Intron Length Evolution in Drosophila

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I present data on the evolution of intron lengths among 3 closely related Drosophila species, D. melanogaster, Drosophila simulans, and Drosophila yakuba. Using D. yakuba as an outgroup, I mapped insertion and deletion mutations in 148 introns (spanning ~30 kb) to the D. melanogaster and D. simulans lineages. Intron length evolution in the 2 sister species has been different: in D. melanogaster, X-linked introns have increased slightly in size, whereas autosomal ones have decreased slightly in size; in D. simulans, both X-linked and autosomal introns have decreased in size. To understand the possible evolutionary causes of these lineage- and chromosome-specific patterns of intron evolution, I studied insertion–deletion (indel) polymorphism and divergence in D. melanogaster. Small insertion mutations segregate at elevated frequencies and enjoy elevated probabilities of fixation, particularly on the X chromosome. In contrast, there is no detectable X chromosome effect on fixations in D. simulans. These findings suggest X chromosome—specific selection or biased gene conversion—gap repair favoring insertions in D. melanogaster but not in D. simulans. These chromosome- and lineage-specific patterns of indel substitution are not easily explained by existing general population genetic models of intron length evolution. Genomic data from D. melanogaster further suggest that the forces described here affect introns and intergenic regions similarly.

Introduction

The genome of Drosophila melanogaster contains over 48,000 introns that must be replicated, transcribed, spliced from precursor mRNAs, and enzymatically degraded (Misra et al. 2002). All of these processes require resources, time, and energy that surely impose costs. Yet, despite the abundance of introns in eukaryotic genomes, their compensating benefits have not been entirely clear because intron sequences have seemed to evolve with little or no functional constraint (Shields et al. 1988; Moriyama and Hartl 1993; Li 1997; Halligan et al. 2004). Recently, however, 2 facts have become clear: introns can encode a variety of functional elements (e.g., regulatory elements, alternative promoters and splicing signals, RNA genes, RNA-editing signals, and sites mediating pre-mRNA secondary structure; [Kirby et al. 1995; Leicht et al. 1995; Bergman and Kreitman 2001; Chen and Stephan 2003; Reenan 2005]), and intron sequence evolution in Drosophila is more constrained than synonymous site evolution (Bergman and Kreitman 2001; Andolfatto 2005; Hadrill et al. 2005; Marais et al. 2005; Halligan and Keightley 2006).

Given that intron primary sequences are constrained, intron lengths might also be constrained. The distribution of intron lengths within and between species is certainly not easily explained by mutational bias in the relative rates of deletion versus insertion: if introns lacked length constraints, then the well-known deletion-biased mutation pressure (Petrov et al. 1996; Petrov and Hartl 1998; Blumenstiel et al. 2002) would cause introns to decay and ultimately disappear. Instead, intron lengths in D. melanogaster range between 44 bp and >70 kb, with a strong mode at 58 bp (Mount et al. 1992; Deutsch and Long 1999; Adams et al. 2000; Comeron and Kreitman 2000; Misra et al. 2002). Introns of ~45 bp are believed to represent a minimum size in D. melanogaster, below which splicing reactions may be compromised (Guo et al. 1993; Talerico and Berget 1994). Deletions that reduce intron length below this minimum size are thus believed to be strongly deleterious (Mount et al. 1992). In addition to direct purifying selection on minimum size, 2 findings suggest that selection influences intron size indirectly as a by-product of selection on functional elements in introns. First, intron sequence divergence is negatively related to intron length, as expected if longer introns comprise more functional elements (Hadrill et al. 2005; Marais et al. 2005). This correlation is strongest for first introns which, being nearest to transcription start sites, tend to be longer and to contain more cis-regulatory elements (Duret 2001; Marais et al. 2005). Second, deletions affecting Drosophila introns are less frequent and smaller than those affecting pseudogenes and “dead-on-arrival” transposons, consistent with functional constraints on intron content (Comeron and Kreitman 2000; Ptak and Petrov 2002; Ometto et al. 2005). Therefore, in addition to selection on minimum size, the presence of functional elements also constrains intron size.

But not all sites in introns encode functional elements. One can therefore ask what population genetic forces influence the evolution of nonfunctional, presumably expendable, sequences in introns? Why, for instance, have some Drosophila species evolved longer introns than others (Moriyama et al. 1998)? It seems unlikely that such species differences exist because the density of functional elements (or the complexity of gene regulation) is greater in one species than in its close relatives. Instead, it seems more plausible that some intron sequence is expendable and that some variation in intron length is due to differences in the amount of such sequence.

Two findings may shed light on the evolution of expendable intron sequence. First, in humans, nematodes, and Drosophila, intron length is negatively correlated with gene expression level (Castillo-Davis et al. 2002; Urrutia and Hurst 2003; Marais et al. 2005), suggesting that selection eliminates expendable sequence in highly expressed genes to minimize the cost and/or rate of transcription (Carvalho and Clark 1999; Castillo-Davis et al. 2002; Urrutia and Hurst 2003). Second, in D. melanogaster, a negative correlation exists between local recombination rate and intron size (Carvalho and Clark 1999; Comeron and Kreitman 2000; Hurst 2003).
The general model of intron length evolution, as weak selection or biased gene conversion, act on insertions or deletions. I find that lineage- and sex chromosome-specific forces, such as weak selection or biased gene conversion, act on insertions. These findings are not easily explained by a single general model of intron length evolution.

