Molecular Evolution of the Sex-Ratio Inversion Complex in *Drosophila pseudoobscura*: Analysis of the *Esterase-5* Gene Region

Christina S. Babcock[1] *and Wyatt W. Anderson*

Department of Genetics, University of Georgia

The Sex-Ratio chromosome in *Drosophila pseudoobscura* is subject to meiotic drive. It is associated with a series of three nonoverlapping paracentric inversions on the right arm of the X chromosome. The *esterase-5* gene region has been localized to section 23 within the subbasal inversion of the Sex-Ratio inversion complex, making *esterase-5* a convenient locus for molecular evolutionary analyses of the Sex-Ratio inversion complex and the associated drive system. A 504-bp fragment of noncoding, intergenic DNA from the *esterase-5* gene region was amplified and sequenced from 14 Sex-Ratio and 14 Standard X chromosomes of *D. pseudoobscura,* and from 9 X chromosomes of its two sibling species, *Drosophila persimilis* and *Drosophila miranda.* There is extensive sequence differentiation between the Sex-Ratio and Standard chromosomal types. The common Standard chromosome is highly polymorphic, while, as expected from either the neutral mutation theory or the selective sweep hypothesis, the rarer Sex-Ratio chromosome has much less within-chromosome nucleotide polymorphism. We estimate that the Standard and Sex-Ratio chromosomes in *D. pseudoobscura* diverged between 700,000 and 1.3 Mya, or at least 2 million generations ago. The clustering of *D. pseudoobscura* Sex-Ratio chromosomes in a neighbor-joining phylogeny indicates a fairly old, monophyletic origin in this species. It appears from these data that Sex-Ratio genes were present prior to the divergence of *D. pseudoobscura* and *D. persimilis* and that both the Standard and Sex-Ratio chromosomes of *D. persimilis* were derived from the Standard chromosome of *D. pseudoobscura* after the inversion events that isolated the *D. pseudoobscura* Sex-Ratio chromosome.

Introduction

The Sex-Ratio chromosomes of *Drosophila* are one of the best-studied cases of meiotic drive (Sandler and Novitski 1957). As their name implies, these chromosomes cause a distortion of the sex ratio in the progeny of males carrying the Sex-Ratio X chromosome. These Sex-Ratio chromosomes have a widespread taxonomic distribution and have been observed in natural populations of species in the subgenus *Drosophila,* including *D. melanica* and *paramelanica* (Stalker 1961), *D. mediopunctata* (De Carvalho, Peixoto, and Klaczko 1989), and *D. quinara* and *testacea* (James and Jaenike 1990). They have also been found in many species in the subgenus *Sophophora,* including the *affinis* subgroup species *D. affinis* (Morgan, Bridges, and Sturtevant 1925) and *athabasca* (Sturtevant and Dobzhansky 1936), as well as the *obscura* group species *D. obscura* (Gershenson, 1928), *subobscura* (Jungen 1967), *azteca, persimilis,* and *pseudoobscura* (Sturtevant and Dobzhansky 1936). The Sex-Ratio genes (sr), which are located on the X chromosome, cause spermiogenic failure and the consequent loss of Y-bearing sperm (Polincasky and Ellison 1970). The spermiogenic failure of Sex-Ratio males in an *obscura* group species was characterized by Jungen and Maurer (1976) in *D. subobscura* by tracing DNA-containing structures such as nuclei and mitochondria during the differentiation of sperm from elongation to maturation. Without direct evidence as to which sperm was Y-bearing, they showed that some sperm of Sex-Ratio males were eliminated during both the elongation and individualization phases of spermatogenesis and that the sperm could also degenerate after individualization.

In *D. pseudoobscura,* males carrying the Sex-Ratio X chromosome (SR^{pse}) produce mostly (95%–99%) daughters. Males carrying a Standard X chromosome not subject to meiotic drive (ST^{pse}) produce nearly equal numbers of male and female offspring. The number of sperm that male *Drosophila* can transfer in a mating depends on the frequency of their matings in the previous day or two, which in turn depends on population density and other factors that determine opportunities for matings (Beckenbach 1978). Because SR^{pse} males are capable of accumulating a sperm load equivalent to ST^{pse} males but containing only X-bearing sperm, the frequency of Sex-Ratio is increased among the population's X chromosomes by the conditional drive favoring the SR^{pse} chromosome.

The genetic mechanism for expression of the Sex-Ratio trait in *D. pseudoobscura* has not been fully characterized. However, the expression of Sex-Ratio requires

[1] Present address: Dept. of Ecology and Evolution, University of Arizona.

Abbreviations: SR^{per}, SR^{pse}, Sex-Ratio X-chromosome in *Drosophila persimilis* and *Drosophila pseudoobscura,* respectively; ST^{per}, ST^{pse}, Standard X-chromosome in *D. persimilis* and *D. pseudoobscura,* respectively; sr, Sex-Ratio genes; *Est-5, esterase-5* locus.

Key words: meiotic drive, Sex-Ratio, *D. pseudoobscura, esterase-5,* phylogenetic analysis, chromosomal inversion.

Address for correspondence and reprints: Wyatt W. Anderson, 310 New College, University of Georgia, Athens, Georgia 30602-1732. E-mail: wyatt@franklin.uga.edu.

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the interaction of at least four loci, which presumably include a drive locus and enhancer regions as in other systems of meiotic drive (Wu and Beckenbach 1983). In many of the species that have the Sex-Ratio system of meiotic drive, SR chromosomes are associated with inversions distinguishing them from the Standard X chromosome (ST). These inversions are not known to be involved directly in the expression of Sex-Ratio but most likely arose secondarily; they act to reduce recombination between the unidentified Sex-Ratio drive locus and its linked drive enhancers (Wu and Beckenbach 1983; Lyttle 1991). In *D. pseudoobscura,* the inversion complex forming the Sex-Ratio gene arrangement is a series of three nonoverlapping paracentric inversions on the right arm of the X chromosome (Dobzhansky 1939). Because the inversions are closely associated with the Sex-Ratio drive system, it has been of much interest to study the evolutionary history of the Sex-Ratio inversion complex as a marker of this meiotic drive system. Previous electrophoretic studies of allozymes (Prakash and Merritt 1972; Prakash 1974; Keith 1983) and studies of reciprocal introgression between *D. pseudoobscura* and *D. persimilis* (Wu and Beckenbach 1983) have suggested extensive genetic differentiation between the two types of X chromosomes.

