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A Selective Sweep Associated With a Recent Gene Transposition in *Drosophila miranda*

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ABSTRACT

In *Drosophila miranda*, a chromosome fusion between the *Y* chromosome and the autosome corresponding to Muller's element *C* has created a new sex chromosome system. The chromosome attached to the ancestral *Y* chromosome is transmitted paternally and hence is not exposed to crossing over. This chromosome, conventionally called the neo-*Y*, and the homologous neo-*X* chromosome display many properties of evolving sex chromosomes. We report here the transposition of the *exuperantia1* (*exu1*) locus from a neo-sex chromosome to the ancestral *X* chromosome of *D. miranda*. *Exu1* is known to have several critical developmental functions, including a male-specific role in spermatogenesis. The ancestral location of *exu1* is conserved in the sibling species of *D. miranda*, as well as in a more distantly related species. The transposition of *exu1* can be interpreted as an adaptive fixation, driven by a selective advantage conferred by its effect on dosage compensation. This explanation is supported by the pattern of within-species sequence variation at *exu1* and the nearby *exu2* locus. The implications of this phenomenon for genome evolution are discussed.

THE evolution of Drosophila karyotypes exhibits a strong conservation of the gene content of chromosomal arms, which are conventionally referred to as "elements" (Muller 1940). In contrast, gene order varies considerably within each element, mostly as a result of paracentric inversions (Patterson and Stone 1952; Stone *et al.* 1960; Powell 1997). Pairs of elements can, however, fuse with each other, creating new karyotypes in different species. When one of the elements in a fusion is a sex chromosome, a new set of sex chromosomes develops, due to physical association of an autosomal element with the original *Y* or *X* chromosome (Patterson and Stone 1952; Powell 1997). The new pairs of sex chromosomes created in this way are often referred to as "neo-sex chromosomes" (Ashburner 1989). Neo-sex chromosomes show different levels of differentiation into sex chromosomes, according to their relative ages (Ashburner 1989; pp. 327–330 in Powell 1997).

An illuminating example of a neo-sex chromosome system is provided by *Drosophila miranda*. In this species, element *C* has fused with the *Y* chromosome, creating a neo-*Y* chromosome; its free homolog constitutes the X_2 or neo-*X* chromosome (Macknight 1939; Steinemann 1982a). The true *X* chromosome of *D. miranda* is homologous to that of *D. pseudoobscura*. This consists of an *X*L arm homologous to element *A*, the ancestral *X* chromosome of Drosophila, and an *X*R arm homologous to element *D* (Ashburner 1989). Consistent with their relatively recent origin ~1–2 million years ago (Wang and Hey 1996; Barrio and Ayala 1997; Bachtrog and Charlesworth 2000; Yi and Charlesworth 2000), the neo-sex chromosomes of *D. miranda* show characteristics of sex chromosomes in an intermediate stage of evolution. Specifically, the neo-*X* chromosome of *D. miranda* has evolved a partial usage of the dosage compensation machinery of the ancient *X* chromosome (Bone and Kuroda 1996; Marín *et al.* 1996; Steinemann *et al.* 1996), while the neo-*Y* chromosome shows a considerable amount of degeneration at both the cytological and genetic levels (Macknight 1939; Das *et al.* 1982; Steinemann 1982a). Several neo-*Y*-linked genes show either drastically reduced levels of expression (Steinemann *et al.* 1993; Steinemann and Steinemann 1998) or complete loss from the neo-*Y* (Steinemann and Steinemann 1999).

Our aim was to investigate DNA sequence variation at genes on the neo-sex chromosomes of *D. miranda* to make inferences concerning the nature of the forces involved in sex chromosome evolution (Yi and Charlesworth 2000). One of the candidate genes pursued for this purpose was *exuperantia1*. In *D. melanogaster*, its homolog *exuperantia* (*exu*) has various developmental functions (Hazelrigg *et al.* 1990; Macdonald *et al.* 1991; Hazelrigg and Tu 1994; Theurkauf and Hazelrigg 1998). The homolog of *exu* in *D. pseudoobscura* was previously examined to study the conservation of its function in the genus Drosophila (Luk *et al.* 1994), revealing that the *exu* gene had duplicated in the ob-

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scura group lineage to give rise to two loci, *exu1* and *exu2*. *Exu1* is orthologous to *exu* in *D. melanogaster*, as inferred from both sequence homology and the specific function of the gene (Luk *et al.* 1994). The function of *exu2* is unclear (Luk *et al.* 1994).

Since *exu* is located on element *C* in *D. melanogaster* (Hazelrigg *et al.* 1990), the expected location of *exu1* in *D. miranda* is on the neo-sex chromosomes, according to the established principle of chromosomal homology in Drosophila. When we investigated the location of *exu1* in *D. miranda*, it violated this expectation and was found to be on *X*L, the ancestral *X* chromosome of Drosophila. Further study revealed that this change is unique to *D. miranda*; the location of *exu1* appears to be well conserved in related Drosophila species. Several characteristics of *exu1* and its paralogous locus *exu2* led us to form a hypothesis that invokes a selective advantage to its relocalization. We used nucleotide sequence variation data from *exu1* and *exu2* of *D. miranda* to test this hypothesis. The pattern of nucleotide variation at the *exu1* locus strongly suggests the action of directional selection at or near this locus. The level of nucleotide variation at *exu2* also shows an unusual pattern, when compared with the expectations of the neutral model of molecular evolution.

MATERIALS AND METHODS

Species and strains: Twelve *D. miranda* lines were used, as described in Table 1 of Yi and Charlesworth (2000). These included 4 lines from British Columbia, Canada, 3 lines from Spray, Oregon, 2 lines from Mather, California, and 2 lines from Mount St. Helens in California. Jerry Coyne provided one strain each of *D. persimilis* (Mather, CA) and *D. subobscura* (Mount St. Helens, CA). A *D. affinis* line from Nebraska (line no. 0141.2; Drosophila Species Stock Center, Bowling Green, OH) was obtained from Rhonda Snook.