Three weak selection models have been offered. In the first model, intron length evolves as a consequence of Hill-Robertson effects (Hill and Robertson 1966; Felsenstein 1974; Gordo and Charlesworth 2001). Hill and Robertson found that natural selection acting at one locus interferes with selection at linked loci (Hill and Robertson 1966; Birky and Walsh 1988; Hey 1999). Recombination reduces linkage among loci, thereby alleviating interference and increasing the efficacy of natural selection. Thus, natural selection may be less effective at loci in low recombination regions of the genome as these experience more interference than loci in high recombination regions (Kliman and Hey 1993; Betancourt and Presgraves 2002; Hey and Kliman 2002; Presgraves 2005). In the first model, Carvalho and Clark (1999) argue that genes in low recombination regions are less able to prevent the accumulation of weakly deleterious insertions. In the second model, however, Comeron and Kreitman (2000) argue that the known deletion-biased mutation pressure would drive introns to smaller sizes; thus, the fact that introns are longer in low recombination regions suggests that insertions enjoy a relative advantage in regions of low versus high rates of recombination. Comeron and Kreitman (2000) suggest that longer introns may be favored in low recombination regions as modifiers that increase recombination rates between adjacent exons and thus alleviate interference acting at many weakly selected sites, for example, synonymous sites (see also Comeron and Kreitman 2002; Qin et al. 2004; Comeron and Guthrie 2005). The third model is also a weak selection model, but it does not attempt to explain the correlation between recombination rate and intron length. By studying the long-term evolution of 15 Drosophila introns, Parsch (2003) inferred that small deletions are commonly fixed by mutation pressure, whereas larger, relatively rare insertions that restore optimal intron size are occasionally fixed by compensatory selection (see also Stephan et al. 1994). Each of these models makes distinct, testable predictions, for example, that insertions are weakly deleterious (Carvalho and Clark 1999), that insertions are weakly favorable in low recombination regions (Comeron and Kreitman 2000), or that optimal intron size is maintained by the long-term balance of mutation pressure and compensatory selection on indel mutations (Parsch 2003).

Here, I study indel polymorphism and divergence in a large collection of introns from D. melanogaster, D. simulans, and D. yakuba to better understand what forces have shaped intron length evolution. I find that lineage- and sex chromosome-specific forces, such as weak selection or biased gene conversion, act on insertions. These findings are not easily explained by a single general model of intron length evolution.

Materials and Methods

The Data

I gathered publicly available DNA sequences for 68 intron-containing genes with polymorphism data in D. melanogaster (genes, sample sizes, and references are provided in Supplementary Table 1, Supplementary Material online). In the analyses presented below, I included genes for which 6 or more chromosomes were sampled from the population. I also gathered homologous sequences from D. simulans and D. yakuba when available; when unavailable, I identified homologous sequences using Blast searches against the D. simulans and D. yakuba genome sequences. If multiple D. simulans and D. yakuba sequences were available, one from each species was chosen arbitrarily. To annotate sequences, I used GadFly version 4.3 of the D. melanogaster genome. All sequences were aligned using DIALIGN2 with default parameters (Morgenstern 1999), followed by hand editing. In total, the study surveys 29.6–31.3 kb of intron sequence, depending on species. I also studied polymorphism and divergence data from ~94 kb of coding sequence from regions flanking the introns. The average number of chromosomes sampled from D. melanogaster is 21.5 (median = 20), with samples ranging from \( n = 6–71 \). For some analyses, I focused on chromosomes sampled from African populations of D. melanogaster, where the average number of chromosomes sampled is 13.6 (median = 12) and samples ranging from \( n = 6–25 \). Polymorphism and divergence data for nucleotide changes were tallied using DnaSP v. 4.0 (Rozas et al. 2003).

To investigate the effects of recombination rate on intron evolution, I used Kliman and Hey’s (1993) “KH93” estimator of local recombination rate that, like other genome-wide estimators (Hey and Kliman 2002), is based on the relationship between the genetic and cytological maps of the D. melanogaster genome. The KH93 estimator is based on the fit of 4- and 5-term polynomials of the genetic and physical maps of each chromosome. KH93 is used here because, in a separate study, I found that it explained more variation in silent nucleotide variability for 98 loci scattered throughout the D. melanogaster genome than 5 alternative recombination rate estimators (Presgraves 2005). In the analyses below, I consider 3 recombination rate classes, low (0–1.664 × 10^{-3} rec/bp/gen), medium (1.664–0.327 × 10^{-3} rec/bp/gen), and high (3.328–4.999 × 10^{-8} rec/bp/gen), that span equal ranges of recombination rates in the genome (Hey and Kliman 2002).

Scoring Indels

The common ancestor of D. melanogaster and D. simulans diverged from D. yakuba ~5–12 MYA and subsequently split into D. melanogaster and D. simulans ~2.5–5 MYA (Li et al. 1999; Tamura et al. 2004). I used D. yakuba sequences as outgroups to map mutations onto the branches of the 3-species phylogeny using parsimony (Akashi 1994) and to classify indel events as either insertions or deletions. Only indels that could be unambiguously classified as insertions or deletions were used in the analyses (see fig. 1). Of 1,076 indel events identified, 951 (88.3%) could be unambiguously mapped onto the branches of the phylogeny as either polymorphic in D. melanogaster.
Polymorphisms and Fixations in *Drosophila melanogaster* and *Drosophila simulans* Introns

<table>
<thead>
<tr>
<th>Lineage</th>
<th>Deletions</th>
<th>Insertions</th>
<th>Deletion bias</th>
<th>Noncoding</th>
<th>Synonymous</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Drosophila melanogaster</em>—polymorphic</td>
<td>73</td>
<td>62</td>
<td>1.18</td>
<td>659</td>
<td>639</td>
</tr>
<tr>
<td><em>Drosophila melanogaster</em>—fixed</td>
<td>79</td>
<td>104</td>
<td>0.76</td>
<td>707</td>
<td>657</td>
</tr>
<tr>
<td><em>Drosophila simulans</em>—fixed</td>
<td>99</td>
<td>37</td>
<td>2.68</td>
<td>674</td>
<td>530</td>
</tr>
</tbody>
</table>
having higher probabilities of fixation in the *D. melanogaster* lineage than in the *D. simulans* lineage.

