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Single-strand conformation polymorphism analysis coupled with stratified DNA sequencing reveals reduced sequence variation in the *su(s)* and *su(w^a)* regions of the *Drosophila melanogaster* X chromosome

MONTSERRAT AGUADÉ[†], WENDY MEYERS[‡], ANTHONY D. LONG[‡§], AND CHARLES H. LANGLEY[‡]

[†]Departament de Genètica, Facultat de Biologia, Universitat de Barcelona, 08071 Barcelona, Spain; [‡]Center for Population Biology and the Section of Evolution and Ecology, University of California, Davis, CA 95616; and [§]Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario, Canada L8S 4K1

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ABSTRACT **Single-strand conformation polymorphism (SSCP) analysis followed by DNA sequencing of stratified subsamples was used to survey DNA polymorphism in the *su(s)* and *su(w^a)* regions in a natural population of *Drosophila melanogaster*. *su(s)* and *su(w^a)* are located near the telomere of the X chromosome, where the rate of crossing over per kilobase of DNA monotonically decreases toward the tip. SSCP was assessed in 12 noncoding segments amplified from the *su(s)* region (3213 bp) and in 8 noncoding segments amplified from the *su(w^a)* region (1955 bp). Sets of segments were multiplexed in a single electrophoretic lane to increase the number of base pairs assayed per lane. Eight segments were monomorphic, and the other 12 segments exhibited two to four SSCP classes. Only four within-SSCP-class DNA sequence differences (a single nucleotide substitution) were observed among 24,360 bp compared within classes. The between-SSCP-class DNA sequence comparisons revealed 27 substitutions and 9 insertion/deletion polymorphisms. The average numbers of substitutional differences per site were 0.0010 and 0.0021 for *su(s)* and *su(w^a)*, respectively. These values are intermediate between those reported for the more distal *y–ASC* region (0.0004) and the more proximal *Pgd* locus (0.0024). This observation is consistent with the prediction of the hitchhiking-effect model—i.e., a monotonic increase in polymorphism as a function of crossing over per kilobase.**

In *Drosophila*, as in many other organisms, the genetic map is reduced relative to the physical map near the centromeres and some of the telomeres. Several studies of *Drosophila melanogaster* (1–7), *D. simulans* (2–5), and *D. ananassae* (8–10) have shown relatively little polymorphism in these regions of little or no crossing over per physical length of the chromosome. These observations are consistent with predictions of the hitchhiking-effect model (11–13), in which rare, selectively favored variants rapidly go to fixation in a population. The particular DNA haplotype surrounding the selectively fixed variant is also swept to fixation; polymorphisms near to the selected site are eliminated. Since the physical size of the fixed region associated with a given selective sweep is greater in regions of low crossing over per physical length than in those of normal levels of crossing over, we can expect the standing level of polymorphism in these regions to be reduced (12). Alternatively, the cumulative effect of deleterious mutants at many linked loci has been proposed as a possible cause of observed reduction in DNA sequence polymorphism in regions of reduced crossing over (14).

Surveys of variation in genomic regions of reduced crossing over have so far focused on domains with no measurable crossing over. Attempts (7, 13) to quantitatively correlate levels of DNA sequence polymorphism with crossing over per unit physical length indicated that more quantitative studies in regions where crossing over can be measured may allow the predictions of the hitchhiking-effect model to be more rigorously tested. To this end, we are surveying DNA sequence polymorphism in several regions near the telomere of the X chromosome of *D. melanogaster*. Detection of all DNA sequence polymorphism in such a region where polymorphism is severely reduced requires much effort. Direct DNA sequencing is the method of choice, since it gives complete information. But that method is not amenable to the rapid survey of large regions and a large number of alleles. A more efficient technique is needed. We decided to apply the single-strand conformation polymorphism (SSCP) procedure (15) to survey variation in two regions, *su(s)* and *su(w^a)*, near the tip of the X chromosome. After alleles were sorted into SSCP classes, the DNA sequences of subsamples (stratified into SSCP classes) were determined. Our survey of >5 kb in 50 X chromosomes from a natural population of *D. melanogaster* shows that SSCP coupled with stratified DNA sequencing is an efficient and reliable method for the study of DNA sequence variation in regions of low polymorphism. The observed values of polymorphism at *su(s)* and *su(w^a)* are intermediate between those observed for more distal and proximal loci.

