Fluctuating Mutation Bias and the Evolution of Base Composition in *Drosophila*

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Received: 4 May 1999 / Accepted: 26 July 1999

Abstract. The idea that the pattern of point mutation in *Drosophila* has remained constant during the evolution of the genus has recently been challenged. A study of the nucleotide composition focused on the *Drosophila saltans* group has evidenced unsuspected nucleotide composition differences among lineages. Compositional differences are associated with an accelerated rate of amino acid replacement in functionally less constrained regions. Here we reassess this issue from a different perspective. Adopting a maximum-likelihood estimation approach, we focus on the different predictions that mutation and selection make about the nonsynonymous-to-synonymous rate ratio. We investigate two gene regions, alcohol dehydrogenase (Adh) and xanthine dehydrogenase (Xdh), using a balanced data set that comprises representatives from the *melangaster*, *obscura*, *saltans*, and *willistoni* groups. We also consider representatives of the Hawaiian picture-winged group. These Hawaiian species are known to have experienced repeated bottlenecks and are included as a reference for comparison. Our results confirm patterns previously detected. The branch ancestral to the fast-evolving *willistoni/saltans* lineage, where most of the change in GC content has occurred, exhibits an excess of synonymous substitutions. The shift in mutation bias has affected the extent of the rate variation among sites in *Xdh*.

Key words: Mutation bias — Nucleotide composition — Nonsynonymous/synonymous rate ratio — Among-site rate heterogeneity — *willistoni* group — *saltans* group

Introduction

The pattern of point mutation is generally thought to have been a negligible source of heritable variation during the diversification of the *Drosophila* genus, initiated around 60 My ago (Fitch and Ayala 1994; Powell and DeSalle 1995). This assumption is based on the observations that (1) nucleotide composition varies little in introns (Shields et al. 1988; Moriyama and Hartl 1993; Kliman and Hey 1994) and other allegedly unconstrained regions, and (2) the pattern of codon usage is fairly homogeneous across species, except when differences can be accounted for by changes in natural selection (Akashi 1995, 1996; Akashi and Schaffer 1997). These studies, however, have been restricted largely to two species, *D. melanogaster* and *D. pseudoobscura*, of the *Sophophora* subgenus, and *D. virilis* of the subgenus *Drosophila*, all three of which have quite similar base compositions (reviewed by Powell 1997).

The idea of the constancy of the pattern of point mutation in the evolution of *Drosophila* has recently been challenged (Rodríguez-Trelles et al. 1999b). The not previously investigated *Drosophila saltans* group exhibits patterns of GC content and codon usage bias markedly different from those previously known in *Sophophora*. The GC content in the third codon position, and to a lesser extent in the first position and the introns, is higher in the *D. melanogaster* and *D. obscura* groups than in the
D. saltans group. Differences are greater for the xanthine dehydrogenase (Xdh) region than for the alcohol dehydrogenase (Adh), superoxide dismutase (Sod), period (Per), and 28S rRNA regions, which are functionally more constrained. In addition, the saltans group shows an increased rate of amino acid substitution in Xdh, with the new replacements occurring preferentially by amino acids encoded by low-GC content codons. These observations are best explained by a shift in the pattern of point mutation that occurred in the ancestor of the saltans lineage, after it split from the lineage that gave rise to the melanogaster and obscura groups (Rodriguez-Trelles et al. 1999b). Alternatively, they can be explained without invoking changes in the mutational spectrum, either by a change in protein function (i.e., positive selection) or by a reduction in the effectiveness of selection (due to diminished population numbers or reduced recombination) that generally counteracts the underlying mutation bias significant [i.e., in the absence of selection, the nucleotide composition of functionally relevant regions would move toward that exhibited by the less constrained regions of the genome, i.e., =65% AT in Drosophila (Shields et al. 1988; Moriyama and Hartl 1993; Kliman and Hey 1994; Petrov and Hartl 1999)]. In light of the available evidence, these competing hypotheses have received less credit, yet no statistical arguments have been so far provided to resolve the issue.