Nonneutral Indel Evolution

The above findings suggest that insertions and deletions may evolve differently. To compare their evolution against a neutral (or nearly neutral) standard, I performed MK tests (McDonald and Kreitman 1991) contrasting the evolution of insertion and deletion mutations with silent (synonymous + intron) point mutations in *D. melanogaster*. The ratio of deletion to silent fixations in *D. melanogaster* does not differ from that for polymorphisms (*G = 0.03, *P* = 0.860; table 1). In contrast, the ratio of insertion to silent fixations is significantly greater than that for polymorphisms (*G = 8.25, *P* = 0.004; table 1). I performed similar contrasts using only synonymous changes as a rough neutral standard because there is increasing evidence that many intron sites are constrained (Andolfatto 2005; Marais et al. 2005) and because there is little evidence for current selection on preferred synonymous codons in the *D. melanogaster* lineage (Akashi 1995; Akashi 1996; McVean and Viera 2001). As above, the ratio of deletions to synonymous mutations fixed does not differ from that for polymorphisms (*G = 0.09, *P* = 0.765; table 1), but the ratio of insertions to synonymous mutations fixed is significantly greater than that for polymorphisms (*G = 8.53, *P* = 0.004; table 1). Similar results hold in contrasts involving deletions and insertions, respectively, with noncoding point mutations in introns (deletions: *G = 0.003, *P* = 0.960; insertions: *G = 7.14, *P* = 0.008; table 1). Insertions in *D. melanogaster* introns thus appear to enjoy greater than neutral probabilities of fixation as if favored by some directional force, such as natural selection or biased gene conversion (Nagylaki 1983).

Indel Evolution at X-linked versus Autosomal Introns

The findings above show that the ratio of deletions to insertions fixed in *D. melanogaster* is lower than that in *D. simulans* (table 1). This species difference holds for the 99 autosomal introns in the data set (*G = 6.53, *P* = 0.011; table 2) but is especially strong for the 49 X-linked introns (*G = 25.61, *P* = 7 × 10^{-7}). Indeed, the ratio of deletions to insertions fixed in *D. melanogaster* is significantly lower for X-linked introns than autosomal ones (*G = 8.42, *P* = 0.004; table 2). There is, however, no such X-autosome difference for indels fixed in the *D. simulans* lineage (*G = 0.09, *P* = 0.761; table 2). These observations show that, in *D. melanogaster*, factors affecting either the mutational process or the substitution process (or both) differ between the X and autosomes.

Importantly, the PDB in *D. melanogaster* does not differ between the X and the autosomes (*G = 1.22, *P* = 0.269; table 2), suggesting that the mutational spectra on the X and the autosomes are not different. Instead, the X and autosomes appear to differ in the probabilities of fixation of either deletions or insertions. There is no significant species difference in the numbers of deletions fixed at X-linked (*χ^2_5 = 3.66, *P* = 0.056; table 2) or autosomal introns (*χ^2_5 = 0.15, *P* = 0.700; table 2). However, *D. melanogaster* and *D. simulans* show strong differences in the numbers of insertions fixed at X-linked introns (*χ^2_5 = 25.33, *P* = 5 × 10^{-7}; table 2) and to a lesser but still significant extent at autosomal ones (*χ^2_5 = 8.47, *P* = 0.004; table 2).

In the *D. melanogaster* lineage, MK tests of insertions versus synonymous mutations show that the fixation probabilities of insertions are significantly elevated for X-linked introns (*G = 8.32, *P* = 0.004; table 2) but not for autosomal ones (*G = 1.35, *P* = 0.245; table 2). MK tests of deletions versus synonymous mutations reveal no evidence of selection on deletions at either X-linked (*G = 0.001, *P* = 0.999; table 2) or autosomal introns (*G = 0.14, *P* = 0.707; table 2). Thus, the elevated probabilities of fixation for insertions in *D. melanogaster* appear to be particularly strong for X-linked introns.

**Table 2**

<table>
<thead>
<tr>
<th>Lineage</th>
<th>X versus A</th>
<th>Deletions</th>
<th>Insertions</th>
<th>Deletion Bias</th>
<th>Noncoding</th>
<th>Synonymous</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Drosophila melanogaster</em>—polymorphic</td>
<td>X</td>
<td>25</td>
<td>27</td>
<td>0.926</td>
<td>216</td>
<td>260</td>
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<tr>
<td></td>
<td>A</td>
<td>48</td>
<td>35</td>
<td>1.371</td>
<td>443</td>
<td>379</td>
</tr>
<tr>
<td><em>Drosophila melanogaster</em>—fixed</td>
<td>X</td>
<td>27</td>
<td>58</td>
<td>0.466</td>
<td>257</td>
<td>278</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>52</td>
<td>46</td>
<td>1.130</td>
<td>450</td>
<td>379</td>
</tr>
<tr>
<td><em>Drosophila simulans</em>—fixed</td>
<td>X</td>
<td>43</td>
<td>15</td>
<td>2.867</td>
<td>233</td>
<td>192</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>56</td>
<td>22</td>
<td>2.545</td>
<td>441</td>
<td>338</td>
</tr>
</tbody>
</table>