MATERIALS AND METHODS

Fifty independent isogenic X chromosome lines of *D. melanogaster* extracted from a North American population (Raleigh, North Carolina) were used (16). Genomic DNA was prepared by either of two methods (17, 18).

Fig. 1 shows the PCR-amplified segments of noncoding sequence, which totalled 3213 and 1955 bp for the *su(s)* and *su(w^a)* regions (not counting the length of the primers), respectively. The 20 pairs of PCR amplification primers (20–22 nt) were each separated by 161–358 bp (Figs. 1 and 2).

The PCR products were labeled by incorporation of [α-[^{35}S]thio]dATP (0.05 μCi/μl, >1000 Ci/mmol; 1 Ci = 37 GBq) in presence of 50 μM dCTP, dGTP, and dTTP and 25 μM dATP. Amplification conditions were 92°C (45 sec) for denaturing, 62°C (45 sec) for annealing (58°C for primer sets 5 and 7), and 72°C (45 sec) for extension over 30 cycles. The products of several PCRs that migrated to different positions in the nondenaturing polyacrylamide gel (Hydrolink MDE gel, J. T. Baker) were chosen to be loaded in the same lane. Electrophoresis was carried out in a 0.6× TBE/0.5× MDE gel in a standard (40 cm × 30 cm × 0.04 cm) "sequencing" chamber with 0.6× TBE in the upper and lower buffer reservoirs. (TBE is 89 mM Tris/89 mM boric acid/2 mM

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Abbreviation: SSCP, single-strand conformation polymorphism.

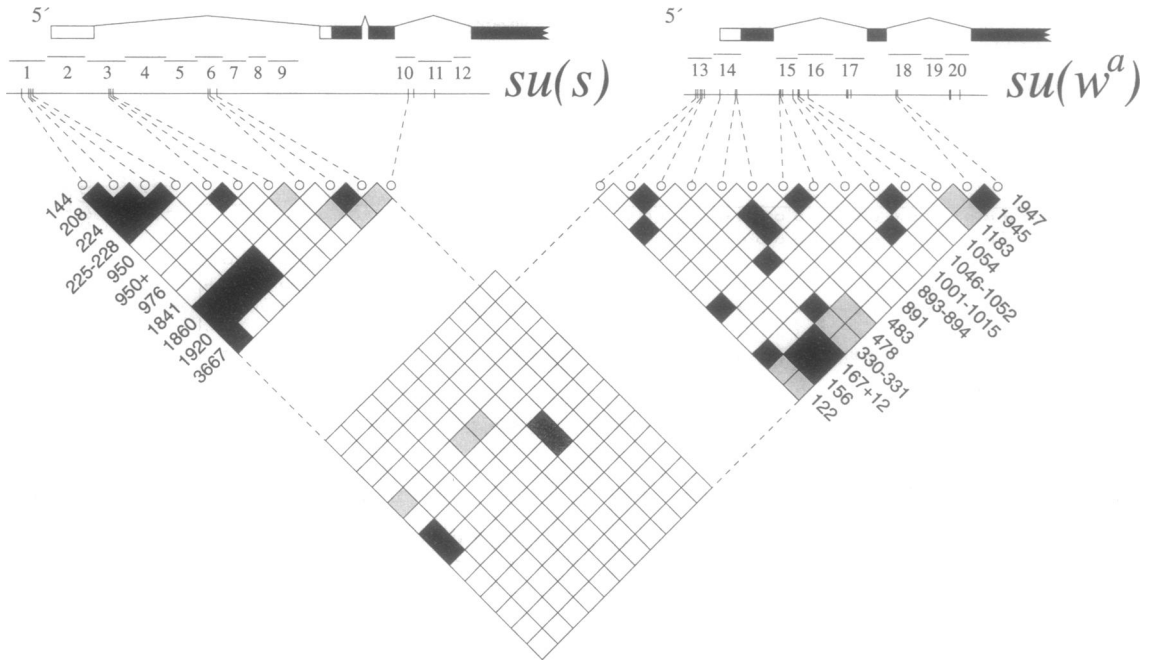

FIG. 1. Map of the *su(s)* and *su(w^a)* regions. The boxes indicate exons (filled boxes are coding). The short bars below the map show the positions of the segments 1–20. The ticks on the line indicate the positions of the polymorphisms detected in the study (see Fig. 3*b*). Below the map is a matrix indicating the statistical significance of linkage disequilibrium: black, $P < 0.005$; dark gray, $P < 0.01$; light gray, $P < 0.05$.