In this paper, we tackle the above hypotheses on quantitative terms. We concentrate on the different predictions that mutation and natural selection make about the ratio of nonsynonymous-to-synonymous substitutions (dN/dS) for lineages. Specifically, if the shift in GC pressure had actually occurred in the ancestor of saltans, it would be reflected more in neutral parts of the genome than in functionally significant parts, so that we would expect a reduced dN/dS ratio in the critical branch; on the contrary, most frequent scenarios of natural selection predict an increase in the dN/dS ratio. We focus on two loci, Adh and Xdh, from which a large number of nucleotide sequences is available. It is possible in this way to choose a balanced data set that includes three representatives of each of the four major species groups of Sophophora (namely, the melanogaster, obscura, willistoni, and saltans groups), with known phylogenetic relationships. The willistoni group is more closely related to the saltans group than to the other species groups, but it has not been investigated before in this respect. Also, we include three Hawaiian species of the picture-winged group (available only for Adh). The species of this Hawaiian group are known to have experienced repeated episodes of relaxed effectiveness of selection due to reduced population numbers (DeSalle and Templeton 1988; Ohta 1993); consequently, these species constitute a valuable reference to evaluate the dN/dS ratios in the branches of interest. We chose the maximum-likelihood approach developed by Yang (1998) (see also Yang and Nielsen 1998) for estimating the nonsynonymous and synonymous substitution rates, which allows the dN/dS ratio to vary among lineages. While time-consuming, maximum-likelihood methods are preferable over conventional methods because they allow us to accommodate more realistic models of evolution (Yang 1998; Yang and Nielsen 1998). Finally, we investigate the effects that fluctuating mutation bias has had on the extent of the among-site rate variation of the Xdh gene.

Materials and Methods

Species and Sequences. We have examined 17 drosophilid species, with known phylogenetic relationships, as shown in Figs. 1, 5, and 6 [these hypotheses are supported by data of several sorts (see Powell 1997; Tatarenkov et al. 1999); for the species of the saltans group, see Rodriguez-Trelles et al. (1999b)]. The Adh and Xdh gene sequences include three representatives of each of the four major species groups of the Sophophora subgenus, as follows. D. melanogaster (GenBank accession numbers X78384 and Y00307 for Adh and Xdh, respectively), D. teissieri (X54118 and AF169401), and D. erecta (X54116 and AF169400) from the melanogaster group; D. pseudoobscura (U64560 and M33977), D. subobscura (X55391 and AF058976-7), Y08237, and D. bifasciata (AF169402-03 for Xdh) from the obscura group; D. willistoni (U95251 and AF093206), D. insularis (U95273 and AF093210), and D. nebulosa (U95275 and AF93213) from the willistoni group; and D. saltans (AF054113 and AF058978), D. sturtiavanti (AF045114 and AF058983), and D. subsaltans (AF045117 and AF058980) from the saltans group. Three Adh sequences from representatives of the Hawaiian picture-winged group of the subgenus Drosophila, namely, D. silvestris (M6321), D. affinisdisjuncta (M37262), and D. picticornis (M63392), and one Xdh sequence from Scaptodrosophila lebanonensis (AF058984), are used as outgroups. Except those from D. melanogaster and D. pseudoobscura, the Xdh sequences have been obtained in our laboratory. The Xdh region investigated includes about half of exon 2 (371 codons), intron 2 (around 60 bp except for D. subobscura, where it is 528 bp long, and D. melanogaster and D. erecta, where it is about 250 bp long), and most of exon 3 (324 codons), or about 52% of the Xdh coding region. Details about the amplification and sequencing primers and strategy are given by Tarri et al. (1998).

The sequences of Adh are obtained from the literature and consist of 135 codons of exon 2. The available Adh sequence from D. bifas-
ciata is incomplete; therefore we have used instead the sequence of D. ambigua (X54813), which is not expected to alter significantly the conclusions of this study.

Coding sequences were aligned using the CLUSTAL W (vs. 1.5) program (Thompson et al. 1994) and no gaps were observed.

**GC Content, Codon-Use Bias, and Amino Acid Composition.** The nucleotide composition of the sequences is determined by the G + C content. As a measure of departure from optimal codon use in nucleotide composition of the sequences is determined by the G + C program (Thompson et al. 1994) and no gaps were observed.

Figure 2 shows the GC-content variation of the Xdh gene regions across species groups for each fourfold degenerate sites (4-F) of genes, and in intron 2 (I2) of Xdh, for the species groups in this study.