Amplification and the cloning of *exu* genes: Primers for amplifying *D. miranda exu1* alleles were designed using regions conserved between *D. melanogaster* and *D. pseudoobscura*. These are 5′ CTC CCC TTT GCC CAT TTT CCA 3′ for the forward primer and 5′ TTA GTT GGT GGC AGC 3′ for the reverse primer [the resulting PCR product corresponds to nucleotides 207–1624 of the *exu1* sequence reported from *D. pseudoobscura* (GenBank accession no. L22554)]. For *exu2* alleles, several primer pairs were designed from *D. pseudoobscura* sequences and those providing the most reliable PCR reactions were chosen. The sequences are 5′ TTT CCA GAT TGT CCA GTT 3′ and 5′ GAG TGC CAT TGC CAG AGC 3′ for forward and reverse primers [the product corresponds to nucleotides 218–1305 of a *D. pseudoobscura* allele (GenBank accession no. L22553)]. For both *exu* loci, 30 cycles of PCR reactions with annealing temperature 54° and extension at 72° were successfully used, following denaturation at 94°. After *exu1* and *exu2* in *D. miranda* were found to be located on *X*L in *D. miranda*, a single male fly from each line was used for PCR templates to generate DNA polymorphism data.

All the sequencing was performed by the ampliTaq FS cycle sequencing method (Applied Biosystems, Foster City, CA), using an ABI 377 sequencer. Additional sequencing primers were designed so that they would be spaced ∼450 bp apart on each strand, and the sequences are available on request from the corresponding author. Both strands were sequenced. The only variant allele from the *exu1* survey was verified through three different full sequencing runs on DNA from three different male flies from the MA32 line. All the sequences obtained from this study are deposited in GenBank (accession nos. AF286098–AF286111).

***In situ* hybridization and genomic Southern blotting:** Preparation and *in situ* hybridization of salivary chromosome squashes from third instar larvae generally followed the protocol described in Montgomery *et al.* (1987), with slight modifications. PCR products from genomic DNA were extracted from a 1% agarose gel using the QIAquick gel extraction kit (QIAGEN, Chatsworth, CA) and then labeled with biotinylated dUTP (Roche Diagnostics) by random primer extension for use as probes. Sites of hybridization were detected by staining with diaminobenzidine and peroxidase (Vector Laboratories, Burlingame, CA), with counterstaining of the polytene chromosomes in 5% Giemsa (Gurr). The banding patterns of polytene chromosomes of *D. miranda* reported by Das *et al.* (1982) were used as the reference point when determining the sites of hybridization. The polytene maps of Anderson *et al.* (1977) were used for *D. pseudoobscura* and *D. persimilis*. The chromosome map of Krimbas (1992) was used for *D. subobscura*, and Miller and Sanger's (1968) polytene chromosome maps were used for *D. affinis*.

High molecular weight genomic DNA isolated using the Puregene DNA isolation kit (Gentra, Research Triangle Park, NC) was used for the Southern analyses. The digoxygenin (DIG) nonradioactive system (Roche Diagnostics) was used for labeling probes and detecting hybridization. The protocol supplied by the manufacturer was generally followed for the Southern analyses, with slight modifications. PCR products prepared in the same way as for *in situ* hybridization and labeled with DIG by random primer extension were used to produce the probes.

Data analysis: Sequences were first aligned using the Sequencher 3.0 program and then edited and aligned manually. Published sequences for *exu1* and *exu2* in *D. pseudoobscura* were used to assign the coding regions for the *exu* alleles of different species. The nucleotide site diversity π, based on the average pairwise differences among alleles (Nei 1987), and the neutral mutation parameter θ, based on the number of segregating sites in the sample (Watterson 1975), were estimated using DnaSP 3.0 (Rozas and Rozas 1999). The DnaSP program was also used to calculate the chi-square value for the Hudson-Kreitman-Aguadé (HKA) test (Hudson *et al.* 1987) and Tajima's *D* statistic (Tajima 1989). Various other analyses were done using the program SITES (Hey and Wakeley 1997). The numbers of synonymous (k_s) and nonsynonymous (k_a) substitutions per nucleotide site in the interspecies comparisons were estimated using the program K-Estimator v 5.3 (Comeron 1999).

RESULTS

Localizations of the *exu* loci: The *exu* gene of *D. melanogaster* is located on polytene band 57A8–10 (chromosome arm *2*R; Muller's element *C*; see FlyBase). To determine whether the location of *exu* on element *C* is ancestral in the species studied here, *in situ* hybridization was performed using amplified *exu1* sequences of *D. pseudoobscura* as probes to polytene chromosomes of several different species (the sequences used are the same for the polymorphism study described below). Element *C* corresponds to chromosome *3* in *D. pseudoob-*

