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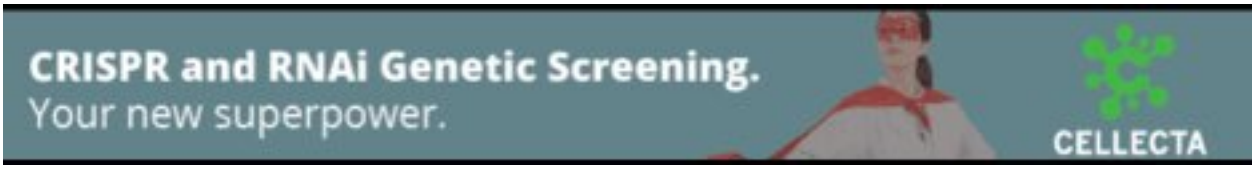

Letter

X chromosomes and autosomes evolve at similar rates in *Drosophila*: No evidence for faster-X protein evolution

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Recent data from *Drosophila* suggest that a substantial fraction of amino acid substitutions observed between species are beneficial. If these beneficial mutations are on average partially recessive, then the rate of protein evolution is predicted to be faster for X-linked genes compared to autosomal genes (the "faster-X" hypothesis). We test this prediction by comparing rates of protein substitutions between orthologous genes, taking advantage of variations in chromosome fusions within the genus *Drosophila*. In members of the *Drosophila melanogaster* species group, the chromosomal arm 3L segregates as an ordinary autosome (i.e., two homologous copies in both males and females). However, in the *Drosophila pseudoobscura* species group, this chromosomal arm has become fused to the ancestral X chromosome and is hemizygous in males. The faster-X hypothesis predicts that protein evolution should be faster for genes on this chromosomal arm in the *D. pseudoobscura* lineage, relative to the *D. melanogaster* lineage. Here we combine new sequence data for 202 gene fragments in *Drosophila miranda* (in the *pseudoobscura* species group) with the completed genomes of *D. melanogaster*, *D. pseudoobscura*, and *Drosophila yakuba* to show that there are no detectable differences in rates of amino acid evolution for orthologous X-linked and autosomal genes. Our results imply that the contribution of the faster-X (if any) to the large-X effect on reproductive isolation in *Drosophila* is not due to a generally faster rate of protein evolution. The lack of a detectable faster-X effect in these species suggests either that beneficial amino acids are not partially recessive on average, or that adaptive evolution does not often use newly arising amino acid mutations.

[The *Drosophila* three and four species alignments are available at http://www.molpopgen.org/krthornt/Data/FastXGR. The sequence data from this study have been submitted to GenBank under accession nos. DQ334053–DQ33425.]

While beneficial mutations are the raw material for adaptive evolution by natural selection, little is known about their nature. For example, it is unclear if most adaptation draws primarily on newly arising mutations (e.g., Braverman et al. 1995), on previously neutral polymorphisms (Dykhuizen and Hartl 1980; Innan and Kim 2004), or on a pool of deleterious polymorphisms in mutation–selection balance (Orr and Betancourt 2001). The intensity of selection felt by a new mutation is scaled by its degree of dominance, h. It is also unknown whether beneficial alleles have on average partially recessive effects on fitness (i.e., their dominance coefficient $h < 0.5$) or are partially dominant ($h > 0.5$) and thus more readily visible to selection when heterozygous. In heterogametic sexual species, genes on the X chromosome generally lack a homologous copy in males (i.e., they are hemizygous). If the average intensity of selection on beneficial mutations is the same on the X chromosome and autosomes, Charlesworth et al. (1987) showed that newly arising partially recessive beneficial mutations have a higher probability of fixation if arising on the X chromosome. Therefore, if mutation rates to beneficial alleles are equal, the rate of adaptive evolution will be higher under these conditions for genes on the X chromosome than for autosomal loci. This predicted phenomenon has been called the "faster-X" effect.