EDTA.) One and one-half microliters of each of the five PCR mixtures was added to 4.5 μl of formamide loading buffer. After 2 min of denaturation at 92°C, the samples were chilled on ice and loaded onto the gel. Gels were run at room temperature for 14 hr at 8 W. Gels were dried directly on Whatman 3MM paper and autoradiographed for 16–24 hr on Kodak XAR film. After the initial SSCP scoring, the mobilities of variant alleles or "classes" were confirmed by rerunning the samples in a different order in which samples with similar mobilities were grouped together.

For the silver staining of SSCP gels the longer glass plate was treated with γ-methacryloxypropyltrimethoxysilane (Sigma M 6514) followed by two ethanol rinses, and the shorter plate was coated with a silanizing solution followed by a distilled water rinse. Sample preparation and electrophoresis were carried out under the same conditions as the radiolabeled case, with the exception that the amplifications were performed with each of the four dNTPs at 50 μM. After electrophoresis, the silanized plate was removed and a Plexiglas frame was placed on top of the large glass plate, sealed with agarose, and clamped with butterfly clips. All staining and rinses were carried out at room temperature on a rotary shaker according to a published protocol (19), with the following modifications and specifics: 30 min in a 0.02% formaldehyde solution, two 20-min rinses with distilled water, 25 min in 200 ml of silver staining solution (prepared by slowly adding 8 ml of 19.4% $AgNO_3$ to 42 ml of freshly made 0.36% NaOH with 2.8 ml of 28–30%, NH_4OH with vigorous agitation, then adding 147.2 ml of distilled water), three 5-min rinses with distilled water, development in 0.02% formaldehyde/0.005% trisodium citrate (≈10 min), a brief rinse with distilled water, and three 15-min washes in distilled water. Gels were then air dried on the glass plates overnight and photographed. The DNA sequence was determined for one strand as described (20).

The DNA sequence polymorphisms detected in this survey involve both nucleotide substitutions and insertion/deletion differences. It appears that all insertion/deletion differences are detected by SSCP (21). The nucleotide substitutions can be assumed to be divided into two categories: differences between SSCP classes and differences between alleles within SSCP classes. The overall average number of pairwise differences per site, $\hat{\pi}^*$, can be estimated as the weighted (by number of comparisons between classes i and j and by number of alignable sites between classes i and j, λ_{sij}) average over the S segments,

$$\hat{\pi}^* = \frac{\frac{\hat{\pi}'}{2}\sum_{s=1}^{S}\sum_{i=1}^{I_s}\lambda_{si}n_{si}(n_{si}-1) + \sum_{s=1}^{S}\sum_{i=1}^{I_s}\sum_{j<i}^{I_s} n_{si}n_{sj}\bar{\delta}_{sij}}{\frac{1}{2}\sum_{s=1}^{S}\sum_{i=1}^{I_s}\lambda_{si}n_{si}(n_{si}-1) + \sum_{s=1}^{S}\sum_{i=1}^{I_s}\sum_{j<i}^{I_s} n_{si}n_{sj}\lambda_{sij}},$$

where I_s is the number of classes in segment s, $\bar{\delta}_{sij}$ is the average number of site differences between sequenced alleles of classes i and j in segment s, n_{si} is the total number of alleles in class i in segment s as determined by SSCP, and

$$\hat{\pi}' = \frac{2\sum_{s=1}^{S}\sum_{i=1}^{I_s}\sum_{j=1}^{n'_{si}}\sum_{k<j}^{n'_{si}}\delta_{sijk}}{\sum_{s=1}^{S}\sum_{i=1}^{I_s}\lambda_{si}n'_{si}(n'_{si}-1)},$$

where δ_{sijk} is the number of site differences between sequenced alleles j and k in class i of segment s, and n'_{si} is the total number of sequenced alleles of the ith class in segment s.

Bootstrap analyses of $\hat{\pi}^*$ [made by selecting a sample (n = 50) of chromosomes with replacement from the total set of 50 surveyed chromosomes; 1000 replicates] showed that the mean of the bootstrapped $\hat{\pi}^*$ was biased from the sample estimate by a factor very close to $(n-1)/n$ (22). The reported values of $\hat{\pi}^*$ were corrected by $n/(n-1)$, and bootstrap confidence intervals, corrected by $n^2/(n-1)^2$, were placed on the estimates of the overall average pairwise differences. Estimates of $\theta = 3N_e\mu$ and its upper confidence value are also shown (23).