Results

**GC Content, Codon Usage, and Amino Acid Composition.** Figure 2 shows the GC-content variation of the Adh and Xdh gene regions across species groups for each codon position and fourfold degenerate sites. Also shown
are the GC-content values of intron 2 of Xdh. The results confirm the trends already reported (Rodríguez-Trelles et al. 1999b); the base composition varies extensively and consistently among the lineages of Sophophora in both the Adh and the Xdh gene regions. The compositional pattern of the D. willistoni group, which had not been investigated before, is very similar to that of D. saltans, its sister clade; the GC content in fourfold degenerate sites and third codon positions, and to a lesser extent in the first position and the Xdh intron, is lower in these two species groups than in the D. melanogaster and D. obscura groups. The Hawaiian species (available only for Adh) show an intermediate GC content. Differences are greater for Xdh than for Adh, suggesting that the former region evolves faster than the later. We examine this possibility by means of a likelihood-ratio test of the null model that both gene regions evolve at equal rates. Thus we have combined the Adh and Xdh data in a single data set for both nucleotides and amino acid sequences. For nucleotides we have considered first and second, and third, codon positions separately, with the substitution model of Hasegawa et al. (1985); for amino acids we used an empirical model based on the matrix of Jones et al. (1992), using amino acid frequencies as free parameters [referred to as the JTT-F model by Yang et al. (1998)]. Setting different rate parameters for Adh and Xdh improves significantly the likelihood over the equal-rates model at the amino acid level (1-df test), with the Xdh protein evolving $1.43 \pm 0.22$ times faster than Adh ($2\ln \Lambda = 6.04; p = 0.013$) and the third codon positions of Xdh evolving $1.61 \pm 0.13$ faster ($2\ln \Lambda = 35.08; p < 0.001$); for first and second positions the difference is marginally significant ($2\ln \Lambda = 3.64; p = 0.056$), with Xdh evolving $1.28 \pm 0.17$ faster than Adh in these codon sites.

If a given locus experiences different mutation pressures in different lineages, then positive correlations should be observed between the GC compositions of the codons and the introns (assuming that the intron base composition reflects the mutational equilibrium of the genome), the stronger the less constrained is the coding region. With Felsenstein’s (1993) contrast test, the GC content of intron 2 correlates significantly with the first ($r = 0.81, p = 0.002$) and the third ($r = 0.84, p = 0.001$) codon positions in Xdh. Moreover, the GC content at fourfold degenerate sites of Xdh, which can reasonably be assumed to be under very weak functional constraints and less affected by sampling error than the intron 2 GC content, also correlates significantly with the first ($r = 0.84, p = 0.001$), second ($r = 0.60, p = 0.04$), and third ($r = 0.96, p < 0.001$) codon positions of Xdh and the third codon positions of Adh ($r = 0.61, p = 0.049$).

Figure 3 represents the frequency of optimal codons (Fop) (Ikemura 1985) across species groups in Adh and Xdh, computed with the set of major codons defined by Akashi (1995) for D. melanogaster. The D. saltans and D. willistoni groups deviate the most from the optimal codon usage among these species, while the Hawaiian representatives exhibit intermediate Fop values. The largest Fop values correspond to the Adh region in the melanogaster and obscura species, and the lowest to the Xdh region in the species of the D. saltans and D. willistoni groups.

Figure 4 plots the proportions of high-GC (group I), intermediate-GC (group II), and low-GC (group III) amino acids against the GC content at fourfold degenerate sites in the Xdh region for the various species. As expected, group I amino acids are less used by species with a low GC content (the saltans and willistoni groups), while the opposite is the case for group III amino acids and, less so, for group II amino acids. The correlation between the group I contrast, f(I), and the GC$_4$ contrast is highly significant ($r = 0.80, p = 0.002$), the correlation of the GC$_4$ and f(III) contrasts is $r = -0.63 (p = 0.02)$, and the correlation between the GC$_4$ and the f(II) contrasts is not significant ($r = -0.30, p = 0.34$). For the more conserved Adh region, an association between amino acid composition and GC content cannot be detected.

Previous analyses with the relative rate test on a reduced data set have shown that the above differences in amino acid composition between the saltans and the melanogaster and obscura species arise as the result of an increased rate of amino acid replacement in the saltans lineage (Rodríguez-Trelles et al. 1999b). This is apparent in Fig. 1, where we see that, since their split from a common ancestor, the number of amino acid substitutions that have occurred in the saltans and the closely related willistoni lineages is conspicuously larger than in the obscura and melanogaster lineages; a likelihood-ratio test against a model without the restriction of rate constancy among lineages clearly leads to the rejection of the molecular clock hypothesis ($2\ln \Lambda = 27.10, p = 0.004, 11$ df).

The GC% compositional statistics for the ancestor of the species in our study (obtained by the maximum-likelihood RateAncestor method of PAML 1.4 (Yang 1997)) are 52.6, 39.2, 71.8, and 72.9 and 61.3, 41.5, 73.1, and 76.9, respectively, for positions 1, 2, 3, and GC$_4$ of
Adh and Xdh, assuming the phylogenies in Figs. 5 and 6. These values are much closer to those of the melanogaster and obscura groups than to those of the willistoni and saltans species (Fig. 2). This observation supports the inference that GC content has evolved faster in the willistoni and saltans group species than in the Sophophora. Furthermore, the fact that the willistoni and saltans species exhibit a quite similar base composition, which is very different from that of their ancestor, indicates that most of the compositional change in the lineage took place before the differentiation of these two species groups, along the branch marked by the arrow in Fig. 1. As mutation and selection have different effects on synonymous and nonsynonymous substitutions, comparison of synonymous and nonsynonymous rates in this branch with those in other branches in the tree may help to elucidate the mechanisms responsible for the switch in base composition that occurred in the willistoni and saltans lineages. Hence, this is the branch of interest in later analyses.