The faster-X theory has been proposed as a possible explanation for several biological phenomena. Among these is the observation in *Drosophila*, and other taxa, that the X chromosome harbors a disproportionately large number of loci causing reproductive isolation between closely related species (called the "large X effect") (for review, see Coyne and Orr 2004). While the weight of current evidence appears to favor the dominance theory (Turelli and Orr 1995) for the large-X effect, faster-X evolution remains a possible, and not mutually exclusive, factor contributing to the large-X effect (Charlesworth et al. 1987; Naveira 2003; Tao and Hartl 2003; Coyne et al. 2004). Recent studies have found that a substantial fraction of amino acid divergence between members of the *Drosophila melanogaster* species group is driven by positive selection. This adaptive amino acid evolution seems to be a general property of most genes (Fay et al. 2002; Smith and Eyre-Walker 2002; Bierne and Eyre-Walker 2004; Sawyer et al. 2003; Andolfatto 2005), as well as specific genes underlying reproductive isolation (Ting et al. 1998; Presgraves et al. 2003; Barbash et al. 2004). This suggests that a major component of the faster-X effect on reproductive isolation could be a byprod-

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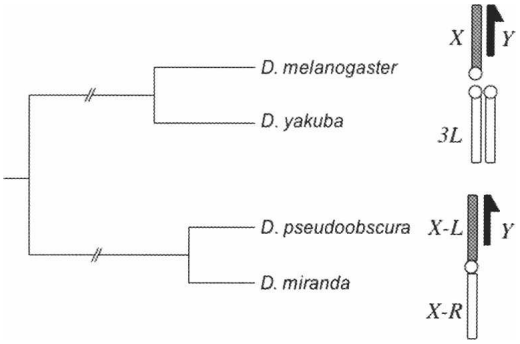

Figure 1. Karyotypes of the *D. melanogaster* and *obscura* species groups. In the *obscura* group, which includes *D. pseudoobscura* and *D. miranda*, a translocation of Muller's element D (white) to the X chromosome (gray) results in a karyotype where autosomal loci in the *melanogaster* group are X-linked in the *obscura* group. This new X chromosome was created ~13 Mya. In this figure, the neo-sex chromosomes in *D. miranda* (which result from rearrangements between the Y chromosome and an autosome) are not shown for simplicity.

uct of an overall faster rate of adaptive protein evolution for genes on the X chromosome. In addition, the X chromosomes of *Drosophila simulans* (Begun and Whitley 2000) and humans (Payseur et al. 2002), as well as the Z chromosome of chickens (Sundström et al. 2004), harbor reduced levels of nucleotide variability compared to expectations based on autosomal genes. These results have been interpreted as evidence for a faster rate of adaptive evolution on the X (or Z) chromosomes. The rationale behind this is that the more frequent fixation of partially recessive advantageous mutations on X (and the shorter time to fixation) will result in a greater reduction in variability at linked neutral sites (i.e., the hitchhiking effect) relative to autosomes.

Two recent studies in *Drosophila* tested for faster protein evolution on X, using DNA divergence data (Betancourt et al. 2002; Counterman et al. 2004). The rationale of these studies is that, if a substantial fraction of amino acid substitutions are beneficial (as mentioned above) and are on average partially recessive, then the rate of protein evolution should be faster for X-linked genes compared to autosomal ones. The rate of protein evolution is usually measured by the rate of amino acid substitutions compared to synonymous substitutions (d_N/d_S), to account for differences in mutation rates among loci. Betancourt et al. (2002) used DNA divergence data between *Drosophila melanogaster* and *Drosophila simulans* for a total of 254 published sequences and did not find a significant difference in rates of protein evolution between X-linked and autosomal genes. However, this test may lack power since genes can vary widely in their level of functional constraint (and therefore in d_N/d_S). Thus, even if a particular gene is more likely to evolve faster when X-linked, the average rate of protein evolution of X-linked genes might not statistically exceed the rate of autosomal ones.