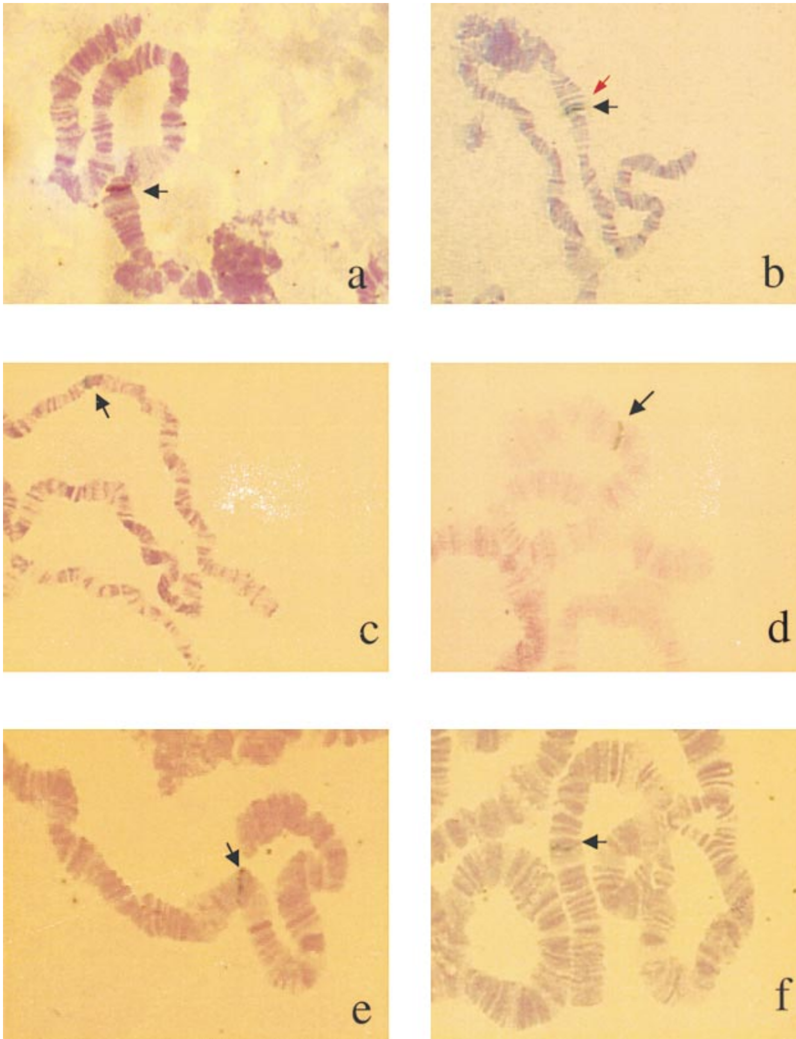

Figure 1.—Localization of each gene probe by *in situ* hybridization to polytene chromosomes. (a) *exu1* probe hybridized to *X*L of *D. miranda*. (b) *exu2* probe hybridized to *X*L of *D. miranda*. In some cells, cross hybridization with *exu1* is detectable, as indicated by a red arrow. The strain of *D. miranda* shown is MA32. (c) *exu1* probe hybridized to *D. pseudoobscura* chromosome 3. (d) *exu2* probe hybridized to *D. pseudoobscura* *X*L. (e) *exu1* probe hybridized to *D. affinis* chromosome *3*. (f) *exu1* probe hybridized to *D. subobscura* chromosome *E*.

scura and *D. persimilis*, but forms the neo-sex chromosome system in their close relative *D. miranda* (see Introduction). The results show that the location of *exu1* on element *C* is indeed conserved in *D. subobscura*, *D. affinis*, *D. pseudoobscura*, and *D. persimilis* (Figure 1; the results for *D. persimilis* are not shown).

In *D. miranda*, however, there was no sign of hybridization to the neo-*X* chromosome. In fact, *exu1* was found on *X*L (Figure 1), at band 43 of Das *et al.* (1982). This location of *exu1* in *D. miranda* was also confirmed by W. W. Anderson (personal communication). It defies the established chromosomal homology in Drosophila (Powell 1997). This new location of *exu1* appears to be fixed in the *D. miranda* lines used in this study (results not shown). The location of *exu2* in *D. miranda* and its sibling species *D. pseudoobscura* and *D. persimilis* was also determined and found to be near the base of *X*L in all three species examined (Figure 1; results for *D. persimilis* not shown), using *in situ* hybridization with *exu2* from *D. pseudoobscura* as the probe. The locations of *exu2* in the three species correspond well to each other, unlike the case of *exu1* (Figure 1). In *D. miranda*, *exu1* and *exu2* appear to be in adjacent polytene bands on *X*L, *exu1* being proximal to *exu2*. In some cells, we were able to observe cross-hybridization of *exu2* to the location corresponding to *exu1* (Figure 1b). In conclusion, the results show that *exu1* in *D. miranda* is not present at its ancestral location on element *C*, but is on element *A*.

The nature and scale of the rearrangement: From the direct observation of polytene chromosomes by means of *in situ* hybridization, there was no evidence that any neo-sex-linked *exu1* alleles exist in the *D. miranda* genome. The status of the ancestral neo-sex-linked *exu1* alleles was further investigated by genomic Southern blotting, using various restriction digestion reactions. Genomic Southern blots from *D. pseudoobscura* show the presence of two *exu* loci, as previously reported (Figure 2A; Luk *et al.* 1994). From *D. miranda*, we observed two strong bands corresponding to the two *exu* loci detectable by *in situ* hybridization (Figure 2A).

Males and females of *D. miranda* show different banding patterns in genomic Southern blots. While the major bands corresponding to the *exu1* and *exu2* loci were generally weaker in males because of their *X*-linkage