The Ratio of Nonsynonymous-to-Synonymous Substitution Rates ($d_N/d_S$) Among Lineages. For practical reasons, because maximum-likelihood methods are computationally highly demanding, the following analyses are conducted on a subset of the sequences identified in Fig. 1 (i.e., we have eliminated one species of each group, generally one of the two most closely species in the group). This is not a serious handicap if we take into account that we are interested in lineage effects, rather than in effects associated with particular species. In addition, we have considered Adh data from the three picture-winged Hawaiian representatives. Previous analyses of the Adh gene in these species have shown an excess of nonsynonymous over synonymous substitutions, which was interpreted to be a consequence of reduced population sizes known for this Hawaiian lineage (DeSalle and Templeton 1988; Ohta 1993). These species provide an invaluable ground to evaluate the $d_N/d_S$ ratios of other lineages.

Figures 5 and 6 show, respectively, the Adh and Xdh phylogenies of the major species groups of Sophophora (and the Hawaiian species for Adh) inferred under the codon-based model, allowing independent $d_N/d_S$ ratios for branches (the “free-ratio” model). Above the branches are shown the numbers of nonsynonymous-to-synonymous substitutions, each per 100 sites of each class. Reference node numbers are shown in circles. According to the codon-based model, the Adh region has 101.15 synonymous and 303.85 nonsynonymous sites (with a total of 405 sites or 135 codons); for Xdh these numbers become, respectively, 534.78 and 1550.22 (with 2085 sites or 695 codons). We can see that synonymous substitutions occur more often than nonsynonymous substitutions, indicating that Adh and Xdh have spent a majority of time under purifying selection during the evolution of Sophophora. Constraining the ratio to be the same for all branches (the “one-ratio” model) we obtain $d_N/d_S$. 

![Fig. 5](image-url)
= 0.072 ± 0.010 for Adh, and dS/dS = 0.053 ± 0.003 for Xdh, which can be interpreted as average dS/dS ratios over all the branches. The difference in log likelihoods between the free-ratio and the one-ratio models is significant for both, Adh (2logΛ = 16.64, p < 0.001, 3 df), and Xdh (2logΛ = 31.42, p < 0.004, 14 df), which means that the differences in dS/dS ratios among lineages are, anyhow, too large to be accounted for by a strictly neutral model.

For the purpose of this study, it is possible to extract the main trends contained in Figs. 5 and 6 using simplified models, intermediate between the simplest (one-ratio) and the most general (free-ratio) models discussed above. For instance, for Adh we chose a five-dS/dS ratio model, which includes one ratio for the species of the willistoni and saltans groups (i.e., from node 5 to the tips; see Fig. 5) and a second one for the branch ancestral to them (between node 1 and node 5), a third ratio for the melanogaster–obscura lineage (from node 1 to the tips), and a fourth and fifth ratio for the Hawaiian species (node 8 to the tips) and the branch ancestral to them (nodes 1 to 8). Similarly, for Xdh we use a four-dS/dS ratio model. The results are presented in Table 1. First, we can see that the highest dS/dS ratio in the table is, by and large, that exhibited by the Hawaiian species in the Adh region (0.197 ± 0.069), which agrees with Ohta’s (1993) results and is consistent with a hypothesis of relaxed constraints driven by reduced population numbers. Second, the branch ancestral to the willistoni–saltans lineage shows the lowest dS/dS ratio in both Adh (0.018 ± 0.012) and Xdh (0.036 ± 0.009), which is, on average, about eight times lower than the ratio shown by the Hawaiian species. Despite the low dS/dS ratio, this branch is the largest of Sophophora, i.e., has accumulated many changes, but these appear to have been overwhelmingly synonymous.

| Gene region | Lineage        | dS/dS ± SE |
|-------------|---------------|------------|
| Adh         | Node 1 · Me & Ob | 0.076 ± 0.019 |
|             | Node 1 · node 5 | 0.018 ± 0.012 |
|             | Node 5 · Wi & Sa | 0.062 ± 0.018 |
|             | Node 1 · node 8 | 0.070 ± 0.029 |
|             | Node 8 · Hawaiian | 0.197 ± 0.069 |
| Xdh         | Node 1 · Me & Ob | 0.046 ± 0.005 |
|             | Node 1 · node 5 | 0.036 ± 0.009 |
|             | Node 5 · Wi & Sa | 0.070 ± 0.006 |
|             | Node 1 · Saltans | 0.039 ± 0.008 |

*a dS/dS estimates are obtained with a five (for Adh) and a four (for Xdh) codon-based model. These two models render a significant improvement of the log-likelihood over the corresponding one-ratio models (2logΛ = 12.64, p = 0.013, 4 df, and 2logΛ = 16.64, p < 0.001, 3 df, respectively). Estimates for Adh are based on 135 codons and the tree shown in Fig. 5. For Xdh we use 695 codons and the tree shown in Fig. 6. Node numbers are those shown in Figs. 5 and 6.