Counterman et al. (2004) proposed a more robust test for faster-X evolution, by comparing rates of protein evolution between orthologous genes that vary in whether they are autosomal or X-linked, taking advantage of chromosome fusions in the genus *Drosophila* (Muller 1940). In members of the *D. melanogaster* species group, the chromosomal arm 3L (Muller's element D) is segregating as an ordinary autosome (i.e., two homologous copies in both males and females). In the *Drosophila pseudoobscura* species group, however, this chromosomal arm has become fused to the ancestral X chromosome (Muller's element A) and is hemizygous in males (see Fig. 1). This test therefore allows a comparison of rates of divergence at orthologous loci that are X-linked in one species group and autosomal in another. Counterman et al. (2004) compared a relatively small number of gene fragments (between 25 and 110) in three or four species of these two groups to test for faster-X evolution and found a marginally significant faster-X effect in one comparison but not the other. Here, we follow a similar approach to test whether the X chromosome shows a higher rate of protein evolution by taking advantage of the X/autosome fusion in two members of the *D. pseudoobscura* species group relative to the *D. melanogaster* species group. We significantly increase the number of genes analyzed by using the genome sequences of *D. melanogaster*, *Drosophila yakuba*, and *D. pseudoobscura* (comparing a total of 2646 genes) and obtaining new sequence information for >200 gene fragments from *Drosophila miranda*.

Results

In *Drosophila* species, chromosome arms are sometimes referred to as Muller elements. In *D. melanogaster*, this labeling begins with the X chromosome (Muller element A), and continues through arms 2L, 2R, and so on. In this study, we focus on elements A and D. In the *D. melanogaster* karyotype, element D is the left arm of the (autosomal) third chromosome, 3L. In *D. pseudoobscura* and *D. miranda*, element D has fused to the ancestral X chromosome (element A) and has thus become the right arm of the X chromosome (chromosome XR) (see Fig. 1.) In this paper, we use the notation X/XL to refer to element A, and 3L/XR to refer to element D, making it clear which element has remained X-linked in all species studied (element A = X/XL), and which has been translocated and only is X-linked in the *pseudoobscura* group (element D = 3L/XR).

Whole chromosome analysis (three-species comparison)

If proteins evolve faster when X-linked, genes located on 3L/XR should show a higher rate of protein evolution (d_N/d_S) in the *D.*

Table 1. Test for faster-X evolution using whole chromosome analysis

A.	*D. pseudoobscura–D. melanogaster* d_N/d_S higher	*D. melanogaster – D. yakuba* d_N/d_S higher (or equal)	
3L/XR	483	1076	1559
X/XL	318	769	1087
			2646
			$P = 0.34$

B.	*D. pseudoobscura* d_N/d_S higher	*D. melanogaster–D. yakuba* d_N/d_S higher (or equal)	
3L/XR	589	970	1559
X/XL	398	689	1087
			2646
			$P = 0.57$

Each cell count gives the number of cases in which d_N/d_S was higher in comparisons between (A) *D. pseudoobscura–D. melanogaster* vs. *D. melanogaster–D. yakuba* and (B) between the *D. pseudoobscura* lineage and the *D. melanogaster–D. yakuba* lineage. *P*-values were obtained by a Fisher's exact test.

Table 2. Test for faster-X evolution using the four-species comparison

	D. pseudoobscura–D. miranda d_N/d_S higher	*D. melanogaster – D. yakuba* d_N/d_S higher (or equal)	
3L/XR	58	46	104
X/XL	57	41	98
			202
			$P = 0.78$

Each cell count gives the number of cases in which d_N/d_S was higher in comparisons between *D. pseudoobscura–D. miranda* vs. *D. melanogaster–D. yakuba*. *P*-values are obtained by a Fisher's exact test.