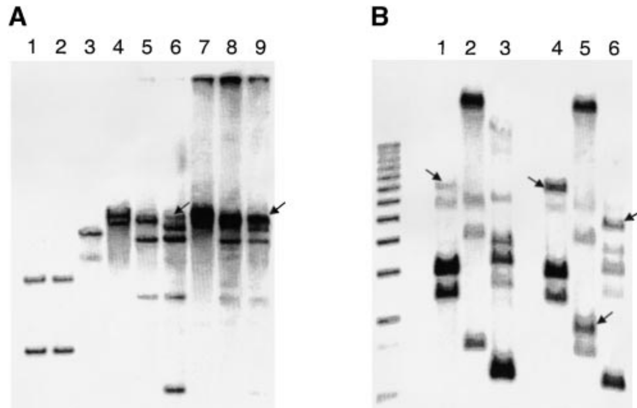

Figure 2.—(A) Genomic Southern blotting results for determining the copy numbers of *exu*-related loci in *D. pseudoobscura* and *D. miranda*. Hybridization was done overnight at 55°. The *exu2* locus of *D. miranda* was amplified by PCR and then gel extracted. To better detect the homologous copies in the genome, a 400-bp fragment of a region that is highly conserved between *exu1* and *exu2* was generated by restriction digestion using *Hae*III and then labeled as described in materials and methods. Lanes 1–3 correspond to the digests of *D. pseudoobscura* genomic DNA. The enzymes used are *Bam*HI (lane1), *Bam*HI and *Sal*I (lane 2), and *Sal*I and *Xba*I (lane 3). Lanes 4–6, digests of *D. miranda* female genomic DNA using the same set of restriction enzymes as in the previous lanes, in the same order; lanes 7–9, digests of *D. miranda* male genomic DNA prepared in the same way. *Bam*HI digests in females (lane 4) show the presence of two bands with strong homology to the probe. The bands with stronger intensity in males are indicated by arrows (compare lanes 6 and 9; the band with the highest molecular weight is stronger in the male lane, while the other bands are relatively weaker in the male lane). This difference also exists between lanes 4 and 7 and between lanes 5 and 8, which are blurred in the male lanes due to the close proximity of the high molecular weight bands. This pattern appeared consistently using other enzymes with overnight restriction digestions, suggesting that it is not an artifact of partial digestion. (B) Differences between female and male genomic DNA Southern blots of *D. miranda* shown against a 1-kb ladder. The probe used for these blots is the *exu1* locus from *D. pseudoobscura* amplified by PCR and prepared in the same way as above. Hybridization was done overnight at 60°. The restriction enzymes used are *Ava*I (lanes 1 and 4), *Bam*HI and *Hin*dIII (lanes 2 and 5), and *Eco*RI (lanes 3 and 6). Multiple bands appear due to internal digestion of target material. Lanes 1–3, *D. miranda* female DNA; lanes 4–6, male genomic DNA of *D. miranda*. In lanes 1 and 4, the same high molecular weight band is stronger in males, while all the other bands are generally weaker in male digests, as was seen in A. This suggests that the high molecular weight band (indicated by arrows) corresponds to the neo-sex-linked alleles, with the neo-*Y* allele possessing higher homology to the probe. In the remaining lanes, male-specific bands with relatively high intensity were detected (arrows in lane 5 and 6). These bands must correspond to the neo-*Y*-linked *exu*-like locus.

(the same amount of total genomic DNA was used for each lane), in each set there was one band with stronger intensity in males, with a much weaker corresponding band in females (Figure 2, A and B). This suggests neo-sex linkage for that particular band, the neo-*X* locus possessing less homology than the neo-*Y*-linked allele to the *exu1* locus used as probe. The intensities of the neo-*X*-linked bands were very low.

Furthermore, we succeeded in finding some enzyme pairs that generate male-specific bands (*i.e.*, neo-*Y*-linked alleles; lanes 5 and 6 in Figure 2B). The neo-*Y*-linked *exu1* allele still appears to possess a relatively high homology to *exu1*, even though it could not be seen by *in situ* hybridization to the polytene chromosomes, probably due to the poor banding pattern of the neo-*Y* chromosome in such preparations.

We wanted to determine the scale of the genomic material including the *exu1* locus that has been duplicated onto the *X* chromosome of *D. miranda*. The following evidence suggests that the observed location of *exu1* has not resulted from a large-scale translocation. Segarra and Aguadé (1992) surveyed the cytological locations of several *X*-linked markers of *D. melanogaster* in obscura group species, including *D. pseudoobscura*, *D. persimilis*, and *D. miranda*. The region of *X*L where the markers used in this study (*per*, *exu1*, and *exu2*; see below) are located corresponds to the three divisions in the polytene map referred to in their survey (*X*L-*1*, *X*L-*2*, and *X*L-*3* from Dobzhansky and Tan 1936). Five markers were localized to this region for all three species (Table 1 in Segarra and Aguadé 1992). Not only were all these markers located in the corresponding bands, their order was perfectly conserved. This excludes the possibility of a large-scale translocation involving element *C* (the neo-sex chromosomes) and element *A* (*X*L) in *D. miranda*.

Another insight comes from the location of *exu2*, which in *D. miranda* is distal but very close to *exu1* (Figure 1). The location of *exu2* is well conserved in the three species surveyed, setting a distal boundary for the segment of genome that is new in *D. miranda*. From these observations, we conclude that the most parsimonious interpretation of the location of *exu1* is that a small section of genome, including the *exu1* locus of *D. miranda*, has been duplicated on *X*L, close to *exu2*. The ancestral neo-*X* site then lost its homology to a substantial degree, while the neo-*Y*-linked site has also lost some homology, so that it cannot be detected by our PCR approach. In the following section, we present molecular population genetic analyses of the *exu1* and *exu2* loci, to infer the underlying evolutionary forces responsible for this unusual phenomenon.

Sequence variation at the *exu1* locus in *D. miranda*: The function of *exu1* in male spermatogenesis, together with the ancestral location of *exu1* on the newly developing sex chromosomes of *D. miranda* and its new location on the true *X* chromosome, have led us to form a "selective" interpretation of this movement of *exu1* (see discussion). In what follows, we refer to this movement as the "transposition" of *exu1*, following Powell (1997), since it probably involves the movement of a small amount of genetic material to another location in the genome. This does not necessarily implicate a transpos-

TABLE 1

Polymorphism summary

	m	n	S	Diversity estimates ($\times 10^{-3}$)						Tajima's D
				θ_t	π_t	θ_r	π_r	θ_s	π_s	
exu1	1423	12	2	0.47	0.23	0	0	1.75	0.86	−1.45
exu2	1012	12	6	1.98	1.27	1.78	1.07	2.45	1.73	−1.37
per[a]	1480	12	18	4.03	4.20	2.82	2.43	4.92	5.10	0.18

m, length of sequence surveyed (in base pairs); n, number of alleles sampled; S, number of segregating sites; θ and π, Watterson's (1975) estimator of $4N\mu$ and the average nucleotide pairwise difference (Nei 1987), respectively. The subscripts t, r, and s mean total sites, replacement sites, and silent sites (synonymous + noncoding sites), respectively.