In this study we compare the SR and ST X chromosomes of *D. pseudoobscura* using a nucleotide sequence analysis of the *esterase-5* gene region. The *Est-5* gene region has been localized to banding section 23 within the small basal inversion of the Sex-Ratio inversion complex (Brady, Richmond, and Oakeshott 1990). Due to the location of this gene region and the reduction of recombination between inverted segments of chromosomes (Sturtevant and Dobzhansky 1936; Dobzhansky and Epling 1948), studies of the *Est-5* gene region can be used to trace the evolutionary history of the Sex-Ratio inversion complex and drive system. Our goals are to determine the extent of nucleotide sequence differentiation between the Sex-Ratio and Standard gene arrangements of *D. pseudoobscura,* to infer relationships among the nucleotide sequences, to estimate of the age of the Sex-Ratio system, and to begin to reconstruct the evolutionary history of the Sex-Ratio inversion complex.

Materials and Methods

Fly Stocks

Twenty-eight strains of *D. pseudoobscura* were used in this study, 14 carrying Sex-Ratio (SR^{pse}) chromosomes and 14 matching strains carrying Standard (ST^{pse}) chromosomes, collected at the same time as the SR^{pse} strains (table 1). Nine sets of matching strains were collected at San Bernardino (SB), Calif., and one set of strains at each of the following locations: Sierra Mountains (SM), Toiyabe National Forest, Calif.; Grand Canyon (GC), Ariz.; Tempe (TE), Ariz.; Albuquerque (AL), N.M.; all of them collected in the late 1980s. One set of strains was collected in 1993 at Black Canyon of the Gunnison (BC), Colo. The SR^{pse} stocks were crossed with a line of *sepia* (*se*) flies according to figure 1 in order to isolate the SR^{pse} chromosome. The recessive allele *sepia* is on the X chromosome and causes a brownish eye color that serves to mark the Standard chromosome. The wild-type eye color of SR^{pse} males made it easy for us to distinguish them from *sepia* males, and the identity of SR^{pse} males was further verified by making test crosses to virgin females and checking for all-female offspring prior to DNA analysis.

Drosophila miranda and *D. persimilis,* the two species most closely related to *D. pseudoobscura,* were included in this study to aid in rooting phylogenetic trees. Three *D. miranda* strains were used, one from the National Drosophila Species Resource Center at Bowling Green State University (0101.0), its geographic origin unknown, and the other two (MA83 and MA23) from a collection at Mather, Calif., at the edge of Yosemite Na-

Table 1
***Drosophila* Chromosomes Used in This Study According to the Site of Origin**

Strain Origin	Sex-Ratio Chromosome	Standard Chromosome
D. pseudoobscura		
San Bernardino, Calif.	SRSB3	XSB3
	SRSB4	XSB4
	SRSB6	XSB6
	SRSB7	XSB7
	SRSB9	XSB9
	SRSB10	XSB10
	SRSB13	XSB13
	SRSB16	XSB16
	SRSB18	XSB18
Sierra Mtns., Calif.	SRSM	XSM
Grand Canyon, Ariz.	SRGC	XGC
Tempe, Ariz.	SRTE	XTE
Albuquerque, N.M.	SRAL	XAL
Black Canyon, Colo.	SRAL	XAL
	SRBC	XBC
D. miranda		
Mather, Calif.		MIRMA83
		MIRMA23
Drosophila Center		MIRDC
D. persimilis		
Mather, Calif.		PER1
		PER2
		PER75
James Reserve, Calif.		PER
		PERJR105
British Columbia	SRPER	

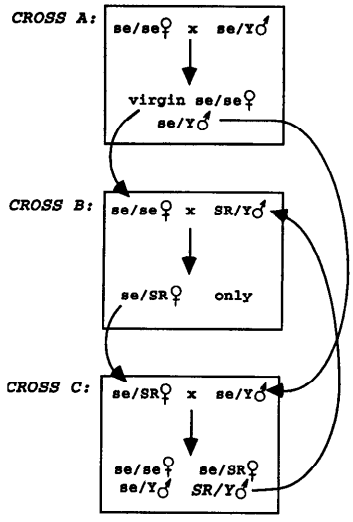

FIG. 1.—Maintenance scheme of Sex-Ratio stocks. SR indicates an X chromosome with the Sex-Ratio gene arrangement and *se* indicates an X chromosome with the *sepia* mutation. Three types of crosses are maintained: the female offspring of cross A are collected as virgins and used in cross B, and the male offspring of cross A are used in cross C; the offspring of cross B are all heterozygous females that are used in cross C; and among the offspring of cross C are Sex-Ratio males readily identified by wild-type eye color. These SR males are used to maintain cross B and also in the sequence analysis of the SR gene arrangement.

tional Park, in 1993. Five ST *D. persimilis* strains were used, three collected at Mather, Calif. (MA1, MA2, and MA75) and two at James Reserve, Calif. (PER and JR105), in the San Jacinto Mountains near Palm Springs, in 1977 and 1993, respectively. Unfortunately, we were only able to locate a single SR strain of *D. persimilis*. This strain, kindly provided by Gary Cobbs, was collected in British Columbia in the late 1980s.

In Situ Hybridization

A study by Brady, Richmond, and Oakeshott (1990) and work in this laboratory have previously localized *Est-5* to section 23 on the right arm of the X chromosome in *D. pseudoobscura*. To confirm that this section of the chromosome is located within one of the SR inversions of *D. pseudoobscura* and to localize *Est-5* in *D. persimilis*, the amplified sequence from the SR^{pse} chromosome of *D. pseudoobscura* strain SRBC was labeled with biotin-1-dUTP by nick translation and hybridized to salivary gland chromosomes of Sex-Ratio/Standard heterozygous larvae. Lim's (1992) protocol for hybridization and detection was followed using the ABC Elite Vector Laboratories Kit.

DNA Extraction

Genomic DNA from the Albuquerque, San Bernardino, and Tempe strains was extracted from freshly ground flies and purified by CsCl density gradient centrifugation according to Bingham, Levis, and Rubin (1981). For the other strains, genomic DNA was extracted from a single male fly following the protocol of Beckenbach, Wei, and Liu (1993).

PCR Amplification and Sequencing

The *Est-5* gene region in *D. pseudoobscura* is comprised of three tandem copies of the *Est-5* gene: *Est-5C, Est-5B,* and *Est-5A* (Brady, Richmond, and Oakeshott 1990). We sequenced a 504-bp fragment of noncoding intergenic DNA, 155 bp upstream of *Est5B,* from amplified PCR products.