Indel Frequencies in African *D. melanogaster* Populations

To study the population frequencies of indels in *D. melanogaster*, I limited the analyses to samples obtained from African populations. This should minimize some of the confounding effects of the recent demographic expansion and heterogeneous sampling schemes of non-African samples (Andolfatto and Przeworski 2001; Glinka et al. 2003; Thornton and Andolfatto 2006). African data were available for 83 introns from 33 genes with at least 6 chromosomes sampled from the population. Because there are too few indel mutations per locus to perform meaningful locus-by-locus analysis, I pooled mutations across loci. Before pooling, all frequencies were rescaled to a common sample size of *n* = 6 (see Materials and Methods). Table 3 shows the frequencies of 5 classes of mutation. There is significant heterogeneity in frequency among classes (Kruskal–Wallis *H* = 18.02, *P* = 0.001), with nonsynonymous mutations segregating at the lowest frequencies, followed by deletion, noncoding and synonymous mutations (table 3). Insertions segregate at significantly higher frequencies than all 4 other classes of mutation (table 3). Two previous surveys of indel frequencies in *D. melanogaster* also found higher population frequencies for
insertions than deletions (Comeron and Kreitman 2000; Ometto et al. 2005).

Comparing the frequency spectra of the different classes of mutation yields similar results. Tajima’s $D_{\text{Taj}}$ and Fu and Li’s $D_{\text{Fu}}$ for deletions are both negative, suggesting an excess of deletions segregating at low frequency (fig. 2 and table 3). In contrast, $D_{\text{Taj}}$ and $D_{\text{Fu}}$ for insertions are both positive, suggesting an excess of insertions segregating at intermediate frequency (fig. 2 and table 3). Although none of the test statistics deviate significantly from the standard neutral model ($P > 0.05$), these tests have little power given small samples like those used here (Simonsen et al. 1995; Akashi 1999). I therefore compared frequency spectra for different mutation classes using the heterogeneity test of Hahn et al. (2002). Here, the relevant contrasts involve comparing the frequency spectra of indel mutations versus putatively neutral mutations (e.g., synonymous mutations). The frequency spectrum of deletions does not differ significantly from those of nonsynonymous, noncoding or synonymous mutations (table 3; $P > 0.05$ for all comparisons). However, the frequency spectrum of insertions is significantly right-shifted with an excess of intermediate frequency variants compared with nonsynonymous mutations (table 3; $P_{\text{Taj}} < 0.001$ for $D_{\text{Taj}}$; $P_{\text{Fu}} < 0.001$ for $D_{\text{Fu}}$), deletions (fig. 2 and table 3; $P_{\text{Taj}} = 0.006$; $P_{\text{Fu}} = 0.010$), noncoding mutations (table 3; $P_{\text{Taj}} = 0.026$; $P_{\text{Fu}} = 0.018$), and synonymous mutations (table 3; $P_{\text{Taj}} = 0.006$; $P_{\text{Fu}} = 0.048$).

Given that there is a significant X-effect on the fixation probabilities of insertions, I performed separate frequency analyses for X-linked and autosomal mutations (table 4). X-linked mutations show significant heterogeneity in frequency among the 5 classes of mutation (Kruskal–Wallis $H = 10.589$, $P = 0.031$), with insertions segregating at significantly higher frequencies than deletion, nonsynonymous, noncoding, and synonymous mutations (Mann–Whitney $P_{\text{MW}} \leq 0.015$ for all contrasts). None of the other classes of mutation differed significantly from one another ($P_{\text{MW}} \geq 0.104$ for all contrasts). These X chromosome findings hold in comparisons of the frequency spectra using heterogeneity tests: insertions have significantly higher $D_{\text{Taj}}$ and $D_{\text{Fu}}$ values than all other classes of mutation ($P \leq 0.046$ for all contrasts), except one ($P_{\text{Taj}} = 0.068$ for the contrast involving insertions and noncoding mutations). Autosomal mutations also show significant heterogeneity in frequency among the 5 mutational classes (Kruskal–Wallis $H = 19.867$, $P = 0.0005$), but the causes differ from those on the X. The only significant differences in frequency among autosomal mutations involve nonsynonymous changes: these segregate at a lower mean frequency than insertions ($P_{\text{MW}} = 0.003$), synonymous mutations ($P_{\text{MW}} < 0.0001$), and noncoding mutations ($P_{\text{MW}} = 0.0004$). Comparing the full frequency spectra of insertions and deletions with the other classes of mutation reveals only one significant contrast: insertions and nonsynonymous mutations have significantly different frequency spectra ($P_{\text{Taj}} = 0.002$ and $P_{\text{Fu}} = 0.006$).

The elevated frequencies found for segregating insertions are consistent with their elevated probabilities of fixation in the D. melanogaster lineage (see above). These data thus further suggest that insertions are currently favored in D. melanogaster, particularly on the X chromosome.

### Indel Lengths

Only nonoverlapping, noncontiguous indel events were used to study indel lengths (see Materials and Methods). Figure 3 shows the distribution of lengths for polymorphic and fixed indels. The distributions are similar to those seen in previous studies of intronic indels (Comeron and Kreitman 2000; Bergman and Kreitman 2001; Parsch 2003; Ometto et al. 2005), with 89.8% of deletions and 92% of insertions being ≤10-bp long. None of the indels corresponds to new transposon insertions or excisions. Table 5 compares the lengths of insertions and deletions. For polymorphisms in D. melanogaster, the sizes of insertions and deletions do not differ (table 5). However, insertion fixations in D. melanogaster are significantly shorter than deletion fixations (table 5). In D. simulans, fixed insertions are also shorter than fixed deletions, although not significantly so (table 5). There are no differences in the lengths of indels.