[a] From Yi and Charlesworth (2000).

able element. We surveyed sequence polymorphism of *exu1* from *D. miranda* to test one of the consequences of a recent fixation of a selectively advantageous variant, a so-called "selective sweep" (Maynard Smith and Haigh 1974; Kaplan *et al.* 1989; Barton 1998), *i.e.*, a very reduced level of variation at the *exu1* locus.

The results of the nucleotide variation survey of *exu1* from 12 lines of *D. miranda* are summarized in Table 1 and Figure 3. We found 1 allele that differs from 11 other identical alleles by two singleton variants. These were both silent, one being a synonymous mutation in the second exon, the other in the intron between exons 2 and 3. They were inferred to be derived, when compared with the outgroup sequence from *D. pseudoobscura*. The level of nucleotide diversity (0.86×10^{-3} for silent sites) is lower than the previously reported lowest estimate of nucleotide diversity from *D. miranda*; the silent site diversity from *hsp82* estimated from 4 alleles was 1.59×10^{-3} (Wang *et al.* 1997). The low diversity for *hsp82* probably reflects a high degree of selective constraint, given its high level of protein sequence conservation in Drosophila (Blackman and Meselson 1986; Wang *et al.* 1997) and high level of codon bias in *D. miranda* (Yi 2000). In contrast, *exu1* exhibits relatively unconstrained evolution (see below).

The level of diversity at *exu1* is much lower than for a more proximal *X*-linked gene previously surveyed from this species, *period* (Wang and Hey 1996; Yi and Charlesworth 2000). For the lines studied here, silent site diversity at *per* was 4.93×10^{-3} (Yi and Charlesworth 2000), which is comparable with a mean of 4.00×10^{-3} for three other *X*-linked loci surveyed (Yi 2000). We obtained a significant HKA test statistic (Hudson *et al.* 1987) when we compared the ratio of silent polymorphism in *D. miranda* to divergence from *D. pseudoobscura* for *exu1* to the corresponding ratio for *per* ($P < 0.02$).

In addition to its extremely reduced level of variation, *exu1* shows an unusual configuration of variation: the two singletons observed are in the same haplotype. Un-

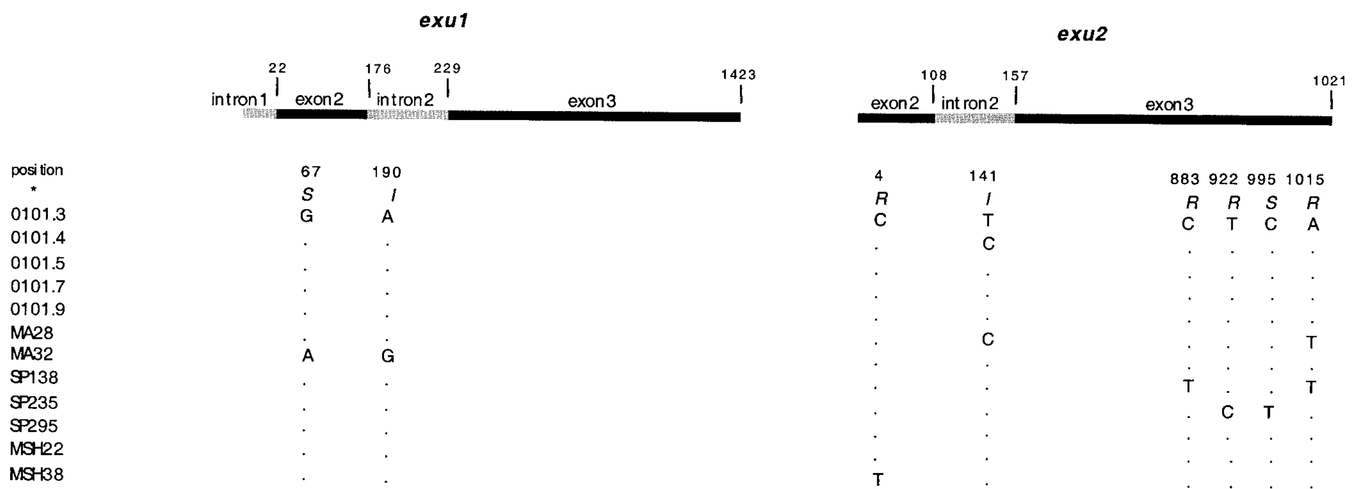

Figure 3.—Polymorphic sites from *exu1* and *exu2* loci in *D. miranda*. The exon/intron structures of the two loci are very similar, as is shown for *exu1*. Sequenced regions are shown as starting from number 1, ending at nucleotides 1423 and 1021, respectively. The endpoints of each exon/intron block are numbered. For *exu2*, the structure of the gene is shown only for the sequenced regions. The orientation of *exu2* relative to *exu1* is not known. Each site has a letter R, S, or I, standing for replacement, synonymous, or intron substitution, respectively, and a number corresponding to the position within the sequenced region.

der the infinite sites model, this must reflect two mutations that occurred on the same external branch of the gene genealogy. We constructed the following test to examine the probability of the presence of two mutations on the same external branch under a neutral genealogy. Neutral genealogies without recombination were simulated according to the standard coalescence scheme (Hudson 1990), and the length of the external branches for all the genes initially present in a given replicate was determined. Two mutation events were assigned randomly to each replicate genealogy, and the cases where the two mutations occur at the same external branch were counted. The probability of observing both mutations on the same external branch, estimated from 100,000 runs with a sample size of 12, was 2.67×10^{-2}. This strongly suggests that the observed configuration of mutations is incompatible with a neutral genealogy.