The design of primers for amplification was based upon the published sequence of the *D. pseudoobscura Est-5* gene region (Brady, Richmond, and Oakeshott 1990). These primers (DE5C3′S1: 5′CGATAAGTCGAGCCTCTCTCTATG3′ and DE5B5′N1: 5′AACCAGTCTCAGGGGGATAGCTCT3′) amplify a 587-bp region of noncoding DNA located between genes *Est-5C* and *Est5B*. As internal sequencing primers, DE5C3′S2 (5′TTCCATTTATGTGCTAGCGG3′) and DE5B5′N2 (5′TTATACAAATATTTGT(A/G)ACG3′) were also designed on the basis of the sequence of Brady, Richmond, and Oakeshott (1990).

PCR amplification was carried out in 50-μl reactions using either 50 ng of CsCl-purified DNA or 1 μl of extracted DNA as template, 50 pM of each end primer, and 2.5 units of Promega Taq polymerase. Thermal cycling took place in either a Perkin Elmer Cetus or M. J. Research programmable thermocycler. Following an initial denaturation of 5 min at 94°C, each of 35 amplification cycles consisted of 94°C denaturation (1 min), 55°C annealing (1 min), and 72°C extension (1 min, 30 s) steps with a final extension of 7 min. Double-stranded product was diluted 150-fold and 5 μl served as template DNA for the asymmetric amplification of each strand. Primer concentrations were at 100:1 ratios and reaction conditions were as described above. Single-stranded product was column-purified using Centricon microconcentrators.

For 31 of the sequences, 7 μl of purified product were used in sequencing reactions following the protocol provided with the Sequenase 2.0 DNA Sequencing Kit (U.S.B.) with snap-cooling on dry ice/ethanol during primer annealing. Six sequences were determined using an automatic sequencer (Applied Biosystems 373A), following the manufacturer's protocol. For all strains both strands were sequenced.

Sequence Analysis

Alignments were made using the Genetics Computer Group (GCG) software package (1990), and differing sites were rechecked on the autoradiographs. The average number of pairwise divergences (K) and aver-

age heterozygosity per nucleotide site (H) were calculated as direct counts of the 469 nucleotides that were shared by all sequences. Estimated times of divergence were calculated using two methods of calibration. One was Caccone, Amato, and Powell's (1987) substitution rate of 1.7% nucleotide substitutions per million years. This estimate was determined from DNA–DNA hybridization studies of single-copy nuclear DNA and thus is likely a conservative estimate for noncoding *Drosophila* DNA. The other calibration was Sharp and Li's (1989) substitution rate of 3.2% sequence divergence per million years along two lineages. This rate was determined from the number of nucleotide substitutions per silent site among 10 *Drosophila* genes and may be the more appropriate rate for estimating divergence times from noncoding sequence data in *Drosophila.*

Relationships among nucleotide sequences were inferred from the GCG alignment of 469 shared bp using PHYLIP version 3.5c (Felsenstein, 1993). Kimura's two-parameter model for nucleotide differences was used to generate a corrected pairwise distance matrix with the transition/transversion ratio set at 2.0 in PHYLIP. Phylogenetic analysis utilized the neighbor-joining method (Saitou and Nei 1987) on the corrected pairwise distance matrix. One hundred replicates for bootstrap analysis were then generated using the SEQBOOT command in PHYLIP. Phylogenetic analysis by the neighbor-joining method was also performed using the Molecular Evolutionary Genetics Analysis (MEGA) package (Kumar, Tamura, and Nei 1993), with essentially the same results.

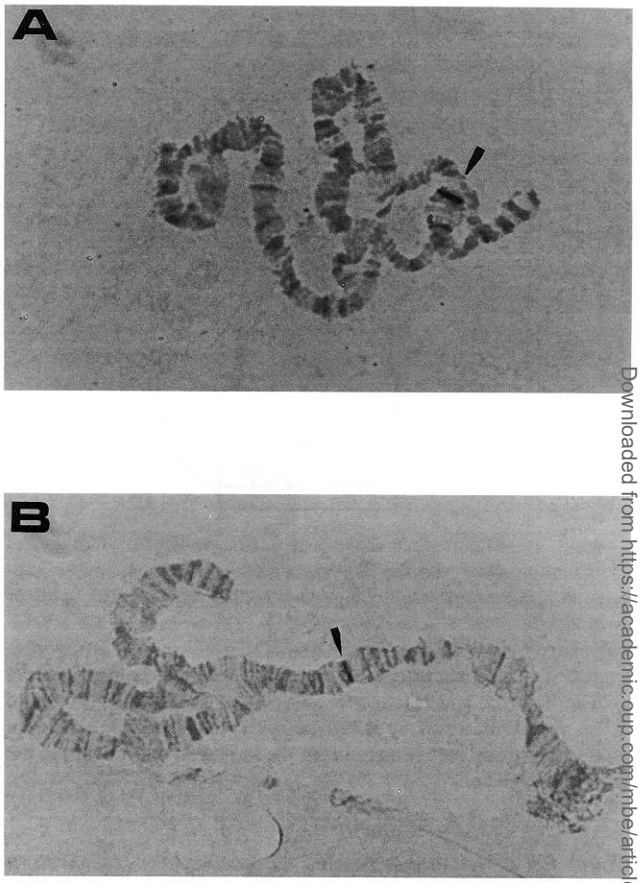

FIG. 2.—In situ hybridization of the *esterase-5* gene region to heterokaryotypes for the Sex-Ratio inversion complex of (*A*) *D. pseudoobscura* and (*B*) *D. persimilis.*

Results and Discussion

Localization of *Esterase-5*

In situ hybridization to *D. pseudoobscura* polytene chromosomes that were heterokaryotypic for the SR^{pse} and ST^{pse} gene arrangements localized the *Est-5* gene region to banding section 23 within the subbasal inversion of the Sex-Ratio inversion complex (fig. 2A). The location of this gene region inside an inversion, coupled with the lack of recombination between inverted gene arrangements in *D. pseudoobscura* (Sturtevant and Dobzhansky 1936; Dobzhansky and Epling 1948) gives us confidence that the *Est-5* gene region can be used to trace the evolutionary history of the Sex-Ratio inversion complex within *D. pseudoobscura.* In situ hybridization to *D. persimilis* showed that *Est-5* is located three banding sections outside of the single *D. persimilis* inversion that is associated with the Sex-Ratio trait in this species (fig. 2B).