pseudooboscura lineage, where this element is X-linked compared to the *D. melanogaster/D. yakuba* lineage, where it is autosomal (see Fig. 1). We used the genome sequences of these three species to test for faster-X evolution using orthologous gene comparisons similar to the three-species test performed by Counterman et al. (2004). To account for lineage-specific effects (e.g., a generally lower rate of amino acid or synonymous evolution in one lineage), it is necessary to contrast d_N/d_S of loci from 3L/XR with genes from another chromosome. Following Counterman et al. (2004), we chose chromosome X/XL for this purpose, whose genes are X-linked in all possible comparisons. We annotated 1087 genes on X/XL and 1559 genes on 3L/XR in the *D. yakuba* genome. Table 1A presents a contingency table comparing d_N/d_S between *D. pseudoobscura* and *D. melanogaster* to d_N/d_S between *D. melanogaster* and *D. yakuba*. The rationale of this test is that, if there is a general faster-X effect on amino acid substitutions, then d_N/d_S will be systematically higher between *D. melanogaster/D. pseudoobscura* than between *D. melanogaster/D. yakuba* for loci on 3L/XR compared to loci on X/XL. Table 1A shows that this is not the case ($P = 0.44$, Fisher's exact test), suggesting that X-linkage does not generally increase the rate of protein evolution.

However, the above test of the faster-X hypothesis could lack statistical power for several reasons. First, the rate of synonymous substitutions d_S is saturated between *D. melanogaster* and *D. pseudoobscura* (Bergman et al. 2002; Richards et al. 2005). This means that there is little information about the true value of d_N/d_S between the two species, and these estimates have wide confidence intervals. Secondly, substitutions on the lineage leading to *D. melanogaster* are counted twice, once in the comparison with *D. pseudoobscura*, and again in the comparison to *D. yakuba* (Fig. 1). The problem of nonindependence can be addressed by directly estimating d_N and d_S per branch on the three-species phylogeny. In Table 1B, we compare the d_N/d_S on the branch leading to *D. pseudoobscura* to the sum of d_N/d_S on the branches leading to *D. melanogaster* and *D. yakuba* for both X/XL and 3L/XR. Again, there is no detectable acceleration of d_N/d_S on the lineage leading to *D. pseudoobscura* for genes on 3L/XR compared to genes on X/XL (Table 1B) ($P = 0.56$, Fisher's exact test). A third factor contributing to a lack of power is the fact that chromosome 3R has been fused to the X chromosome in *D. pseudoobscura* for a relatively short time period (~13 million years [Myr]) compared to the total tree length (50–110 Myr) (Richards et al. 2005). Thus, the "background noise" of amino acid substitutions accumulating on this chromosome before it became an X chromosome might be too large to detect the faster-X effect using this type of comparison.

Paired comparisons between four species

Given the large amount of "background noise" expected in the three-species comparisons, a more robust test of the faster-X hypothesis is to compare rates of substitution among orthologous loci that have historically always been X-linked in one comparison and autosomal in another (see Counterman et al. 2004). In *D. pseudoobscura* and its close relative *D. miranda*, the X chromosome is metacentric with two large, euchromatic arms (labeled XL and XR). In *D. melanogaster* and *D. yakuba*, the X is acrocentric and homologous to XL, and the autosomal arm 3L is homologous to the right arm of the *pseudoobscura/miranda* X. We sequenced and annotated 98 loci on X/XL, and 104 genes on 3L/XR in *D. miranda*, to test whether X-linked genes show a higher rate of protein evolution compared to autosomal genes. Table 2 summarizes the results of the paired comparison of the d_N/d_S analysis as a contingency table. The fraction of comparisons where the rate of protein evolution d_N/d_S was greater in *D. pseudoobscura–D. miranda* than in *D. yakuba–D. melanogaster* was 0.58 for X/XL and 0.55 for 3L/XR. This difference is not statistically significant, using a Fisher's exact test ($P = 0.78$) (see Table 2). The distributions of the d_N/d_S ratios are shown in Figure 2. Kolmogorov-Smirnov tests reveal no differences in the distributions, either between species pairs or between chromosome arms.