Sequence variation at the *exu2* locus of *D. miranda*: We were also interested in the pattern of molecular variation at the *exu2* locus in *D. miranda*, because of its close linkage to *exu1*. We found six polymorphic sites (Table 1 and Figure 3). The level of nucleotide diversity appears to be reduced compared to the more proximal gene, *per* (Table 1), although this is not significant on the HKA test. Among the six segregating sites, four were singletons. All the low frequency variants were derived, when compared with the outgroup sequence from *D. pseudoobscura.* Interestingly, the two sites that occurred twice in the population show all four possible haplotypes, consistent with the occurrence of recombination among the informative sites. Two estimators of the population recombination rate $2N_er$ for the locus (where N_e is the effective population size for autosomal loci and r is the recombination rate in females), C (Hudson 1987) and C_{HRM} (Wall 2000), are rather different (60.9 and 16.8, respectively). An estimate of 16 was obtained from P. Fearnhead's unpublished algorithm, which incorporates an importance sampling scheme (cited in Wall 2000). These differences no doubt partly reflect the large variances of the estimates, but may also be due to violation of the neutral equilibrium assumption on which the estimators are based. The excess of singleton variants, observed in the case of *exu2*, results in a deflated pairwise nucleotide difference (Nei 1987) compared to the θ estimate based on the number of segregating sites (Watterson 1975), leading to a negative Tajima's D (Tajima 1989). The excess of mutations on the external branches of the gene genealogy also results in values of Fu and Li's D^* and F^* (Fu and Li 1993) of -1.11 and -1.33, respectively. The probability of obtaining a value equal to or less than the observed Tajima's D (-1.37) under the neutral model with no recombination is $\sim$9% by coalescent simulation. With recombination, as clearly happens at *exu2*, these tests are conservative, since the true variance is then less than under the assumption of no recombination (Fu and Li 1993; Tajima 1993a). We conducted a coalescent simulation to evaluate the probability of obtaining the observed value of Tajima's D with the lower estimate of $2N_er = 16$ (see above); the probability of obtaining a Tajima's D of -1.37 is $<4\%$. This suggests that the observed configuration of variant sites is incompatible with neutrality. We also performed Fu's F_s test (Fu 1997) to assess the significance of the excess of rare haplotypes. The probability of observing four singletons conditioned on six haplotypes was $<5\%$.

Divergence of the protein-coding regions: The sequenced regions of *exu1* and *exu2* include most of exon 2 and exon 3 of each gene (Figure 3). Within each of the three sibling species, the divergence between *exu1* and *exu2* was $\sim$16% at the DNA level. The frequencies of substitutions per synonymous and nonsynonymous sites for *exu1* and *exu2* between the three sibling species, *D. miranda*, *D. pseudoobscura*, and *D. persimilis*, are summarized in Table 2. The observation that the ratio of nonsynonymous to synonymous divergence for the two *exu* loci is <1 for all the three comparisons implies that the functional products of the *exu* loci are under some selective constraints. The observed levels of synonymous site divergence at these two loci are among the highest for all genes compared between *D. miranda* and *D. pseudoobscura* (Yi 2000).

For nonsynonymous sites, *exu1* appears to be more constrained than *exu2*. For example, the nonsynonymous divergences (k_a) for *exu2* between *D. miranda* and each of its sibling species lie outside the 95% confidence intervals for the corresponding estimates for *exu1*. The rate of molecular evolution of the *exu* genes between *D. pseudoobscura* and *D. persimilis* was further tested by a relative rate test (Tajima 1993b), using the sequences of *D. miranda* as an outgroup. The numbers of synonymous substitutions on the two lineages did not depart from the null hypothesis of a constant rate of evolution ($P > 0.09$). To assess whether the observed pattern of higher nonsynonymous site substitutions at *exu2* was statistically significant, we investigated the distributions of the k_a and k_s values using a "hybrid" sequence of *exu1* and *exu2* to generate a null distribution of differences assigned randomly to each locus, as previously described (Yi and Charlesworth 2000). From this analysis, the probability of observing a k_a of 0.046 between *D. miranda* and *D. pseudoobscura* (the estimate for *exu2*) from 10,000 replicates was <0.047, suggesting a faster rate of amino acid substitution for *exu2* in this lineage. All other comparisons were nonsignificant (results not shown).

Exu1 of *D. pseudoobscura* has been shown to function in both oogenesis and spermatogenesis, while the function of the duplicated *exu2* locus is unclear (Luk *et al.* 1994). The faster rate of protein evolution at the *exu2* locus could be the consequence of a newly acquired function for *exu2*, following its duplication onto XL. But the high frequency of newly derived replacement site polymorphisms for *exu2* in *D. miranda* (four out of six

TABLE 2

Divergence at the protein-coding regions of the *exu1* and *exu2* loci

	mir-pse	*mir-prs*	*pse-prs*
exu1			
(1353 bp)			
k_s	0.070	0.049	0.025
	[0.044, 0.134]	[0.020, 0.090]	[0.007, 0.062]
k_a	0.031	0.033	0.007
	[0.013, 0.039]	[0.014, 0.040]	[0.001, 0.013]
k_a/k_s	0.44	0.67	0.29
	[0.25, 0.84]	[0.34, 1.46]	[0.12, 0.98]
exu2			
(945 bp)			
k_s	0.076	0.076	0.029
	[0.039, 0.117]	[0.039, 0.121]	[0.009, 0.056]
k_a	0.046	0.043	0.009
	[0.030, 0.064]	[0.028, 0.061]	[0.003, 0.018]
k_a/k_s	0.61	0.57	0.31
	[0.34, 1.26]	[0.32, 1.29]	[0.06, 0.99]

95% confidence intervals are shown in brackets. *mir*, *D. miranda*; *pse*, *D. pseudoobscura*; *prs*, *D. persimilis*.

segregating sites) in fact suggests a less constrained mode of evolution for this locus.