The Data Set

Among the 37 sequences compared there were four insertions/deletions. The lengths of the resulting gaps varied from 4 bp to 19 bp (see sequence alignment in fig. 3). One of the gaps was variable in length. Only six of the sequences, all from SR^{pse} chromosomes, had the 4 bp at position 400–404, whereas ST^{pse} from strain XSB9 was missing these 4 bp plus an additional 2 bp. It is interesting that 5 bp (nucleotides number 160–164) were absent from all of the SR^{pse} chromosomes but present in all of the ST^{pse} chromosomes and in the chromosomes of the outgroup species. These data lead us to hypothesize that these bases were present in the ancestral chromosome and that the deletion of these 5 nucleotides was captured by the SR^{pse} inversion.

Ignoring gaps, there were 469 shared base pairs in the 504-bp fragment that was sequenced. Of the 469 shared sites, 27 were polymorphic among the *D. pseudoobscura* chromosomes sequenced. The distribution of these polymorphic sites was not uniform across the fragment studied ($\chi^2 = 38.47$; $df = 18$; $P < 0.005$), as 60% of the polymorphic sites occurred within 100 bp in the central region of study (see fig. 4). A sequence analysis of the *Est-5B* coding and associated flanking regions

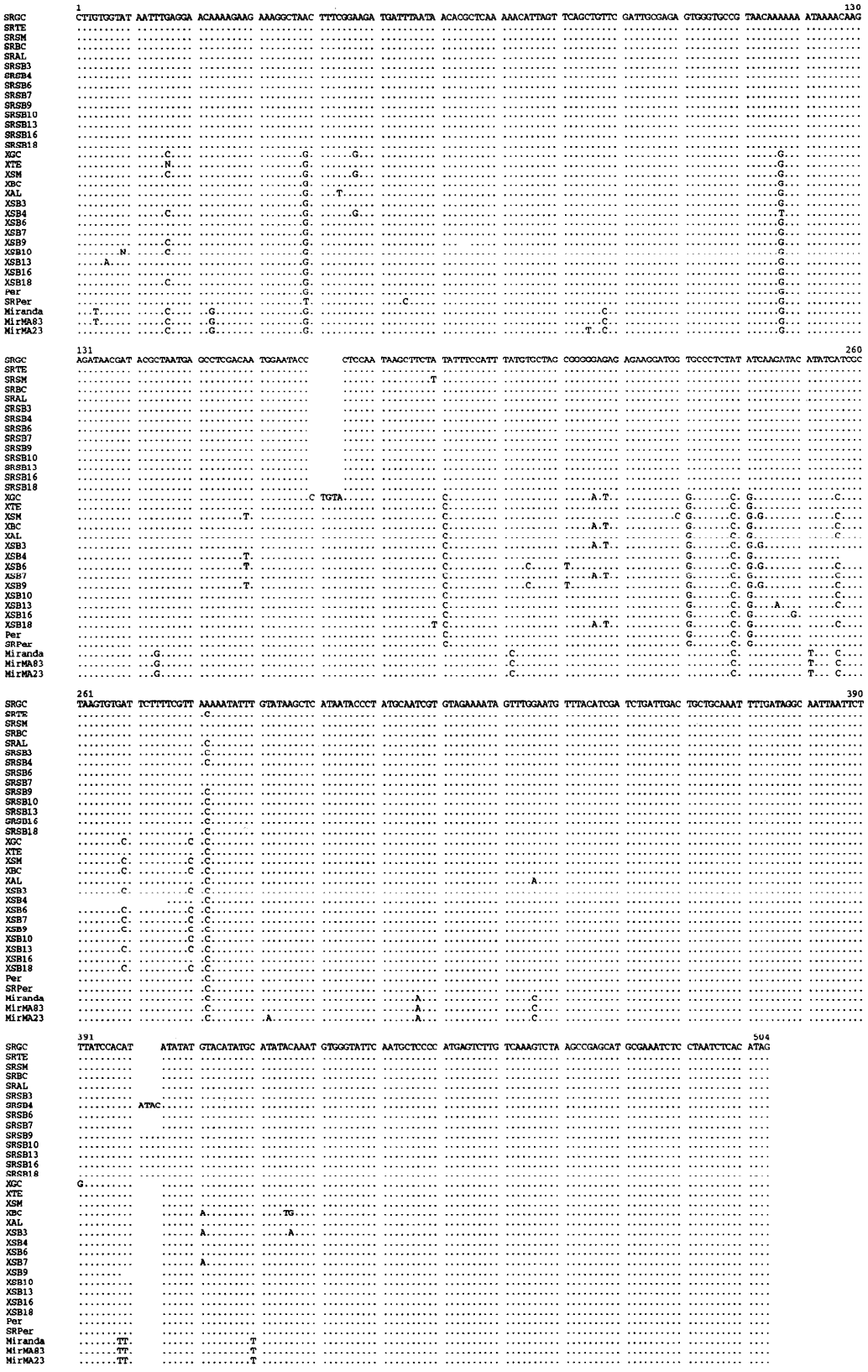

FIG. 3.—Alignment of sequenced region corresponding to positions −659 to −155 upstream of *Est-5B*. Dots (.) indicate sequence identity in other sequences compared with SRGC. This region was amplified by PCR from the genomic DNA of 37 *Drosophila* strains and then sequenced. Names of sequences are defined in table 1.

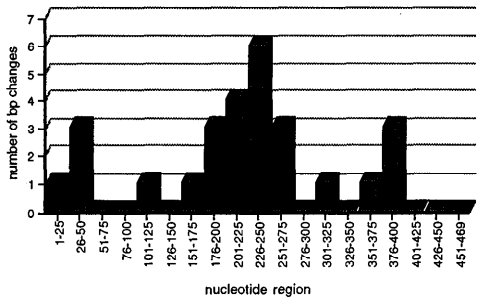

FIG. 4.—Nonrandom distribution of variable sites in different segments of the sequenced *esterase-5* region ($\chi^2 = 38.47$; $df = 18$; $P < 0.005$). *Drosophila pseudoobscura* sequences only are considered, and all gaps have been ignored.

from SR^{pse} and ST^{pse} lines of *D. pseudoobscura* (our laboratory, unpublished data) showed an increase in the proportion of substitutions in the flanking regions when compared to the coding region, indicating that there is less functional constraint acting on the flanking regions of *Est-5B*. The nonuniform pattern of substitution in the intergenic segment we studied suggests that this region of DNA may nonetheless be under some selective constraints, a possibility that is not surprising because this region is located upstream of the functional copy of *Est-5* and may contain regulatory information (Brady, Richmond, and Oakeshott 1990). Interestingly, there is a palindromic region (ATATG-CATAT) of 10 bp located at positions 415–424 that was conserved among the *D. pseudoobscura* chromosomes but not the *D. miranda* chromosomes. The perfect palindrome discussed by Brady, Richmond, and Oakeshott (1990), our sites 407–418, contained one site substitution in three of the *D. pseudoobscura* lines surveyed here. Mutations in the homologous region of *D. melanogaster* appear to have decreased levels of enzyme activity, illustrating the potential regulatory function of this region (Oakeshott, Healey, and Game 1990).