Integration of divergence and expression data

The analyses presented above are only concerned with differences in rates of divergence in X-linked versus autosomal genes, and suggest no difference between the two. However, such large-scale analyses may obscure faster-X evolution that may be restricted to certain classes of genes, such as X-linked loci that are male biased in expression, which are known to evolve at higher rates than female-biased or non-sex-biased genes (Zhang et al. 2004). Since a faster-X would be driven by selection in males (in the XY sex chromosome system), it follows that genes expressed specifically in males may be preferred targets for male-specific adaptive protein evolution on the X. Of the 2646 genes that we annotated on X and 3L in *D. yakuba*, we identified 436 as significantly sex biased in expression, using gene expression data from

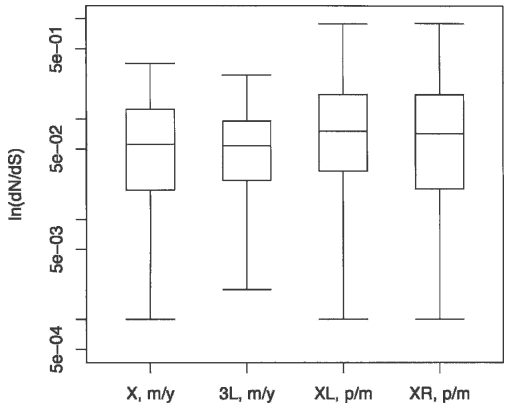

Figure 2. Summaries of the distribution of d_N/d_S ratios for the 202 loci analyzed in the four-species comparison. Rectangles surround the quantiles of the distribution (25-th, 50-th, and 75-th percentiles), with lines extending out to the 2.5-th and 97.5-th percentiles. (m/y) d_N/d_S between *D. melanogaster* and *D. yakuba*; (p/m) d_N/d_S between *D. pseudoobscura* and *D. miranda*.

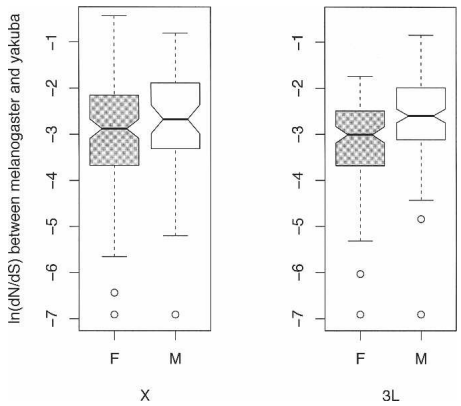

Figure 3. Boxplot of divergence between *D. melanogaster* and *D. yakuba* for genes that are significantly sex-biased in expression. The boxplots show the quartiles of the distribution of d_N/d_S, with a notch at the median value and whiskers extending to three times the interquartile distance. (M) Significantly male-biased; (F) significantly female-biased; (X) X-linked loci; (3L) loci on chromosome arm 3L.

Parisi et al. (2003). On the X, there were 52 male-biased and 132 female-biased genes. On 3L, there were 142 male-biased and 110 female-biased genes. Repeating the three-species comparison using only male- or female-biased genes does not change any of our conclusions. That is, we find no evidence for a faster-X effect for either class of genes (data not shown). Interestingly, however, male-biased genes always appear to evolve faster than female-biased genes. Figure 3 shows boxplots of d_N/d_S between *D. melanogaster* and *D. yakuba* for male- and female-biased genes on chromosome arms X and 3L. On the X chromosome, there is no significant difference in rates of evolution between male- and female-biased genes (pairwise Wilcoxon rank sum test, $P = 0.17$). However, male-biased genes on the autosomes evolve faster than female-biased, autosomal genes (pairwise Wilcoxon rank sum test, $P = 5e-06$). Despite this interesting difference, sex linkage has no effect on the rates of evolution of male-biased genes in these data (pairwise Wilcoxon rank sum test, $P = 0.79$). Significantly faster evolution of male-biased genes on the autosomes, if anything, contradicts the predictions of the faster-X model.