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**Table 3**

<table>
<thead>
<tr>
<th>Mutation Class</th>
<th>Observed</th>
<th>Mean Frequency (SE)</th>
<th>Tajima’s $D$</th>
<th>Fu and Li’s $D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsynonymous changes</td>
<td>66</td>
<td>1.788 (0.168)$^a$</td>
<td>-0.753</td>
<td>-1.005</td>
</tr>
<tr>
<td>Deletions</td>
<td>21</td>
<td>1.952 (0.305)$^{ab}$</td>
<td>-0.436</td>
<td>-0.546</td>
</tr>
<tr>
<td>Noncoding changes</td>
<td>253</td>
<td>2.221 (0.086)$^b$</td>
<td>-0.028</td>
<td>0.014</td>
</tr>
<tr>
<td>Synonymous changes</td>
<td>272</td>
<td>2.279 (0.084)$^b$</td>
<td>-0.016</td>
<td>0.085</td>
</tr>
<tr>
<td>Insertions</td>
<td>25</td>
<td>2.760 (0.254)$^c$</td>
<td>0.699</td>
<td>0.989</td>
</tr>
</tbody>
</table>

Note.—Classes with different superscripts differ significantly by Mann–Whitney test.

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Fig. 2.—Frequency distributions of insertions and deletions in introns of African populations of *Drosophila melanogaster*.
fixed in *D. melanogaster* and *D. simulans* (insertions: \( P_{MW} = 0.739 \); deletions: \( P_{MW} = 0.132 \)). There are no X–autosome differences in the lengths of polymorphic or fixed indels (\( P_{MW} \geq 0.288 \) for all contrasts).

### Intron Size Evolution

For the 148 intron sequences studied here, those in *D. simulans* are significantly shorter than their homologous sequences in *D. melanogaster* (Wilcoxon signed rank test, \( P = 0.005 \); Comeron and Kreitman 2000; Ometto et al. 2005) and in *D. yakuba* (\( P = 0.05 \)). Intron sequences in *D. melanogaster* do not differ from those in *D. yakuba* (\( P = 0.143 \)). Fourteen of the 148 introns surveyed were not sequenced across their entire lengths. Excluding these and using only the 134 fully sequenced introns yields similar results: intron lengths in *D. simulans* (mean ± standard error [SE] = 191.7 ± 29.3; median = 68.3) are significantly shorter than those in *D. melanogaster* (mean ± SE = 195.8 ± 30.2; median = 70.0; \( P = 0.013 \)) and in *D. yakuba* (mean ± SE = 207.0 ± 31.9; median = 70.5; \( P = 0.023 \)). Intron lengths in *D. melanogaster* do not differ from those in *D. yakuba* (\( P = 0.082 \)).

I tested whether total intron length evolution is at equilibrium by combining information on the numbers and sizes of indels fixed in the *D. melanogaster* and *D. simulans* lineages across introns. (There are too few indel events per intron to perform a meaningful intron-by-intron analysis.) A lineage at equilibrium should show little or no net change in intron length, with the loss of intron sequence by deletions being balanced by the addition of intron sequence by insertions. In the rough calculations that follow, I assume that the lengths of nonoverlapping, noncontiguous indels are representative of all indels. Overall, the history of indel substitutions appears to conform to the equilibrium expectation in *D. melanogaster*, but not in *D. simulans*. In *D. melanogaster*, 79 deletions and 104 insertions have been fixed, eliminating (on average) 5.2 bp and adding 4.0 bp, respectively, thereby causing a net gain of ~6 bp of intron sequence. In *D. simulans*, the analogous calculation reveals a net loss of ~280 bp of intron sequence. However, these naïve calculations ignore the X–autosome differences in indel evolution in *D. melanogaster*. (There is no such X–autosome difference in *D. simulans*; see above.) On the X chromosome, deletions and insertions are 6.3 bp and 3.4 bp, respectively; on the autosomes, deletions and insertions are 4.5 bp and 4.8 bp, respectively. Repeating the above calculations separately for X-linked and autosomal introns shows that X-linked introns have gained ~32 bp and autosomal ones have lost ~13 bp.

In the lineages connecting *D. yakuba* to the common ancestor of *D. melanogaster* and *D. simulans*, 540 indels were fixed in 117 introns from 57 genes. Although these indels cannot be classified as deletions or insertions without

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**Table 4**

Mean Frequency of Mutations Segregating on the X Chromosome and the Autosomes in African Populations of *Drosophila melanogaster* (sample scaled to \( n = 6 \))