Of the polymorphic sites, 21 were shared between the SR^{pse} and ST^{pse} gene arrangements of *D. pseudoobscura,* while there were six fixed differences for one or more nucleotide type. The outgroup species *D. miranda* shared three of the fixed-site differences with the ST^{pse} chromosomes and an equal number of fixed-site differences with the SR^{pse} chromosomes. Thus, our data do not allow us to infer whether SR^{pse} or ST^{pse} is the ancestral chromosomal type.

Nucleotide Diversity

The average number of pairwise differences (K) among the 28 *D. pseudoobscura* chromosomes we studied was 7.02. The mean heterozygosity was 1.5% with a standard error of 0.5%; some chromosomes were identical in sequence while others diverged by as much as 3.2%. This level of sequence divergence is comparable

Table 2
Average Number of Pairwise Differences (K) and Heterozygosity per Nucleotide Site (H) ± Its Standard Error (SE)

Comparison	*n*	K	H ± SE
D. pseudoobscura			
Sex-Ratio vs. Sex-Ratio ..	91	0.63	0.0013 ± 0.0012
Standard vs. Standard	91	5.75	0.0123 ± 0.0049
Sex-Ratio vs. Standard ...	196	10.56	0.0230 ± 0.0068
All X chromosomes	378	7.02	0.0150 ± 0.0050
D. persimilis			
Sex-Ratio vs. Standard ...	5	3.0	0.0063 ± 0.0037
D. miranda			
Standard vs. Standard	3	2.0	0.0043 ± 0.0024
D. miranda vs. Sex-Ratio ...	42	14.76	0.0324 ± 0.0081
D. miranda vs. Standard	42	17.05	0.0363 ± 0.0086

NOTE.—Total number of sites compared: $n = 469$; heterozygosity per nucleotide site: $H = K/N$; n = number of comparisons.

to that found in other gene regions of *D. pseudoobscura.* Heterozygosity estimates from restriction site data for the *Amy* and *Adh* regions are 1.81% and 2.6%, respectively, while the mean heterozygosity estimated from sequence analysis of the Xdh region is 1.2% (Schaeffer, Aquadro, and Anderson 1987; Aquadro et al. 1991; Riley, Kaplan, and Veuille 1992).

There is a substantial amount of nucleotide diversity, 2.30%, between the SR^{pse} and ST^{pse} chromosomal types (see table 2). However, within 91 pairwise comparisons of the SR^{pse} chromosomal sequences the average heterozygosity was 0.13%, and many of the SR^{pse} sequences were identical. For the 14 SR^{pse} chromosomes studied there were only three different sequence patterns, which we have designated as separate alleles. There does not appear to be any geographical pattern associated with the sequence identity among SR^{pse} chromosomes. For example, one allele, represented nine times in our sample, was found at the San Bernardino and Albuquerque sites. A second allele, represented four times in our sample, was found at the San Bernadino, Grand Canyon, and Black Canyon sites. In contrast, only two of the 14 ST^{pse} alleles were identical in sequence.

The average heterozygosity in 91 comparisons of the ST^{pse} sequences was 1.23%, which is 10-fold greater than that among SR^{pse} sequences. This increased level of heterozygosity is not unexpected for either (or both) of two reasons. First, when all mutations are neutral, the expected value of average heterozygosity is directly proportional to population size (Kimura 1968; Nei 1987, *pp.* 256–257). Because there is little recombination between chromosomes (Sturtevant and Dobzhansky 1936), each inverted gene arrangement can be considered to be

a "population." In natural populations of *D. pseudoobscura* the SR^{pse} chromosome is present in frequencies of 0–25%; thus, on average there are approximately six to seven times more ST^{pse} chromosomes than SR^{pse} chromosomes (Sturtevant and Dobzhansky 1936). Assuming all mutations are neutral and invoking the neutral theory, an increased amount of variation among ST^{pse} chromosomes compared to that among SR^{pse} chromosomes is expected due to the greater number of ST^{pse} chromosomes.

A second explanation for the differences in heterogeneity of the *Est-5* region within each chromosomal type is that selective sweeps may have decreased the level of variation among the SR^{pse} chromosomes. If there is a strongly selected allele at one locus, the neutral alleles at linked loci may be affected due to the hitchhiking effect (Maynard Smith and Haigh 1974; Kaplan, Hudson, and Langley 1989; Begun and Aquadro 1991; Stephan, Wiene, and Lenz 1992). The hitchhiking model predicts that in gene regions with low recombination rates, a strongly selected allele can sweep throughout populations and effectively reduce heterozygosity at linked neutral sites. The size of the region affected depends upon the strength of selection and the level of recombination (Stephan, Wiene, and Lenz 1992). For example, highly reduced variation has been found at the base and the tip of the X chromosome in several *Drosophila* species where crossing-over is heavily restricted at the centromeric and telomeric regions (Aguadé, Miyashita, and Langley 1989; Stephan and Langley 1989; Begun and Aquadro 1991, 1995). It is conceivable that the Sex-Ratio loci are under strong selection and that they are associated with very low levels of recombination due to the presence of the inversion complex and the low frequencies of SR^{pse} chromosomes found in natural populations. Perhaps then, a selective sweep occurred whenever new a Sex-Ratio form with a higher level of meiotic drive replaced a less efficient Sex-Ratio form. Selective sweeps may have happened several times in the evolution of the Sex-Ratio chromosome as each inversion was added to the complex, and they could be the source of the decreased variation among the *Est-5* alleles of SR^{pse} chromosomes. Although both the neutral theory and the selective sweep hypothesis fully explain the data, they are not mutually exclusive and both could have operated.

Our nucleotide sequence data are consistent with previous electrophoretic data from the X chromosome (Prakash and Merritt 1972; Prakash 1974; Keith 1983). Electrophoretically, the SR^{pse} and ST^{pse} gene arrangements differ significantly from each other at all four X chromosome loci that were studied; *esterase-5, adult acid phosphatase-6, phosphoglucomutase-1,* and *octanol dehydrogenase-3* (Prakash 1974). Keith's (1983) series of five sequential electrophoretic conditions characterizing the *Est-5* locus revealed 41 alleles in the ST^{pse} gene arrangement. Only three alleles were present in the SR^{pse} gene arrangement. In addition to the lack of genetic diversity, none of the alleles were shared between SR^{pse} and ST^{pse}, indicating extensive genetic differentiation between the two gene arrangements.