Discussion

If beneficial amino acid substitutions are partially recessive on average and if selection acts primarily on newly arising mutations, then the rate of protein evolution should be higher for an X-linked gene compared to an autosomal one (Charlesworth et al. 1987). Here, we find no evidence that orthologous loci evolve faster when X-linked than when autosomal (Tables 1 and 2). This result may reflect on properties of beneficial mutations and adaptive evolution. First, adaptive protein evolution might not be common enough to be detectable using genomic data. If only a very small fraction of all amino acid changes observed between species is driven by positive selection, than the background noise introduced by neutral amino acid substitutions accumulating uniformly on different chromosomes might overshadow any possible faster-X effect. This scenario, however, is not supported by recent analysis from *Drosophila* (Bustamante et al. 2002; Fay et al. 2002; Smith and Eyre-Walker 2002; Sawyer et al. 2003; Bierne and Eyre-Walker 2004; Andolfatto 2005). By contrasting the amount of amino acid polymorphism to amino acid divergence, various studies have concluded that more than one-third (and up to 90%) of amino acid differences observed between species were, in fact, driven to fixation by positive selection between species of the *D. melanogaster* species group. Thus, adaptive protein evolution appears to be common enough to be detectable in genome-wide scans.

Secondly, adaptation may not often use newly arising mutations, but instead may act on standing genetic variation. The alleles selected may have been previously neutral (Dykhuizen and Hartl 1980; Innan and Kim 2004) or previously deleterious and maintained at mutation–selection equilibrium. For such alleles, Orr and Betancourt (2001) showed that evolution from mutation–selection balance always proceeds more slowly at X-linked than autosomal genes, independent of how dominant a mutation is. Thus, if adaptive evolution primarily occurs from amino acids that were previously deleterious and segregating at mutation–selection balance, the rate of protein evolution should actually be lower on the X chromosome compared to autosomes. Finally, beneficial amino acid mutations might not be recessive on average, but their average effect may be close to additive. In that case, no faster-X effect would be expected (Charlesworth et al. 1987).

Differences in mutation rates between the X chromosome and autosomes affect the ability to detect a faster-X effect. In mammals, for example, the rate of substitution at nonsynonymous, synonymous, and non-coding sites is lower on X than on the autosomes (McVean and Hurst 1997; Malcolm et al. 2003; Lu and Wu 2005), but whether a faster-X effect is observed depends on whether synonymous or non-coding sites are used as the neutral benchmark (Lu and Wu 2005). In contrast to mammals, studies of different *Drosophila* species pairs suggest that there is no evidence that substitution rates differ significantly between the X chromosome and autosomes (Bauer and Aquadro 1997; Betancourt et al. 2002; Bartolome et al. 2005) (see comparison between *D. melanogaster* and *D. yakuba* in Table 3). Therefore, a difference in substitution rates among chromosomes is unlikely to account for the lack of a faster-X effect in our data. In *Drosophila* species, the X chromosome shows a higher degree of codon usage bias than the autosomes (Comeron et al. 1999; Singh et al. 2005), but codon bias does not appear to correlate with estimates of d_S for

Table 3. Means and 95% CI (in parentheses) of point estimates for d_N, d_S, and d_N/d_S in the four-species comparison

	X/XL		3L/XR	
	mel/yak	mir/pse	mel/yak	mir/pse
Mean d_N	0.032 (3e-04–0.14)	0.007 (0–0.035)	0.025 (3e-04–0.108)	0.006 (0–0.027)
Mean d_S	0.348 (0.206–0.588)	0.058 (0.013–0.106)	0.314 (0.189–0.507)	0.048 (0.018–0.112)
Mean d_N/d_S	0.090 (0.001–0.360)	0.166 (0.001–0.895)	0.080 (0.002–0.274)	0.140 (0.001–0.902)

the maximum likelihood method used here (Dunn et al. 2001). However, it is unlikely that differences in codon bias have had a significant impact on our analysis. First, the lack of evidence for differences in substitution rates between the X chromosome and autosomes suggests that the higher codon bias on X is not having a significant effect on long-term rates of divergence. Second, if higher codon bias leads to decreased rates of synonymous substitution (lower d_S), then our analysis would have been biased toward finding a faster-X effect, because the reduction in d_S inflates estimates of d_N/d_S, as is observed in mammals (Lu and Wu 2005).