Tajima's D statistic (24) was calculated over all alleles on the assumption that every allele in a given SSCP class had the

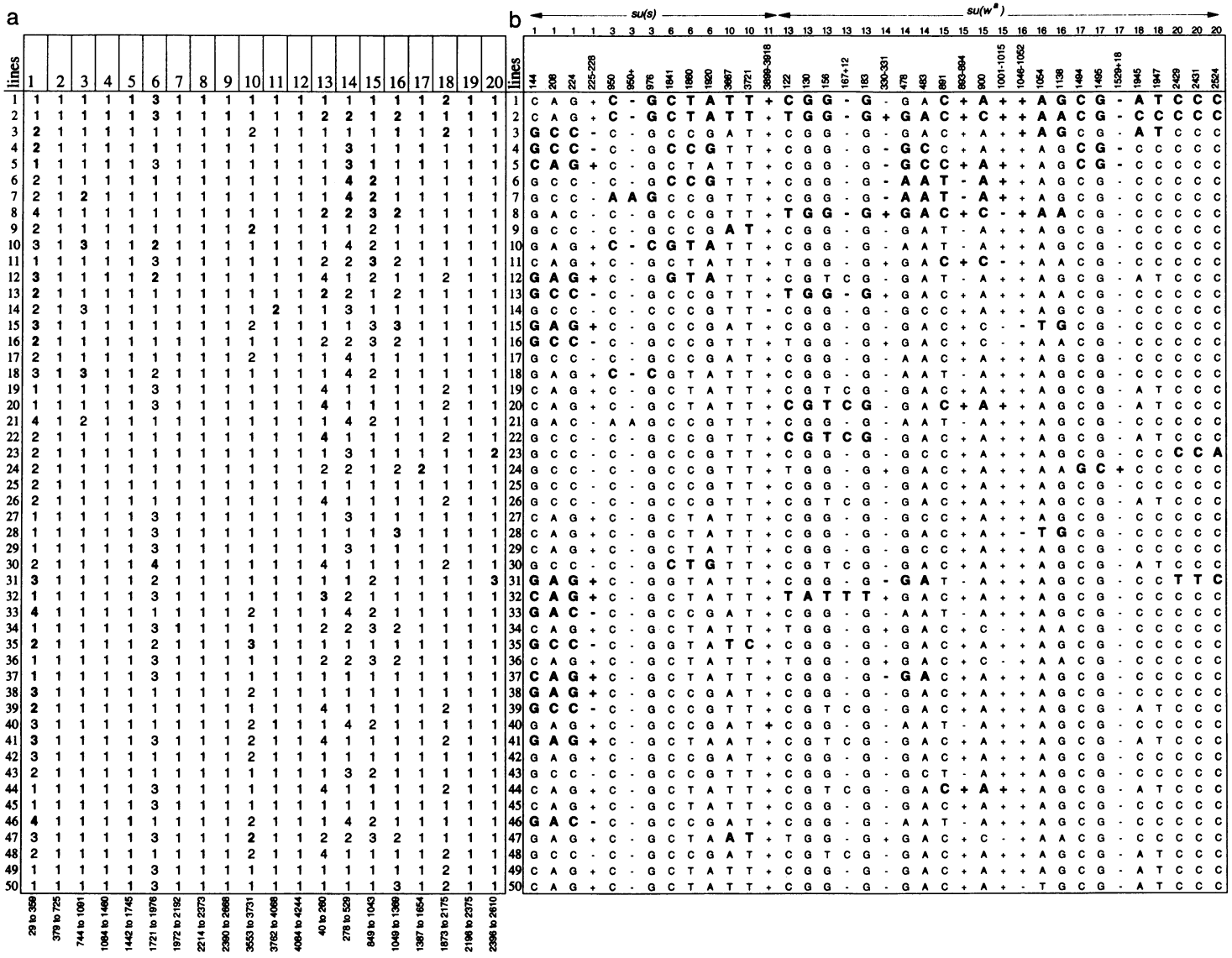

FIG. 2. Distribution of SSCP classes and DNA sequences among 12 segments of the *su(s)* region and 8 segments of the *su(w^a)* region in a sample of 50 X chromosome lines (first column) from a natural *D. melanogaster* population in North Carolina (see text and Fig. 1). (*a*) SSCP classes for the various segments (along the top). Bold numbers indicate alleles for which the DNA sequence is also available. The numbers at the bottom of the column indicate the first and last base pairs proximal to the primers. (*b*) Observed (bold) and inferred DNA sequence of the 50 X chromosome lines at the 27 sites (along the top) found to be polymorphic. In the case of the single within-SSCP-class polymorphism at site 900 the inferred nucleotide is set to the "consensus" (A) within that class. The bases in each column designated by a simple integer (sequence positions from the coordinates in GenBank, accession numbers M57889 and X06589) indicate the substitutional variant found (or inferred) for that allele. In the cases such as 225–228, those particular bases are missing for lines indicated by a minus sign. An A in column 950+ indicates that an A residue is inserted at position 950. The presence of a minus sign, C, or T in column 167+12 indicates the published sequence, an insertion of TTGCTCAACTTG, or a similar insertion of TTGCTCAATTTG, respectively. A plus sign in column 1529+18 indicates the presence of a tandem duplication of bases 1529–1546.

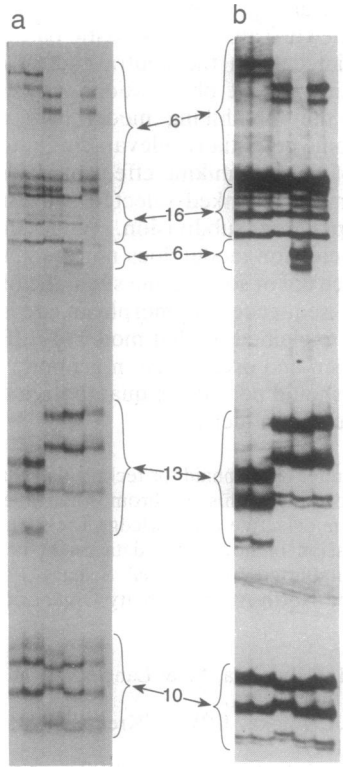

FIG. 3. (*a*) Autoradiograph of five *D. melanogaster* X chromosome lines (left to right, 8, 13, 41, 35, and 32) subject to SSCP analysis using ^{35}S labeling of PCR products of four segments. (*b*) Photograph of the same five samples when SSCP gels were silver stained. The positions of bands associated with various segments (nos. 6, 10, 13, and 16) are indicated. Each amplification product shows three or four bands. Two classes or "phenotypes" are shown for segment 16, 3 classes for segments 6 and 10, and 4 classes for segment 13. Segment 16 is not present for line 32 in *a*.