DISCUSSION

Occurrence of transpositions in the genome of Drosophila: Even though conservation of the content of homologous chromosomal arms across the genus Drosophila has been revealed repeatedly by several different approaches (Patterson and Stone 1952; Powell 1997), a few cases where the rule does not hold true have also been found. For example, Kress (1993) found that the cytological locations of Larval glue protein genes (*Lgp-1* and *Lgp-3*) in *D. virilis* and their homologs in *D. melanogaster* did not conform to the established chromosomal homologies. The same was true for tRNA genes in four different Drosophila species in two different subgenera (Tonzetich *et al.* 1990). Steinemann (1982b) noted that the cytological locations of 5S rRNA and histone genes were not conserved between *D. melanogaster* and *D. miranda*. A probe containing the histone genes hybridized to two different polytene bands in *D. miranda*; moreover, one of the two sites detected by this probe was not conserved in *D. pseudoobscura*. The location of the Larval serum protein genes (*Lsp-1* α, β, γ) also showed an unusually complex evolutionary history when investigated by *in situ* hybridization in 14 different Drosophila species (Brock and Roberts 1983). In several cases, the copy numbers and cytological locations varied between species. When the genomic region encoding a cluster of maltase genes in *D. virilis* was examined, the location was in a different chromosome than for the homologous gene cluster in *D. melanogaster* (Vieira *et al.* 1997).

Both the mechanisms and the evolutionary implications of these cases remain the topic of speculation. None of the above authors was able to reject the possibility of chromosomal rearrangement as the mechanism of movement of genes between arms, due to the lack of detailed comparative cytological maps in most cases. However, they all noted that it is an unlikely explanation; most scenarios invoke fixation of several chromosomal rearrangements, even in the most parsimonious pathway. For example, to account for the dispersal of *Lsp-1* genes in the melanogaster subgroup, three translocations or three fusions and pericentric inversions were required (Brock and Roberts 1983). Tonzetich *et al.* (1990) proposed a transposable element-mediated transposition to explain their observations.

Perhaps we should focus on the common characteristics of the genes showing nonconservation of chromosomal locations to elucidate the causative mechanism. Both the 5S rRNA genes and the histone genes that showed nonconservation (Steinemann 1982b; Felger and Pinsker 1987) are tandemly repeated genes (Powell 1997). tRNA genes and the *Lgp* genes are members of gene families that share homologies. The same is true for the *exu1* and *exu2* genes (see results). This suggests ectopic exchange events involving some homology between donor and target site as a possible mechanism of gene transposition in the Drosophila genome. Characterization of the flanking regions of *exu1* and *exu2* in *D. miranda* should shed light on this possibility.

A possible selective advantage for the transposition of *exu1* to the *X* chromosome: The ancestral location of *exu1* inferred from the other species is on Muller's element *C* (see results), which corresponds to the neo-sex chromosomes of *D. miranda* (MacKnight 1939; Steinemann 1982a). As described in the Introduction, these chromosomes show several characteristics of developing sex chromosomes. Namely, the neo-*Y* chromosome shows a considerable level of degeneration throughout the chromosome, while the counterpart neo-*X* chromosome shows a corresponding level of dosage compensation (Strobel *et al.* 1978; Steinemann and Steinemann 1998, 1999).

The function of *exu1* inferred from data on *D. melanogaster* and *D. pseudoobscura* not only includes the proper localization of maternal mRNA but also normal spermatogenesis. The role of *exu* in spermatogenesis appears to be critical, as all *exu* mutants in *D. melanogaster* are completely male sterile in the hemizygous state (Hazelrigg *et al.* 1990). The deterioration of the ancestral neo-*Y* copy of *exu1* as a result of *Y* chromosome degeneration would thus be expected to have had a deleterious effect on males, since most lethal and detrimental mutations in Drosophila have deleterious heterozygous effects (Crow and Simmons 1983). A transposition of *exu1* from the neo-*X* chromosome to *X*L,

possibly as a result of an ectopic exchange event involving *exu2*, might thus confer a fitness advantage on males, provided that the presence of an extra copy of *exu1* does not impair the fitness of females too much. The spread of a duplication of this kind is analogous to the spread of a sexually antagonistic *X*-linked allele that causes a disadvantage to females but an advantage to males (Rice 1984, 1987; Charlesworth *et al.* 1987). Male carriers of the postulated duplication of *exu1* would have a degenerate copy on the neo-*Y* chromosome and a functional copy on the neo-*X* chromosome. With weak selection, the selective advantage of the duplication in males must just exceed any disadvantage to its heterozygous female carriers, if it is to spread (Charlesworth *et al.* 1987). Once the duplication is established in the population, there is a selective advantage to loss of the ancestral copy from the neo-*X* chromosome, since fitness will be enhanced by restoration of the normal level of gene product, assuming that *exu1* is dosage compensated so that males do not suffer from a reduction in its copy number. This is analogous to the scenario that has been proposed for the evolution of dosage compensation in mammals and nematodes (Charlesworth 1978, 1996; Jegalian and Page 1998), which requires down-regulation of *X*-linked genes in females following an increase in their activity in both sexes.

This scenario leads to several interesting predictions. First, we might expect to see more cases of gene transposition from the neo-sex chromosomes to other chromosomes in *D. miranda*, if the above hypothesis is correct. In fact, we have observed one other case of possible transposition. One of our candidate marker genes on Muller's element *C*, *deadpan*, also showed an unusual location in relation to that expected from chromosomal homology. The *2*R gene *deadpan* is the only known autosomal denominator in the sex determination system of *D. melanogaster* (Younger-Shepherd *et al.* 1992), and we would therefore expect a significant deleterious effect of any loss of activity of the neo-*Y* copy in *D. miranda*. *In situ* hybridization showed that the location of *deadpan* on element *C* is preserved in the sibling species *D. pseudoobscura* and *D. persimilis* (results not shown). In *D. miranda*, *deadpan* has two locations, one corresponding to the ancestral location on element *C* (band 34B of the drawn map of Das *et al.* 1982) and the other one on chromosome *2* (element *E*; band 54B of the drawn map of Das *et al.* 1982; results not shown). This is consistent with the *deadpan* locus being at an earlier stage of the process than *exu1*, the new location representing a recent transposition.