<table>
<thead>
<tr>
<th>Location</th>
<th>Mutation Class</th>
<th>Observed</th>
<th>Scaled Mean Frequency (SE)</th>
<th>Tajima’s ( D )</th>
<th>Fu and Li’s ( D )</th>
</tr>
</thead>
<tbody>
<tr>
<td>X chromosome</td>
<td>Nonsynonymous changes</td>
<td>26</td>
<td>1.846 (0.258)</td>
<td>−0.480</td>
<td>−0.579</td>
</tr>
<tr>
<td></td>
<td>Deletions</td>
<td>5</td>
<td>1.200 (0.200)</td>
<td>−0.710</td>
<td>−1.161</td>
</tr>
<tr>
<td></td>
<td>Noncoding changes</td>
<td>93</td>
<td>1.968 (0.117)</td>
<td>0.094</td>
<td>−0.108</td>
</tr>
<tr>
<td></td>
<td>Synonymous changes</td>
<td>153</td>
<td>2.203 (0.111)</td>
<td>−0.069</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Insertions</td>
<td>14</td>
<td>2.714 (0.322)</td>
<td>0.818</td>
<td>1.152</td>
</tr>
<tr>
<td>Autosomes</td>
<td>Nonsynonymous changes</td>
<td>40</td>
<td>1.750 (0.223)</td>
<td>−0.913</td>
<td>−1.232</td>
</tr>
<tr>
<td></td>
<td>Deletions</td>
<td>16</td>
<td>2.188 (0.379)</td>
<td>−0.314</td>
<td>−0.246</td>
</tr>
<tr>
<td></td>
<td>Noncoding changes</td>
<td>160</td>
<td>2.369 (0.116)</td>
<td>−0.099</td>
<td>0.086</td>
</tr>
<tr>
<td></td>
<td>Synonymous changes</td>
<td>119</td>
<td>2.378 (0.130)</td>
<td>0.053</td>
<td>0.192</td>
</tr>
<tr>
<td></td>
<td>Insertions</td>
<td>11</td>
<td>2.818 (0.423)</td>
<td>0.491</td>
<td>0.622</td>
</tr>
</tbody>
</table>

*NOTE.—* Classes with different superscripts differ significantly by Mann–Whitney test.
Drosophila melanogaster and D. simulans. Indels leaving D. yakuba with longer sequences (299) than D. melanogaster and D. simulans have been fixed significantly more often than those leaving D. yakuba with shorter sequences (241; $\chi^2 = 6.23, P = 0.013$). Thus, D. yakuba has either fixed more insertions or fewer deletions than the D. melanogaster–D. simulans common ancestor. Indel events leaving D. yakuba with longer sequences (mean: 10.6 ± 2.10 bp; $n = 280$) do not differ in length from those leaving D. yakuba with shorter sequences (mean: 6.8 ± 0.96 bp; $n = 194$; $P_{MW} = 0.375$; lengths estimated from nonoverlapping, noncontiguous indels). Combining information on the number and mean size of scoreable indel fixations shows that D. yakuba possesses ~521 bp of excess intron sequence relative to the inferred ancestor of D. melanogaster and D. simulans.

Noncoding DNA Sizes in the D. melanogaster Genome

If the indel fixation profile of D. melanogaster is representative of its deeper evolutionary history, then X-linked introns might be expected to be longer than autosomal ones. To test this possibility, I compared the intron lengths of all introns in Release 4.2.1 of the D. melanogaster genome (for loci with alternative transcripts, one was arbitrarily chosen for the analysis). As Figure 4 shows, X-linked introns (mean = 1005 ± 51; median = 83; $n = 5,950$) are significantly longer than autosomal ones (mean = 844 ± 18; median = 70; $n = 33,479$; $P_{MW} < 2.2 \times 10^{-16}$). To test whether the X–autosome difference in intron length extends to other noncoding DNA, I also compared the lengths of intergenic regions between the X and the autosomes of D. melanogaster. X-linked intergenic regions (mean = 5,779 ± 288; median = 1169; $n = 2003$) are significantly longer than autosomal ones (mean = 4,804 ± 111; median = 820; $n = 9,946$; $P_{MW} < 2.8 \times 10^{-11}$), and there is a good correlation among chromosome arms between the lengths of introns and intergenic regions (fig. 5). Interestingly, intron and intergenic region lengths also appear to covary among autosomal arms. These observations suggest that similar chromosome arm–specific forces influence the evolution of length variation in introns and intergenic regions (Comeron and Kreitman 2000; Ometto et al. 2005).

**Discussion**

Evolutionary geneticists have uncovered many differences between the genomes of D. melanogaster and D. simulans that suggest that D. simulans maintains a tidier genome. The D. simulans genome, for instance, features fewer transposable elements (Dowsett and Young 1982; Viera et al. 1999; Boulesteix et al. 2005), fewer polymorphic inversions (Aulard et al. 2004), shorter microsatellite arrays (Amos et al. 2003), less heterochromatin (Lohe and Roberts 1988; Sage and Csink 2003), greater preferred synonymous codon usage (Akashi 1996), lower ratios of replacement to silent polymorphism (Aquadro et al. 1988; Andolfatto 2001), and higher rates of crossing over (Sturtevant 1929). In addition to these, the present findings confirm that D. simulans has smaller introns than D. melanogaster (Comeron and Kreitman 2000; Ometto et al. 2005).

**Table 5**

Mean Length of Insertions and Deletions

<table>
<thead>
<tr>
<th></th>
<th>Deletions</th>
<th>Insertions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>$&lt;10,\text{bp}$</td>
</tr>
<tr>
<td>Drosophila melanogaster—polymorphic</td>
<td>5.81 (1.55, 48)</td>
<td>89.6</td>
</tr>
<tr>
<td>Drosophila melanogaster—fixed</td>
<td>5.23 (0.70, 48)</td>
<td>87.5</td>
</tr>
<tr>
<td>Drosophila simulans—fixed</td>
<td>3.9 (0.49, 71)</td>
<td>91.5</td>
</tr>
</tbody>
</table>

* ($\pm$ SE, n).

**Fig. 4.**—X-linked introns are longer than autosomal ones in Drosophila melanogaster ($\pm$ 1 SE).

**Fig. 5.**—Intron and intergenic region lengths are correlated among chromosome arms in the Drosophila melanogaster genome.
It is tempting to speculate that many of these differences, including in intron length, follow from the inferred difference in species effective population size ($N_e$): because $D. simulans$ has a putatively larger $N_e$, natural selection may be more effective than in $D. melanogaster$, thereby maintaining a more streamlined genome (Aquadro et al. 1988; Akashi 1996).