The Average Rate of Evolution of Xdh and Its Degree of Among-Site Rate Variation. We have shown that the Xdh region evolves faster in the willistoni–saltans lineage than in the lineage consisting of the melanogaster and obscura species (also see Rodrı́guez-Trelles et al. 1999b). Now we are interested in ascertaining whether this difference in the average evolutionary rate among lineages is reflected in the pattern of among-site rate variation within the gene. This approach differs from previous attempts at exploring this question, which have focused on several genes across a single lineage. Indeed, previous studies have found that within a lineage fast-evolving proteins exhibit a lower degree of among-site rate variation than slowly evolving proteins (Kumar 1996; Zang and Gu 1998).

We have focused on both the degree of among-site rate variation (the subject of previous analyses) and the rate correlation over adjacent sites. The variation and dependence of substitution rates over sites are described with the autodiscrete gamma model of Yang (1995), which is based on a serially correlated gamma distribution (setting eight equal-probability categories of rates). The gamma distribution involves a shape parameter, α (>0), which is inversely related to the extent of rate variation among sites. For nucleotide data the substitution pattern is accommodated by the general reversible model (Yang 1994), which for Xdh produces a better fit than simpler models (Rodrı́guez-Trelles et al. 1999b). For amino acids we use the JTT-F empirical model (Jones et al. 1992; Yang et al. 1988). In a coding sequence, rates at sites three nucleotides apart are highly correlated, so that the correlation between rates for two adjacent sites is weakened (Yang 1995). Therefore, in order to estimate the correlation among neighboring sites from nucleotides (ρ), we have first removed the large-scale variation allowing different rate parameters for codon positions in the model. Model parameters are estimated by maximum likelihood, and their relevance for describing the data is evaluated by means of likelihood-ratio tests.
Estimates of the among-site rate variation ($\alpha$) and the rate correlation among adjacent sites ($\rho$) for lineages are shown in Table 2. As expected from the differences in the average rate of evolution of $Xdh$ among lineages, $\alpha$ values are largest, meaning that the degree of among-site rate variation is lowest, in the fast-evolving $willistoni$–$saltans$ lineage at both the nucleotide ($0.425 \pm 0.039$ vs $0.312 \pm 0.030$) and the amino acid ($0.426 \pm 0.048$ vs $0.186 \pm 0.030$) levels; on the other hand, $\rho$ shows the opposite pattern: it is largest in the comparatively slowly evolving $melanogaster$–$obscura$ lineage ($0.669 \pm 0.113$ vs $0.439 \pm 0.113$ and $0.551 \pm 0.142$ vs $0.450 \pm 0.113$, at the nucleotide and amino acid levels, respectively). From normal-deviate tests [e.g., $z = (\alpha_1 - \alpha_2)/\text{SQRT}(\text{SE}_1^2 + \text{SE}_2^2)$], using standard errors (SEs) computed by the curvature method in the likelihood analysis (see Yang 1997)], the difference in $\alpha$ values between the two lineages is significant ($p < 0.02$ and $p < 0.03$, for nucleotides and amino acids, respectively); for $\rho$ the standard errors of the estimates are larger, and the differences are not significant ($p < 0.11$ and $p < 0.58$, for nucleotides and amino acids, respectively). While the tree topology is the same for the two species sets (i.e., $willistoni$–$saltans$ and $melanogaster$–$obscura$), it can be argued that our results might be affected by tree length differences among lineages, attributable to chronological differences. In fact, $\alpha$ tends to be underestimated for closely related sequences and overestimated for highly diverged sequences (Zhang and Gu 1998). This asymptotic bias, however, occurs when the number of sequences is small [four or less (Zhang and Gu, 1998)]. Moreover, the $willistoni$ and $saltans$ lineages are likely to be more closely related to each other than $melanogaster$ is to $obscura$ (Throckmorton 1975), which would generate a bias in the opposite direction to the observed $\alpha$ values. Finally, enlarging the time scale by combining the two data sets yields estimates that are intermediate between those for the two lineages separately (see Table 2).