Second, if gene transposition or duplication can be used as a way of achieving dosage compensation, then similar phenomena might be observed in species where there is no apparent evidence of the evolution of chromosome-wide dosage compensation, such as birds (Baverstock *et al.* 1982) and butterflies (Johnson and Turner 1979; Suzuki *et al.* 1998, 1999).

Third, we expect to see the footprint of selection at the molecular level for the regions surrounding the recently transposed genes; *i.e.*, we expect to observe the effects of a selective sweep. Theoretical investigations of the selective sweep model make at least two predictions at the molecular level: reduced variability at sites near the target of selection (Maynard Smith and Haigh 1974; Kaplan *et al.* 1989; Barton 1998) and a frequency spectrum at these sites that is skewed toward rare variants (Braverman *et al.* 1995; Simmonsen *et al.* 1995). In our case, the target of selection is postulated to be the transposed copy of *exu1*.

The effect of a putative selective sweep on the nucleotide variability at *exu1* and *exu2*: As shown above (results), the pattern of molecular variation at *exu1* strongly suggests a recent fixation event driven by natural selection. The observed low level of variability is of a truly exceptional kind, when compared with other known estimates of nucleotide diversity in Drosophila, except for regions of drastically reduced recombination (Moriyama and Powell 1996). This dramatic reduction in diversity and the haplotype structure are both in accord with the adaptive fixation of the transposed *exu1* allele. It is also possible that rapid protein sequence evolution of the *exu1* gene after its transposition could have caused the low level of variation. The divergence statistics from the coding region, however, do not suggest a very high rate of replacement relative to silent substitutions, nor does *exu1* seem to be evolving faster than *exu2* at the protein level (Table 2).

The nucleotide variation at *exu2* also shows an unusual pattern: a lower level of variation compared to a nearby locus (*per*) and an excess of singletons. Incorporating the modest amount of recombination inferred from the polymorphism data (see results), the observed pattern of excess of singletons is significantly incompatible with the neutral model. These results suggest that the *exu2* locus has also recently experienced the effect of a selective sweep, possibly associated with the spread of the *exu1* transposition.

Under the model of selective fixation of the transposition, the presumptive "single" *X* chromosome carrying the transposed *exu1* gene has reached fixation in the population, eliminating all *X* chromosomes without the newly duplicated *exu1* gene, as is consistent with the inferred ancestry of the variant sites at *exu1*. If the fixation was recent enough compared to the effective population size, the genealogy after the fixation would resemble a so-called "star phylogeny" (Berry *et al.* 1991). In this case, the expected number of polymorphic sites in a sample of size n is

$$S = n\mu t, \quad (1)$$

where μ is the neutral mutation rate and t is the time since the sweep event. The age of the sweep, scaled

relative to the effective population size for autosomal genes, τ, can thus be estimated as

$$\tau = \frac{3S}{n\theta} \quad (2)$$

for an *X*-linked locus with no sexual selection. Using the θ estimate for silent sites from the *per* locus, this leads to an estimate of 0.08 N_e generations since the putative sweep event. Assuming that the number of segregating sites at *exu1* is Poisson distributed, an upper bound for this estimate is obtained as the τ that gives a probability ≥ 0.05 of obtaining two or fewer segregating sites. This leads to a value of 0.3 N_e generations. Since the estimated N_e of *D. miranda*, based on silent site diversity, is about half that of *D. melanogaster*, *i.e.*, $\sim$500,000 (Yi 2000), these estimates suggest a sweep event of not >150,000 generations ago. This should not be taken as precise, as there are several possible sources of error, but is at any rate in accord with a fairly recent sweep.

An alternative possibility to selective fixation of the *exu1* transposition is that the observed transposition was fixed in *D. miranda* by genetic drift, recently enough that it has not yet recovered its standing level of variation (Tajima 1990). The following argument shows that this scenario is unlikely. First, if the fixation of the duplication preceded the most recent common ancestor of the sample, the neutral shape of the genealogy would not be affected, which is inconsistent with the result of the neutrality test of haplotype structure (see results). On the other hand, if the root of the gene genealogy lies within the period of spread of the duplication, the shape of the genealogy depends on the rate of change of the population size of the duplicated *exu1* class. From Kimura's (1970) formula for the distribution of the fixation time of a neutral variant by random genetic drift, even the fastest such fixation that occurs with any significant probability takes on the order of N_e generations. This means that the mean geometric rate of change of population size of the *exu1* population size is of the order of $1/N_e$, which is negligibly small. Again, the genealogy of the samples must resemble a neutral one.

The shape of the genealogy could also have been significantly distorted by other factors, such as a recent population expansion, which would account for the excess of singletons at *exu1* and *exu2* in *D. miranda*. More nucleotide variation surveys of *D. miranda* are probably necessary to test this hypothesis rigorously, but there is currently no evidence for such an effect from surveys of six autosomal and *X*-linked loci (Yi 2000; Yi and Charlesworth 2000). On the other hand, if there is population subdivision in *D. miranda*, the significance level for the departures from neutrality for the genealogies at the *exu* loci would be difficult to evaluate. Currently, most DNA polymorphism data from *D. miranda* give little evidence of genome-wide population subdivision (Yi 2000; Bachtrog and Charlesworth 2000), suggesting that we can have confidence in our conclusions. Finally, we cannot rule out the possibility that *exu1* and *exu2* have experienced a selective sweep independent of the transposition event, although this is a much less parsimonious explanation of the data than the one we propose. An investigation of the patterns of polymorphism in regions flanking the *exu* loci would shed light on the location of the target of selection.

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