But at least for intron lengths, this effective size hypothesis cannot explain the data. The species difference in intron lengths is not attributable to differences in indel size (table 5) or to differences in the rate at which deletions are fixed (table 1) but is instead caused by differences in the rate at which insertions are fixed: $D. melanogaster$ has fixed more insertions than $D. simulans$. Although small insertions might increase the cost, or slow the rate, of transcription (Castillo-Davis et al. 2002; Urrutia and Hurst 2003), there is no indication that such putatively deleterious insertions are invisible to natural selection in $D. melanogaster$. Rather, small insertions—particularly those on the X chromosome—appear more likely to be fixed in $D. melanogaster$. It is important to distinguish this finding from previous ones that, using restriction map variability in $D. melanogaster$, detected evidence for selection against insertions [Golding et al. 1986; Aquadro et al. 1988; Tajima 1989]; these earlier studies, having limited indel size resolution [Aquadro et al. 1988], detected only large insertion events probably associated with transposable elements. Similar evidence for erratic, species-specific shifts in indel fixations in noncoding regions has been noted before: Kreitman and Ludwig (1996) found striking evidence for lineage-specific fixations of insertions in the more recent past.

Indel Evolution in $D. melanogaster$ Introns

Two lines of evidence indicate that insertions are favored in the $D. melanogaster$ lineage: 1) MK tests reveal greater than neutral probabilities of fixation for insertions and 2) polymorphic insertion frequencies are significantly elevated relative to other classes of mutation, including putatively neutral ones. The latter finding is in good agreement with those of Ometto et al. (2005), despite our use of different data sets. The elevated population frequencies of insertion mutations suggest that whatever forces drove the excess of insertion fixations in $D. melanogaster$’s deeper past have continued into its more recent past.

For reasons that are not clear, the evidence that insertions are favored in $D. melanogaster$ introns is particularly strong on the X chromosome. In principle, the X could differ from autosomes in mutational spectra, the efficacy of selection and/or rates and biases in gene conversion–double-stranded break repair (Singh et al. 2005). The PDB provides some insight into the relative rates of deletion and insertion mutations: although the PDBs for X-linked (1.371) and autosomal introns (0.926) do not differ significantly, a mutation profile difference cannot be excluded from these limited data. However, even if there is a difference in indel mutation profiles, it cannot explain the significantly different ratios of deletions to insertion fixed at X-linked (0.466) and autosomal (1.130) introns.

Therefore, some force appears to have enhanced the fixation probabilities of insertions on the X chromosome. If that force is natural selection, then the pattern could imply that the beneficial fitness effects of newly arising insertions are on average recessive (Charlesworth et al. 1987).

Biased gene conversion is another force that may favor insertions, leaving patterns indistinguishable from those of weak selection (Nagylaki 1983; Marais 2003; Webster and Smith 2004; Galtier et al. 2006). In $D. melanogaster$, only one experimental study (as far as I am aware) has tested for indel-associated biases in gene conversion. By monitoring the repair of transposon-induced double-strand breaks (DSBs) in exon 6 of the white gene, Johnson-Schlitz and Engels found that sequences with insertions had substantially higher efficiency as templates for DSB repair than those with deletions and, remarkably, those with wild-type sequences: conversion frequencies for insertion, deletion, and wild-type templates were 40.3–52.7%, 6.3–8.5%, and 18.6%, respectively (Johnson-Schlitz and Engels 1993).

In their experiments, then, insertions enjoyed a transmission advantage. Whether their results reflect properties general to DSB repair or specific to repair of transposon-induced breaks is not known. Nevertheless, this experimental work shows the potential for biased repair to act as a directional force influencing the fates of indel mutations and is consistent with the population genetic results presented above.

Why might insertions on the $D. melanogaster$ X chromosome enjoy increased probabilities of fixation compared with those on the autosomes or to those in $D. simulans$? The many autosomal inversions segregating at appreciable frequencies in African populations of $D. melanogaster$, but not $D. simulans$ (Lemeunier and Aulard 1992), could contribute to this species- and chromosome-specific pattern in 2 ways. First, in inversion heterozygotes, rates of crossing over are suppressed within inverted regions; second, the suppression of crossing over on the autosomes by inversions increases rates of crossing over on the X—the so-called interchromosomal effect (Lucchesi 1976). Together these effects lead to a relatively elevated rate of crossing over on the X chromosome (Begun 1996; Andolfatto 2001). If small insertions are for some reason weakly favorable in the $D. melanogaster$ lineage, then the elevated rates of crossing over on the X chromosome could increase the efficacy of natural selection on the X. (For other causes of X–autosome differences in the efficacy of selection, see Charlesworth 2001). Alternatively, if small insertions are weakly favored by a biased gene conversion–gap repair process whose rate is correlated with crossing over, then the elevated rates of crossing over on the X chromosome could enhance transmission of small insertions on the X. The latter scenario is appealing in that it could simultaneously explain the X- and the lineage-specific effects.