Table 2. $Xdh$ among-site rate variation ($\alpha$) and correlation ($\rho$) in different lineages

| Model          | Parameter | Wi & Sa | Me & Ob | All species |
|----------------|-----------|---------|---------|-------------|
| Among nucleotide sites $\alpha$ | $0.425 \pm 0.039$ | $0.312 \pm 0.030$ | $0.401 \pm 0.023$ |
| Among nucleotide sites $\rho$   | $0.439 \pm 0.113$ | $0.669 \pm 0.113$ | $0.460 \pm 0.070$ |
| Among amino acid sites $\alpha$ | $0.426 \pm 0.101$ | $0.186 \pm 0.048$ | $0.433 \pm 0.056$ |
| Among amino acid sites $\rho$   | $0.450 \pm 0.113$ | $0.551 \pm 0.142$ | $0.485 \pm 0.080$ |

*a* All parameters contribute significantly to describe the variation of the sequences, as inferred from the comparison of the models with the corresponding nested submodels by means of likelihood-ratio tests (1-df tests; results not shown). Estimates are obtained with the general reversible model (Yang 1994) for nucleotides and the empirical model of Jones et al. (1992) (as modified by Yang et al. 1998) for amino acids. Among-site rate variation is assumed to follow an autodiscrete gamma process with eight categories of rates. Different rate parameters for codon positions are allowed in nucleotide models for $\rho$.

**Discussion**

**GC-Content Differences: Fluctuating Mutation Bias vs Natural Selection.** Interspecific differences in nucleotide composition and codon usage bias among lineages within the *Sophophora* subgenus similar to those investigated herein for $Adh$ and $Xdh$ are also found in the *Sod*, *Per*, and 28SrRNA gene regions (Rodríguez-Trelles et al. 1999b); they appear to be the case for other sequenced regions as well [e.g., the $Ddc$ and $amd$ regions (Andri Tatarenkov personal communication)]. The occurrence of such compositional patterns across several unlinked regions, scattered throughout the genome (see Rodríguez-Trelles et al. 1999b), suggests that these patterns reflect genomewide differences between lineages. Extensive variation in GC content is not unique to *Drosophila*; large differences in nucleotide composition have been long known among the genomes of bacterial species (Lee et al. 1956) and between regions of the mammalian genome [the so-called isochores, discovered by Bernardi et al. (1985; Bernardi 1995)].

In both respects, nucleotide composition and codon usage bias, the species of the *willistoni* group, which had not been previously investigated, show a pattern similar (but less extreme) to the pattern exhibited by the *saltans* species, as might be expected because these two species groups are more closely related to each other than to the *melanogaster* and *obscura* groups (Throckmorton 1975). The changes in GC composition can be attributed to an AT increase in the lineage ancestral to the *willistoni* and *saltans* groups after its split from the lineage that gave rise to the *melanogaster* and *obscura* groups [≈55 Mya (Fitch and Ayala 1994; Tatarenkov et al. 1999)]. The shift in nucleotide composition is associated with an accelerated change in the amino acid composition of relatively low-constrained proteins such as $Xdh$.

At least three competing hypothesis may account for these observations: (i) positive selection triggered, for example, by a change in protein function or a shift in metabolic efficiency; (ii) a reduction in the effectiveness of selection caused by either a reduction in population numbers, a reduction in the rate of recombination, or a relaxation of constraints; and (iii) a shift in the pattern of point mutation. According to the first two hypotheses we should observe an increase in the nonsynonymous-to-synonymous substitution rate ratio in the ancestor of the *willistoni* and *saltans* groups; specifically, if positive selection had been responsible for the GC changes, we would expect a $d_{NS}/d_S$ ratio significantly greater than one, because natural selection would have favored the fixation of nonsynonymous mutations over the neutral prediction; on the other hand, if the GC changes had been...
caused by a reduction in the effectiveness of selection, we should observe a $d_{s}/d_{g}$ ratio less than or equal to one but still greater than the average ratio observed in other *Drosophila* lineages. On the contrary, the branch ancestral to *willistoni* and *saltans* exhibits the lowest $d_{s}/d_{g}$ ratio in both the Adh and the Xdh trees (Figs. 5 and 6, Table 1). This is what one would expect if a shift in the pattern of point mutation had actually occurred some time after the split of that branch ancestral to the *willistoni/saltans* lineage, because mutation bias would affect more the least constrained parts of the genome (i.e., synonymous sites) than the functionally significant parts (i.e., nonsynonymous sites) (Sueoka 1988). That the observed AT increase is not due to reductions in population size is further corroborated by noting that the $d_{s}/d_{g}$ ratio obtained for the Hawaiian *Drosophila* in the Adh region is ~11 times higher than for the *willistoni/saltans* lineage (Table 1). High $d_{s}/d_{g}$ ratios have previously been noticed for Hawaiian species (Ohta 1993), which were attributed to diminished effectiveness of natural selection due to their known history of severe bottlenecks (DeSalle and Templeton 1988; Ohta 1993). However, it seems reasonable to infer that if reduced efficiency of natural selection had been the cause of the even larger change in GC content observed in the *willistoni/saltans* lineages, the $d_{s}/d_{g}$ ratio for this lineage should not be far lower than for the Hawaiian species, as is actually the case.

