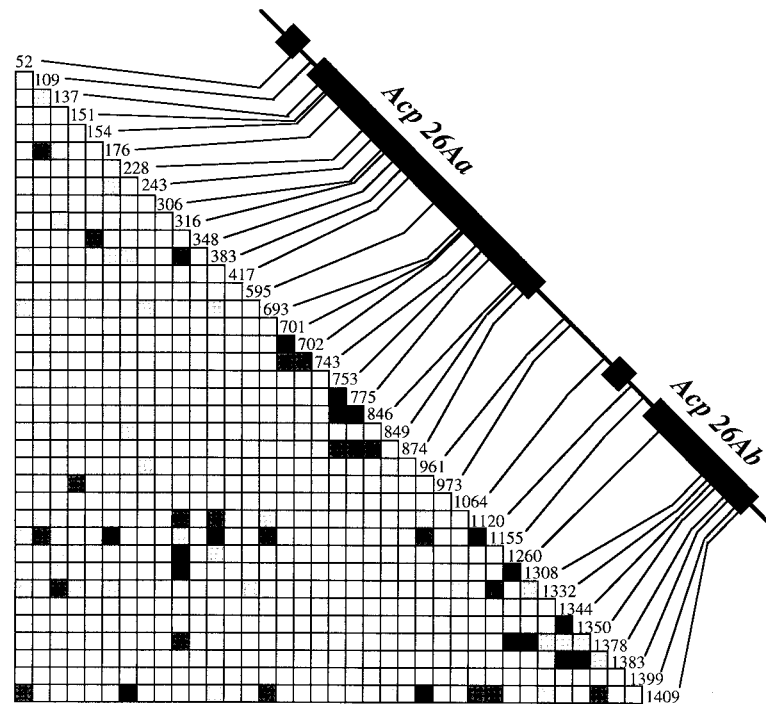


FIG. 3.—Linkage disequilibria in all pairwise comparisons among the 60 segregating sites in the *Acp26A* region. Data are based on all 49 sequences. The locations of the segregating sites relative to the two structural genes are shown on the diagonal. The levels of significance by Fisher's exact test are shown by different shades. Black, $P < 0.001$; dark gray, $0.001 < P < 0.01$; light gray, $0.01 < P < 0.05$.

disrupting gene frequency spectrum, while weaker selection may leave a different signature by dragging nearby variations to higher-than-expected frequencies without causing fixation.

In inferring the derived nucleotide in figure 2, we use the sequences from the three sibling species as outgroups and apply the parsimony principle. The question is how well parsimony holds in this data set. We shall compare the two types of nucleotide sites, $(C \leftrightarrow T) = [T, T, T]$ and $(C \leftrightarrow T) \leftrightarrow [A, A, A]$, where $()$ contains the polymorphic nucleotides of *D. melanogaster*, $[]$ contains the nucleotides of the three sibling species, $=$ denotes no change, and \leftrightarrow denotes a nucleotide change. (The nucleotides are chosen merely for demonstration.) The former set is usually inferred to have experienced a T-to-C change, as shown. If parsimony is violated, then C may in fact be the ancestral type which changed to T twice during evolution, i.e., $(T \leftrightarrow C) \leftrightarrow [T, T, T]$. Given the same number of changes, this set should be half as frequent as $(T \leftrightarrow C) \leftrightarrow [R, R, R]$, where R is either A or G. Since we observed no site with this latter type of nucleotide set, the parsimony inference seems adequate in our analysis.

In performing the statistical tests, we combine the data from five populations. This should not affect our interpretation. For the *D* statistics, Tajima (1993) has shown that the degree of population differentiation observed in our sample would not affect the results much at all. Similarly, Fu's studies (1996, see his fig. 1) suggest that the entire frequency spectrum is not greatly affected by population structure or sampling scheme.

In conclusion, positive selection on the amino acid sequence of *Acp26Aa* may have been a constant feature

during the evolutionary history of the *D. melanogaster* subgroup. The selective coefficient for each amino acid substitution is likely to be small, at most two to three orders of magnitude stronger than the effect of genetic drift.

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