The faster-X hypothesis was originally proposed to explain the disproportionately large effects of loci on the X chromosome to species differences. Such large X effects have been reported in interspecific crosses in studies of mating behavior, morphology, and hybrid sterility (Coyne and Orr 2004), suggesting a special role for X in species differentiation. Another evolutionary phenomenon connected with the sex chromosomes is Haldane's rule (Haldane 1922), which states that it is nearly always the heterogametic sex that is inviable or infertile in interspecific hybrids. While the favored explanation of Haldane's rule is based on the recessivity of genetic incompatibilities (Turelli and Orr 1995; Coyne and Orr 2004), the faster-X effect has been proposed as an additional contributing factor (Charlesworth et al. 1987; Naveira 2003; Tao and Hartl 2003; Coyne et al. 2004). The present analysis provides strong evidence that rates of adaptive evolution, as measured by d_N/d_S, do not differ substantially between X-linked and autosomal loci. This implies that the disproportionately large contribution of the X chromosome to species differences and Haldane's rule is not adequately explained by the faster-X model (in the context of protein evolution).

In addition, several recent polymorphism studies in *Drosophila* (Begun and Whitley 2000; Andolfatto 2001), chickens (Sundström et al. 2004), and humans (Payseur et al. 2002) have found reduced levels of polymorphism on X (or Z) chromosomes relative to autosomes. This finding has often been interpreted as evidence for a higher rate of adaptive evolution on the X chromosome relative to the autosomes due to recessive beneficial mutations, wiping out linked variability more frequently on X (i.e., greater genetic hitchhiking on X). Again, the lack of evidence of a general faster-X effect for amino acid substitutions, at least in *Drosophila*, is inconsistent with this interpretation. Indeed, alternative explanations such as demographic effects (Wall et al. 2002) or other confounding factors such as chromosomal inversions and different effective population sizes in males and females (Andolfatto 2001; Charlesworth 2001) have been suggested as alternative explanations for patterns of variation on X versus autosomes, at least in *Drosophila*.

Methods

Primer design

Primers were designed using the annotation from the "freeze1" assembly of the *D. pseudoobscura* genome project (Richards et al. 2005). Loci were selected on the basis of having at least one exon of >1000 bp in length. Based on the exon annotations (B. Bettencourt, pers. comm.), we designed PCR and internal sequencing primers by an automated procedure using the primer3 software (Rozen and Skaletsky 2000) for every annotated exon fitting our length criterion. We chose 120 loci from both the XL and XR arms of *D. pseudoobscura* for sequencing in *D. miranda* without regard for any feature other than exon length (i.e., functional annotations, gene names, etc., were ignored).

PCR and sequencing

Genomic DNA was extracted from a single male of *Drosophila miranda* (line MSH22) using the Puregene DNA extraction Kit (Gentra). PCR reactions were performed in 50-µL volumes, purified using Exo-SAP treatment, and sequenced using ABI BigDye version 3 (Perkin Elmer). The phred/phrap/consed (Ewing and Green 1998; Ewing et al. 1998; Gordon et al. 1998) software suite was used to automate the base-calling and assembly, leading to a preliminary assembly of the data. We used BLASTN (Altschul et al. 1990) against our *D. pseudoobscura* coding sequence (CDS) database, to confirm that we had sequenced what we expected, and used the BLAST alignments as guides for manually confirming every base call in *D. miranda* that differed from *D. pseudoobscura*. Low-quality regions were trimmed manually. Sequences have been deposited in GenBank under accession nos. DQ334053–DQ33425.