Further evidence supporting the extensive genetic differentiation between the two chromosomal arrangements comes from studies of reciprocal introgression between the sister species *D. pseudoobscura* and *D. persimilis* (Wu and Beckenbach 1983). The ST^{pse} chromosome in *D. pseudoobscura* is homosequential to the SR^{per} chromosome in the sibling species *D. persimilis.* Because hybrid females between these two species are fertile, Wu and Beckenbach were able to introgress the SR^{per} X of *D. persimilis* into *D. pseudoobscura* and, reciprocally, the ST^{pse} X of *D. pseudoobscura* into *D. persimilis.* Mutant alleles are available for marking several regions along the ST^{pse} chromosome in *D. pseudoobscura.* Recombination between the homosequential ST^{pse} and SR^{per} led to chromosomes with one or several marked segments of the ST chromosomes recombined with the SR chromosomes. When the male recombinant offspring were tested for the presence of the SR trait, at least four regions covering all three of the SR^{pse} inversions were determined to be necessary for expression of the Sex-Ratio phenotype. This finding indicates the presence of at least four sr loci and provides supporting evidence for extensive genetic differentiation between the SR^{pse} and ST^{pse} gene arrangements in *D. pseudoobscura.*

It is interesting to note that, by comparison to *D. pseudoobscura,* the sibling species *D. miranda* and *D. persimilis* seem to have lower levels of within-species polymorphism. In the three chromosomes of *D. miranda* studied, two were identical in sequence and the third differed by only 3 of the 469 sites. Surprisingly, all five of the ST^{per} chromosomes of *D. persimilis* were identical in sequence, and the SR^{per} chromosome of *D. persimilis* differed from them by only 2 nucleotides.

Estimated Divergence Time

Prakash and Merritt (1972) suggested that the SR^{pse} gene arrangement is probably quite old. Their idea is motivated by several factors. First, the Sex-Ratio chromosome has a wide geographic distribution and has been found in natural populations throughout much of the species range (Dobzhansky and Epling 1944, *pp.* 96–101; Anderson, Dobzhansky, and Kastritsis 1967). Second, although there are seasonal fluctuations of SR^{pse} in some localities (Dobzhansky 1943; Epling, Mitchell, and Mattoni 1957), studies of natural populations have suggested fairly stable frequencies of the Sex-Ratio gene

arrangement in other localities over a 30-year period (Dobzhansky 1958). Third, if each inversion arose as a single event in evolutionary history, the accumulation of three nonoverlapping inversion differences between the ST^{pse} and SR^{pse} chromosomes suggests a substantial time since common ancestry.

Assuming a constant rate of substitution, we estimated the time at which the two chromosomal types diverged, based upon the sequence divergence information presented here for the subbasal inversion of the Sex-Ratio complex. Using Caccone, Amato, and Powell's (1987) substitution rate of 1.7% sequence divergence per million years along two lineages, the estimated time of divergence between the SR^{pse} and ST^{pse} chromosomes is 1.3 million years. Using this calibration rate, the estimated time of common ancestry for *D. pseudoobscura* and *D. miranda* is 2.0 Mya or about 6 million generations, assuming three generations per year, whereas the estimated time of common ancestry for *D. pseudoobscura* and *D. persimilis* is only 0.7 Mya or about 2.1 million generations. These estimates are congruent with the estimates of Aquadro et al. (1991) that place the *D. miranda/D. pseudoobscura* divergence at 2.1 Mya and the *D. persimilis/D. pseudoobscura* divergence at as little as 0.5 Mya. However, Caccone, Amato, and Powell's (1987) calibration is based upon a substitution rate estimated from DNA–DNA hybridization studies of single-copy nuclear DNA and consequently may be slower than expected for the noncoding region studied here. The substitution rate estimated by Sharp and Li (1989) for synonomous base pair differences only is likely to be more appropriate for noncoding sequence data. Using Sharp and Li's (1989) calibration of 3.2% nucleotide substitutions per million years, we estimate that the SR^{pse} and ST^{pse} gene arrangements shared a common ancestry 700,000 years ago.

Due to the nonuniform distribution pattern of nucleotide substitutions (fig. 4), we suggested earlier that this region of study may be under some selective constraint. Consequently, neither Caccone, Amato, and Powell's nor Sharp and Li's substitution rates is ideal for the calculation of divergence times. However, we can take these estimates as upper and lower bounds and suggest a fairly ancient window of time between 0.7 and 1.3 Mya for the divergence of the two gene arrangements. With confidence we can then state that these two inversion types have been separated for at least 2 million generations.

Our estimate for divergence time of the Sex-Ratio inversion polymorphism is similar to the estimate for the divergence of the third chromosome inversion polymorphism in *D. pseudoobscura.* Using restriction site data for the *amylase* gene region, Aquadro et al. (1991) estimated that the Standard and Tree Line inversion families diverged 1.7 Mya and that the Standard and Santa Cruz inversions diverged 1.0 Mya. Both the X and third chromosome inversion systems are fairly old, and *D. pseudoobscura* has been polymorphic for both of these inversion systems for several million generations.

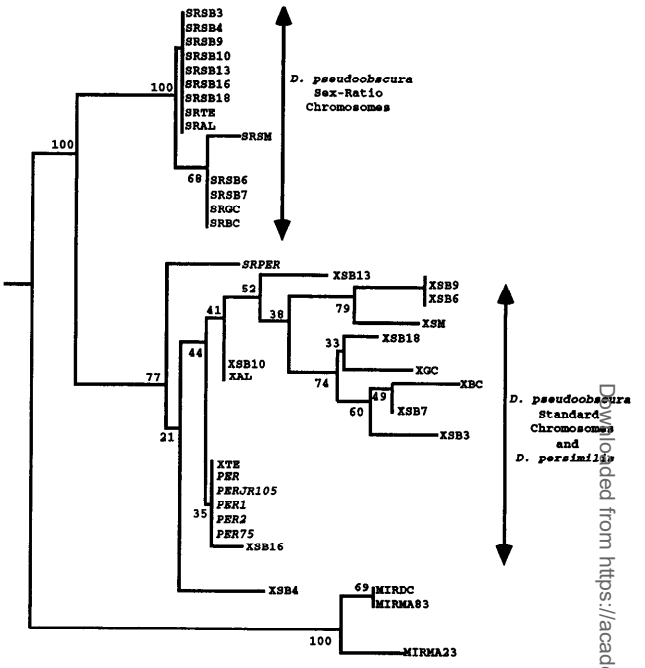

FIG. 5.—Phylogenetic tree of the amplified and sequenced *esterase-5* region from 37 *Drosophila* strains constructed by neighbor-joining using Phylip 3.5. Taxa are named as in table 1. Numbers indicate the percentage of time each branch was joined together under bootstrap analysis. *D. persimilis* strains are in italics and arrows indicate the Sex-Ratio and Standard chromosomal clusters of *D. pseudoobscura.*