The previous inferences regarding the $d_{s}/d_{g}$ ratios can potentially be confounded by several factors. First, our likelihood analysis assumes that nucleotide frequencies have remained more or less the same during the course of evolution (i.e., the stationarity assumption). This assumption is made by virtually all methods and models currently used for phylogenetic analysis; consequently, taxa with similar composition are clustered together regardless of their true phylogenetic relationships. Stationarity does not hold in our case (see also Rodríguez-Trelles et al. 1999b). It should be noted, however, that maximum-likelihood methods appear to be particularly robust to violations of the stationarity assumption and that the purpose of our analysis is not phylogenetic; rather, we start from phylogenetic relationships that are known in advance. Second, our likelihood analysis assumes that the $d_{s}/d_{g}$ ratio is constant over all codon sites in *Adh* and *Xdh*. This assumption is expected to lead to underestimates of $d_{g}$, as nonsynonymous (or amino acid) substitution rates are variable among sites, and hence to lead to underestimates of the $d_{s}/d_{g}$ ratio. From the analysis of the among-site rate variation in *Xdh*, however, rates are more variable in the *melanogaster/obscura* lineage ($\alpha = 0.312$ and $\alpha = 0.186$, from the nucleotide and amino acid sequences, respectively; see Table 2) than in the *willistoni/saltans* lineage ($\alpha = 0.425$ and $\alpha = 0.426$; see Table 2). Consequently, the underestimation problem should be larger in the former lineage than in the latter, which means that the observed differences in the $d_{s}/d_{g}$ ratios among lineages might be even larger yet, in the direction predicted by the mutation shift hypothesis. And third, synonymous substitution rates could be underestimated due to multiple hits, which would lead to overestimates of the $d_{s}/d_{g}$ ratios. However, this overestimation should be more acute in the *willistoni/saltans* ancestral lineage, because it is this branch that exhibits the largest compositional change. Again, this factor would increase the differences in the $d_{s}/d_{g}$ ratios among lineages, precisely in the direction expected from a shift in the pattern of point mutation.

Our results of high $d_{s}/d_{g}$ ratios for the Hawaiian species (relative to the *melanogaster* and *obscura* groups) in *Adh* coincides qualitatively with former analyses of this same gene by Ohta (1993). Nevertheless, ours and Ohta’s (1993) estimates of the $d_{s}/d_{g}$ ratios differ considerably from each other. For example, after correcting for multiple substitutions, Ohta (1993) obtains an average $d_{s}/d_{g} = 0.630$ for the Hawaiian *Drosophila*, whereas the corresponding estimate from our analysis is only 0.197. Discrepancies of this sort can be accounted for, in part, by disparities in the data sets (e.g., Ohta considers the entire *Adh* gene region, and she does not use *D. affinisjuncta*). More importantly, however, might be the differences in the analytical procedures utilized. Ohta (1993) uses the approximate method for pairwise sequence comparisons of Nei and Gojobori (1986), unlike the codon-based likelihood models used here. But Yang and Nielsen’s (1986) method tends to underestimate $d_{g}$ and overestimate (less so) $d_{g}$, yielding $d_{s}/d_{g}$ ratios that are larger than those obtained by maximum likelihood (Yang and Nielsen 1998).

We have discussed the role of natural selection in determining base biases in terms of protein function. Yet positive selection might occur in a different way: a high GC content might be selected for a whole genome or DNA region. Corresponding replacements of amino acids in polypeptide chains might, then, be the result of selection, rather than shifts in the frequencies of transitional and transversional sites in the DNA region. Corresponding replacements of amino acids in polypeptide chains might, then, be the result of selection, rather than shifts in the frequencies of transitional and transversional sites in the DNA region. Consequently, the underestimation problem should be larger in the former lineage than in the latter, which means that the observed differences in the $d_{s}/d_{g}$ ratios among lineages might be even larger yet, in the direction predicted by the mutation shift hypothesis. And third, synonymous substitution rates could be underestimated due to multiple hits, which would lead to overestimates of the $d_{s}/d_{g}$ ratios. However, this overestimation should be more acute in the *willistoni/saltans* ancestral lineage, because it is this branch that exhibits the largest compositional change. Again, this factor would increase the differences in the $d_{s}/d_{g}$ ratios among lineages, precisely in the direction expected from a shift in the pattern of point mutation.
for the high GC content in the thermophilic bacteria (Kagawa et al. 1984) and, also, in isochores of warm-blooded vertebrates (Bernardi et al. 1985; 1988). However, a recent study has failed to find a correlation between the optimum growth temperature and the GC content of thermophilic bacteria (Galtier and Lobry 1997); also, a globin pseudogene from a GC-rich isochore was found to evolve faster than its paralogue from an AT-rich isochore, which is unexpected if thermal adaptation at the DNA level is governing the substitution process in the former (Francino and Ochman 1999). These observations bring into question whether thermal stability of the DNA is the evolutionary factor responsible for the GC-constant variation in bacteria and among the isochores of the mammalian genome. This hypothesis cannot account well for the large GC-content variation among Drosophila lineages either. In Drosophila, solar heating of necrotic fruit may expose larvae to temperatures above 45°C even in temperate latitudes (Feder 1996). However, differences in GC content do not fit the biogeography of the species we have investigated: the highest GC content occurs in the obscura-group species, which evolved in the cold and temperate climates of the Palearctic and Neartic regions (Powell 1997), whereas the lowest GC content is found in the saltans-group species, which evolved in tropical and subtropical regions (Powell 1997).