Alignment, annotation, and analysis for paired comparisons

We annotated the coding regions of the finished contigs using the GeneWise tool (http://www.ebi.ac.uk/Wise2/), which is a comparative annotation program on which many of the *D. pseudoobscura* gene models are based. We used the peptide sequence from the *D. pseudoobscura* sequence to annotate the *D. miranda* coding sequence.

We obtained scaffolds from the April 2004 freeze of the *D. yakuba* genome sequencing project from the Washington University Genome Sequencing Center (http://genome.wustl.edu/projects/yakuba; R. Wilson pers. comm.). First, we generated *melanogaster/miranda/pseudoobscura* alignments using DIALIGN 2.2 (Morgenstern 1999), with the option to align based on CDS translations. We used the *D. melanogaster* CDS sequences (Adams et al. 2000) that were used to annotate the *D. pseudoobscura* genome (B. Bettencourt, pers. comm.). These alignments were manually edited and trimmed in order to find the portion of the *melanogaster* CDS homologous to the region sequenced in *miranda*. The resulting homologous fragments from *melanogaster* were used to search the *D. yakuba* contigs using BLASTN. While translated BLAST programs (i.e., TBLASTX) would be preferable, these species are closely related at the sequence level (Takano 1998), and obtaining nucleotide alignments directly from BLAST was useful for our purposes.

For both *miranda/pseudoobscura* and *melanogaster/yakuba* comparisons, final alignments were made using peptide alignments generated with DIALIGN 2.2, and the CDS sequences were aligned with the tranalign tool in the EMBOSS software suite (http://www.emboss.org), which uses the peptide alignments as guides.

Whole chromosome analysis

Using CDS sequences for *D. melanogaster* loci for which gene models exist in the annotation of the "freeze1" assembly of the *D. pseudoobscura* genome (Richards et al. 2005), we performed BLASTN searches against the *D. yakuba* contigs. Using the BLASTN results for all *D. melanogaster* CDS sequences mapping to X/XL and 3L/XR, we extracted the region of the best BLASTN hit in *D. yakuba* plus 6000 bp on either side. The *D. melanogaster* peptide was then used to predict the gene structure in *D. yakuba* using the GeneWise tool. We were able to obtain 1087 loci on X/XL and 1559 on 3L/XR for which we were able to align *D. melanogaster, D. yakuba,* and *D. pseudoobscura*. The three-species

alignments were performed on the peptide alignments using DIALIGN and tranalign, as described above.

Estimation of divergence parameters

Estimates of the number of amino acid differences per site (d_N) and the number of synonymous differences per site (d_S) were calculated using the likelihood methods implemented in the PAML software package (Yang 1997). Estimates based on the three-species phylogeny were performed using codeml, allowing d_N/d_S to vary across branches (i.e., each lineage can have its own d_N/d_S value). For these analyses, it is important that we are comparing orthologous sequences rather than paralogs (e.g., gene duplications). In *Drosophila*, 90% of *D. melanogaster* gene models were successfully assigned orthologs in *D. pseudoobscura* (Richards et al. 2005). As our identification of genes in *D. yakuba* was based on the set of *melanogaster/pseudoobscura* orthologs, we have high confidence that we are, indeed, comparing orthologs. It is formally possible that some of the genes sequenced from *D. miranda* are recent duplications in that species, rather than true orthologs. However, recent duplications (as measured by d_S) appear to be relatively rare in *Drosophila* species (e.g., Thornton and Long 2002). Furthermore, we observed no "heterozygous" base calls in our sequencing traces, which we would have expected to find if our PCR had amplified closely related sequences.

Expression data

Data of sex-biased gene expression were taken from the Supplemental information of Parisi et al. (2003). We used the GFF annotation files from Releases 3 and 4 of the *D. melanogaster* genome (http://www.flybase.org) in order to map the gene names and synonyms from Parisi et al. to the "CG" numbers that we used as locus labels in this study. We were able to map 7309 of the genes studied in Parisi et al. to CG numbers. We used the same measure of significant sex-biased expression as Parisi et al. to identify sex-biased genes on chromosomes X/XL and 3L/XR.

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