Sequence Relatedness

A neighbor-joining tree produced by PHYLIP 3.5 is presented in figure 5. Similar trees were produced by neighbor-joining using the MEGA package. There is extensive branching and long branch lengths in the ST^{pse} clade of this phylogeny, while within the SR^{pse} cluster there is little evidence for differentiation between chromosomes. As discussed above (in Nucleotide Diversity), these findings are not unexpected and can be explained by either the neutral theory or the selective sweep hypothesis.

Bootstrap analysis strongly supports the deep branches dividing the SR^{pse} and ST^{pse} clades, as the node connecting these two clades is present in all 100 replicates. From the sequence phylogeny, we infer that the basal inversion of the Sex-Ratio gene arrangement arose only once in evolutionary history, and we infer that the entire SR^{pse} gene arrangement, and the associated meiotic drive system, have a monophyletic origin in *D. pseudoobscura.* It is also evident from the phylogeny that the Sex-Ratio system in *D. pseudoobscura* is fairly

old and that the basal inversion in *D. pseudoobscura,* containing the *Est-5* region, arose prior to the split of *D. pseudoobscura* and *D. persimilis.* This situation is similar to the emergence of t-haplotypes in *Mus,* another example of meiotically driven chromosomes associated with inversions. DNA analysis of t-haplotype and wild-type chromosomes suggests that they diverged from a common ancestor at least before the *musculus–domesticus* split 1–2 million years ago (reviewed in Lyttle [1991]).

Drosophila persimilis is a closely related sibling species of *D. pseudoobscura.* There is a single inversion difference between the SR^{per} and ST^{per} chromosomes of *D. persimilis* and, interestingly, the SR^{per} chromosome in *D. persimilis* is homosequential to the ST^{pse} chromosome in *D. pseudoobscura.* In situ hybridization to heterokaryotypic polytene chromosomes demonstrates that the *Est-5* gene region used in this study is not contained within the inversion associated with SR^{per} in *D. persimilis* (fig. 2b). While it is unlikely that this region is in complete linkage disequilibrium, suppression of recombination often extends well beyond the ends of inversions due to the physical disruption of synapsis (Roberts 1976, *pp.* 118–119). Beckenbach (1981) studied the linkage relationship between *Est-5* and several visible mutants and demonstrated that *Est-5* lies between *forked* and *short.* The recombination frequency between these two markers in *D. persimilis* heterokaryotypes (SR^{per}/ST^{per}) is very low, with only 4.4% recombinants observed in a total of 1,170 chromosomes and in fact is not significantly different (and even numerically smaller) than the map distance measured in *D. pseudoobscura* heterokaryotypes (SR^{pse}/ST^{pse}) (Sturtevant and Dobzhansky 1936). Although we cannot be certain that recombination did not occur between alleles of ST^{per} and SR^{per}, *Est-5* is potentially informative regarding the relationship of the ST^{per} and SR^{per} chromosomes within *D. persimilis,* and we believe it remains an informative locus for studying the relationship of X chromosomes between *D. persimilis* and *D. pseudoobscura.*

Unfortunately we were only able to include a single SR^{per} chromosome in our analysis. However, when we look at the relationship of *D. persimilis* chromosomes in the neighbor-joining tree presented in figure 5, we find that both ST^{per} and SR^{per} cluster with the ST^{pse} chromosomes, the SR^{pse} chromosomes of *D. pseudoobscura* having diverged much earlier, as discussed above. The clustering of *D. persimilis* chromosomes with ST^{pse} is supported by the presence of bp 160–164 in the *D. persimilis* and ST^{pse} chromosomes, and by the absence of these base pairs in all SR^{pse} chromosomes. Also, an examination of the character state of the six differences that are fixed for one or more nucleotide between the ST^{pse} and SR^{pse} chromosomes (sites 49, 117, 191, 231, 239, 241), reveals that both SR^{per} and ST^{per} of *D. persimilis* share the character state of the ST^{pse} chromosomes.

Of the SR^{per}, ST^{per}, and ST^{pse} chromosomes that form a clade, the SR^{per} chromosome evidently branches off first (with a supporting bootstrap value of 77), while the branch lengths leading to the ST^{per} chromosomes are relatively short and the nodes not well supported by bootstrap analysis. To analyze this clade more closely, we have listed nucleotide sites that are polymorphic on the ST^{pse} chromosomes and compared them with the ST^{per} and SR^{per} chromosomes of *D. persimilis* in table 3. At nucleotide site 117 the ST^{per} and SR^{per} chromosomes have the same character state, G, as do most of the ST^{pse} chromosomes, while these chromosomes all differ from SR^{pse}, which has character state A. Guanine appears to be the ancestral state at this site because it is shared with the outgroup species *D. miranda,* and there was most likely a G to A base pair change along the lineage leading to the SR^{pse} chromosomes and a G to T base pair change in the XSB4 lineage. More important, however, is the fact that at all 22 of the other sites that are polymorphic on the ST^{pse} chromosomes, both ST^{per} and SR^{per} always have the character state of the more distantly related SR^{pse} chromosome of *D. pseudoobscura.* This character state analysis along with the phylogenetic analysis suggests that SR^{per} is the outgroup to all ST chromosomes of both *D. persimilis* and *D. pseudoobscura.*

The relationship of ST^{per} chromosomes is not as clearly defined. Although the bootstrap support is weak, the ST^{per} chromosomes of *D. persimilis* appear to be more closely related to the ST^{pse} chromosomes of *D. pseudoobscura* than to SR^{per}, and, in fact, they are identical to one *D. pseudoobscura* strain (XTE). The indication that the ST^{per} gene arrangement is more closely related to the ST^{pse} gene arrangement than to other *D. persimilis* chromosomes is puzzling, and it raises the possibility that the species divergence of *D. persimilis* occurred very early in the differentiation of the ST^{pse} chromosomes. Additional data on SR^{per} chromosomes are needed to test this hypothesis.