Mutation Pressure and the Degree of Among-Site Rate Heterogeneity. The profile of conservative and variable regions of any given gene reflects a unique interplay between variable functional constraints and mutation rates that can also change over short regions. As a consequence of this balance, substitution rates are variable from site to site and also correlated because sites within the same region share a broad causal background (Yang 1995). Former analyses (Kumar 1996) have shown that the extent of among-site rate variation along a given gene covaries with the average rate of evolution of the gene itself, with slowly evolving genes having a higher degree of among-site rate variation than fast-evolving genes. This pattern occurs independently of any assumption about the underlying distribution of the substitution rate (most frequently used is the gamma distribution) and appears to be a general feature of molecular evolution (Zhang and Gu 1998). These studies have focused on different genes within the same lineage, but it is not known whether this pattern also holds for a given gene across different lineages. Here we have provided evidence that this can actually be the case, at least for the Xdh gene.

In light of the negative correlation between the average evolutionary rate of a gene and its degree of among-site rate variation pointed out by other studies (Kumar 1996; Zhang and Gu 1998), the finding of a significantly lower degree of among-site rate variation of the willistoni/saltans lineage than in the melanogaster/obscura lineage provides additional support for the hypothesis that the former lineage is evolving faster. Both phenomena, i.e., variation in the rate of amino acid substitution among lineages and changes in the extent of the rate variation among sites in Xdh, can be explained as a consequence of fluctuating mutation bias (Sueoka 1962, 1988).

Sueoka (1993) postulated that for a set of nucleotides for which the pattern of point mutation is at equilibrium with base composition and selective constraints, the mutation frequency per gene will be much lower than the frequency observed immediately after a shift in the pattern of point mutation takes place. To illustrate this principle, we can imagine a hypothetical sequence with 100% G content and for which the pattern of spontaneous mutation is such that G never mutates toward A, T, or C. In this extreme scenario we would not observe variation even if all sites were neutral, i.e., sites would be invariant not because of functional constraints, but simply because variation cannot occur. It is worth noting that, under the assumption that mutations occur directionally at random, the previous reasoning would lead to overestimate functional constraints. However, a change in mutation bias (e.g., from G to A in our example) will provoke a burst of mutations in such a way that many unconstrained sites that were “frozen” with regard to variation will “unfreeze” until the new composition equilibrium is reached. It follows that the rate of replacement of quasi-neutral amino acids should increase, which is what we have observed for the amino acid replacements occurring in Xdh during the evolution of the willistoni/saltans lineage. Moreover, taking into account that the extent of among-site rate variation varies inversely with the proportion of neutral sites in a gene [i.e., the larger the proportion of neutral sites, the lower the extent of among-site rate variation (Zhang and Gu 1998)], the α value for Xdh should be larger in willistoni/saltans than in melanogaster/obscura, which is precisely what we found. In support of this interpretation of the α values, the estimated proportion of neutral sites [defined as those sites for which \( d_{s}/d_{a} = 1 \), obtained with the codon-based likelihood model of Nielsen and Yang (1998)] is significantly larger in the willistoni/saltans lineage (0.223 ± 0.017) than in melanogaster/obscura (0.159 ± 0.014) (\( p = 0.003 \), from the normal-deviate test).

Acknowledgments. We are indebted to Carlos Machado and Emile Zuckerkandl for valuable suggestions. F.R.-T. has received support from Ministerio de Educacio´n y Cultura (Spain) (Contrato de Reincor- poración and Grant PB96-1136 to A. Fontdevila). This research was supported by Grant GM42397 from the National Institutes of Health to F.J.A.

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