Testing Models of Intron Evolution

Carvalho and Clark (1999) and Comeron and Kreitman (2000) both document a negative correlation between intron length and recombination rate in the $D. melanogaster$ genome but arrive at different interpretations for the pattern. Note that recombination rate does not appear to influence the indel mutation profile, as the deletion bias of indel
polymorphisms does not differ among low (1.44), medium (1.47), and high (0.97) recombination environments (see also Comeron and Kreitman 2000; Blumenstiel et al. 2002). Under the first model (Carvalho and Clark 1999), insertions are weakly deleterious and hence more likely to go to fixation in regions of low recombination; however, the findings above show that insertions in *D. melanogaster* behave as moderately favorable, not deleterious. Under the second model (Comeron and Kreitman 2000), insertions are selectively favored as enhancers of recombination, particularly in low recombination regions of the genome; however, insertions in *D. melanogaster* appear to be favored in high not low recombination regions: MK tests for indels versus synonymous mutations show a significant excess of insertion fixations in high, and nearly so in medium, recombination regions (table 6). The elevated probabilities of fixation for insertions in higher recombination environments suggests either that: 1) insertions are specifically favored in these regions but not in low recombination regions; 2) insertions are weakly favored throughout the genome but effectively neutral in low recombination regions; or 3) gene conversion is correlated with rates of crossing over (Marais 2003; but see Langley et al. 2000; Andolfatto and Wall 2003), and gap repair proceeds more efficiently from insertion-bearing templates (Johnson-Schlitz and Engels 1993). Unfortunately, very little experimental information exists concerning the rates and biases of gene conversion processes as a function of rates of crossing over in the *Drosophila* genome. Indeed, recent findings suggest that, at least in *Drosophila*, rates of gene conversion are not correlated with rates of crossing over in the expected way (Langley et al. 2000; Andolfatto and Wall 2003).

Under the third model (Parsch 2003), deletion-biased mutation pressure is balanced by favorable, compensatory insertions that restore optimal intron size (Stephan et al. 1994). Though the findings in *D. melanogaster* are consistent with this model (Ometto et al. 2005), it does not account for the reduction of intron size in *D. simulans*. Indeed, none of the 3 general models of intron evolution easily accounts for the species- and chromosome-specific behavior of insertion mutations seen in this study.

### Table 6

**Recombination Influences Fixation of Indel Mutations in *Drosophila melanogaster***

<table>
<thead>
<tr>
<th>Recombination Environment</th>
<th>Deletions</th>
<th>Insertions</th>
<th>Deletions</th>
<th>Insertions</th>
<th>Fixed</th>
<th>Total</th>
<th>G</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13</td>
<td>9</td>
<td>29</td>
<td>22</td>
<td>73</td>
<td>0.031</td>
<td>0.860</td>
<td></td>
</tr>
<tr>
<td>Medium&lt;sup&gt;c&lt;/sup&gt;</td>
<td>25</td>
<td>17</td>
<td>20</td>
<td>28</td>
<td>90</td>
<td>2.873</td>
<td>0.090</td>
<td></td>
</tr>
<tr>
<td>High&lt;sup&gt;d&lt;/sup&gt;</td>
<td>35</td>
<td>36</td>
<td>30</td>
<td>54</td>
<td>155</td>
<td>2.918</td>
<td>0.088</td>
<td></td>
</tr>
<tr>
<td>Low&lt;sup&gt;e&lt;/sup&gt;</td>
<td>13</td>
<td>101</td>
<td>29</td>
<td>200</td>
<td>343</td>
<td>0.114</td>
<td>0.736</td>
<td></td>
</tr>
<tr>
<td>Medium&lt;sup&gt;f&lt;/sup&gt;</td>
<td>25</td>
<td>263</td>
<td>20</td>
<td>234</td>
<td>542</td>
<td>0.116</td>
<td>0.734</td>
<td></td>
</tr>
<tr>
<td>High&lt;sup&gt;g&lt;/sup&gt;</td>
<td>35</td>
<td>275</td>
<td>30</td>
<td>223</td>
<td>563</td>
<td>0.044</td>
<td>0.834</td>
<td></td>
</tr>
<tr>
<td>Low&lt;sup&gt;h&lt;/sup&gt;</td>
<td>9</td>
<td>101</td>
<td>22</td>
<td>200</td>
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<tr>
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<td>17</td>
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<td>28</td>
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<td>542</td>
<td>3.812</td>
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</tr>
<tr>
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<td>36</td>
<td>275</td>
<td>54</td>
<td>223</td>
<td>588</td>
<td>7.097</td>
<td>0.008</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Genes binned into 3 groups spanning equal ranges of recombination rates; see Materials and Methods.

<sup>b</sup> In low recombination regions, indels occurred in 17/20 genes and in 21/45 introns.

<sup>c</sup> In medium recombination regions, indels occurred in 21/23 genes and in 36/67 introns.

<sup>d</sup> In high recombination regions, indels occurred in 19/25 genes and in 28/41 introns.

### Summary and Conclusions

There is a remarkable convergence among several disparate observations in *D. melanogaster*: the population genetic data show that X-linked insertions are favored (they segregate at elevated frequencies and enjoy elevated probabilities of fixation); experimental data show that DSB repair at a X-linked locus is biased in favor of insertions (Johnson-Schlitz and Engels 1993); and genomic data show that X-linked introns and intergenic regions are longer than autosomal ones. Intron lengths in other taxa also show X chromosome effects. In *Caeonorhabditis elegans*, a “positive” correlation between intron length and recombination rate has been documented for all chromosomes except the X (Prachumwat et al. 2004). In humans, a comparison of paralogous intron pairs found that X-linked introns were longer than autosomal ones (Cardazzo et al. 2003). These results, like several other recent findings (e.g., Kern and Begun 2005; Singh et al. 2005), highlight the need for a better understanding of lineage- and chromosome-specific mutation and repair biases influencing patterns of genome evolution.

### Supplementary Material

Supplementary Table 1 is available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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