Evolution of the Sex-Ratio System

Wu and Hammer (1991) have provided an extensive review of the molecular evolution of meiotic drive systems, including Sex-Ratio in *D. pseudoobscura.* Because there is some evidence that *D. persimilis* and *D. pseudoobscura* can hybridize in nature, Wu and Hammer (1991) suggested that perhaps it is "... possible that the X_r (*our* SR^{pse}) of *D. pseudoobscura* came from *D. persimilis* by introgression, and subsequently acquired its inversions." Although our analysis of Sex-Ratio in *D. persimilis* is based on only one SR^{per} chro-

Table 3
Individual Sites of the *D. persimilis* SR (SRPER) and ST (PER) Chromosomes Compared with the Polymorphic Sites of the *D. pseudoobscura* ST Chromosomes (X. . .) and One Representative of the *D. pseudoobscura* SR Chromosomes (SRGC)

Nucleotide no.	6	1 6	4 4	4 7	1 1 7	1 5 9	1 9 0	2 0 5	2 1 1	2 1 6	2 1 8	2 3 0	2 4 3	2 4 6	2 4 9	2 5 6	2 6 9	2 8 0	3 3 6	3 9 1	4 1 1	4 2 5	4 2 6
SRGC	**G**	**G**	**C**	**A**	**A**	**A**	**A**	**T**	**C**	**G**	**G**	**G**	**C**	**G**	**A**	**A**	**A**	**T**	**G**	**T**	**G**	**A**	**C**
XGC	G	C	C	G	G	A	A	T	C	A	T	G	C	G	A	C	C	C	G	G	G	A	C
XTE	G	N	C	A	G	A	A	T	C	G	G	G	C	G	A	A	A	T	G	T	G	A	C
XSM	G	C	C	G	G	T	A	T	C	G	G	C	G	G	A	C	C	C	G	T	G	A	C
XBC	G	G	C	A	G	A	A	T	C	A	T	G	C	G	A	C	C	C	G	T	A	T	G
XAL	G	G	T	A	G	A	A	T	C	G	G	G	C	G	A	C	A	T	A	T	G	A	C
XSB3	G	G	C	A	G	A	A	T	C	A	T	G	G	G	A	A	C	C	G	T	A	A	A
XSB4	G	C	C	G	T	T	A	T	C	G	G	G	C	G	A	A	—	T	G	T	G	A	T
XSB6	G	G	C	A	G	T	A	C	T	G	G	G	G	G	A	C	C	C	G	T	G	A	C
XSB7	G	G	C	A	G	A	A	T	C	A	T	G	C	G	A	C	C	C	G	T	A	A	C
XSB9	G	C	C	A	G	T	A	C	T	G	G	G	G	G	A	C	C	C	G	T	G	A	C
XSB10	G	C	C	A	G	A	A	T	C	G	G	G	C	G	A	C	A	C	G	T	G	A	C
XSB13	A	G	C	A	G	A	A	T	C	G	G	G	C	A	A	C	C	C	G	T	G	A	C
XSB16	G	G	C	A	G	A	A	T	C	G	G	G	C	G	G	A	A	T	G	T	G	A	C
XSB18	G	C	C	A	G	A	T	T	C	A	T	G	C	G	A	C	C	C	G	T	G	A	C
Per	**G**	**G**	**C**	**A**	**G**	**A**	**A**	**T**	**C**	**G**	**G**	**G**	**C**	**G**	**A**	**A**	**A**	**T**	**G**	**T**	**G**	**A**	**C**
SRPER....	**G**	**G**	**C**	**A**	**G**	**A**	**A**	**T**	**C**	**G**	**G**	**G**	**C**	**G**	**A**	**A**	**A**	**T**	**G**	**T**	**G**	**A**	**C**

mosome, our evidence indicates that the Sex-Ratio inversion complex in *D. pseudoobscura* was acquired prior to the split of these two sibling species. If indeed the origin of SR chromosome in the species ancestral to *D. pseudoobscura* and *D. persimilis* predates the species divergence, then the SR^{pse} chromosome did not introgress from *D. persimilis.*

One can hypothesize a simple scenario for the evolution of Sex-Ratio chromosomes within *D. pseudoobscura.* As suggested by the model of modifier genes proposed by Wu and Beckenbach (1983), the sr alleles, although not the SR^{pse} gene arrangement, may have been present in the ancestral population that later split to give *D. pseudoobscura* and *D. persimilis.* Only rarely in this ancestral population would the sr alleles have been expressed together because they were not yet linked by inversions. The three inversions of the SR^{pse} gene arrangement probably arose in three independent events. When heterozygous, each inversion reduced recombination between the sr genes within it, and each successive inversion reduced recombination over a larger part of the X chromosome. Once the inversions were present, the combined effect of the several sr alleles caused meiotic drive to occur, and the SR^{pse} chromosome increased to noticeable frequencies in the ancestral population.

However, the evolution of the Sex-Ratio system in *D. persimilis* is much more difficult to understand. Our evidence indicates that both gene arrangements in *D. persimilis* are derived from the ST^{pse} chromosome in the ancestral species, the X chromosome now most frequent in *D. pseudoobscura.* The ST^{pse} chromosome is homosequential to the SR^{per} chromosome, and ST^{per} differs from SR^{per} by a single inversion. To hypothesize the evolution of sr alleles in *D. persimilis,* we must propose a different mechanism than the one proposed for *D. pseudoobscura* because the inversion separating the two X chromosomes in *D. persimilis* is on the ST^{per} gene arrangement. Wu and Beckenbach (1983) suggested that the occurrence of an inversion on either chromosome reduces recombination between the inverted chromosomes and is sufficient for the evolution of SR chromosomes possessing all of the sr genes at the several loci necessary for the SR trait to be expressed. We are not sure that this reduction of recombination *between* ST^{per} and SR^{per} is sufficient to explain how the sr alleles became fixed on the uninverted SR^{per} chromosome as there would still be recombination *within* the population of uninverted chromosomes that would separate the sr alleles. If SR^{pse} were acquired prior to the species divergence, the scenario proposed by Wu and Beckenbach (1983) does not fully explain how the sr alleles remaining in the ST^{pse} gene arrangement of the *pseudoobscura–persimilis* ancestral species were tied together to form the homosequential SR^{per} gene arrangement in *D. persimilis* after the two species diverged. Our molecular data have provided new information on the origins of the Sex-Ratio system of meiotic drive and the associated inversions, but intriguing questions still remain concerning the evolution of this complex system in *D. persimilis.*

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