DNA sequence observed in the class members which were actually sequenced [ignoring the polymorphism at 900 in $su(w^a)$]. Linkage disequilibria between the polymorphisms were inferred under the same assumption. The statistical significance of linkage disequilibrium was assessed by application of Fisher's exact test to all pairs of polymorphisms which were not unique.

RESULTS

Fig. 3 shows the results of SSCP analysis for the PCR products of four isogenic *D. melanogaster* genomic DNAs, each amplified with four different sets of oligonucleotide primers. Fig. 3*a* shows the autoradiograph of the ^{35}S-labeled PCR products. Fig. 3*b* is a photograph of a similar SSCP gel (unlabeled PCR products) that was silver-stained. Both procedures gave reliable and comparable results.

Fig. 2*a* presents results of the survey of the 50 X chromosomes of *D. melanogaster* for SSCP polymorphism in the 20 segments of the $su(s)$ and $su(w^a)$ regions. Eight segments (nos. 2, 4, 5, 7, 8, 9, 12, and 19) were scored as monomorphic. The remaining 12 segments revealed two to four different "classes" or conformational morphs, which showed mobility shifts ranging from $\approx$1 mm to several centimeters.

To assess the power of our SSCP protocol to detect DNA sequence variation, we chose to evaluate homogeneity within and diversity between SSCP allelic classes by determining the DNA sequences of several members (bold in Fig. 2) of each class. Fig. 2*b* shows the sequence variation (determined and inferred) underlying the SSCP classes.

The DNA sequence analysis revealed that shifts in SSCP mobility due to single nucleotide substitutions or simple insertion/deletion differences ranged from <1 mm (e.g., segment 16, line 13 versus 41, and segment 10, line 32 versus 35 in Fig. 3) to one or more centimeters (e.g., segment 6, line 35 versus 41, and segment 13, line 13 versus 35, in Fig. 3). Some "suspected" differences scored in the initial SSCP analysis proved not to be repeatable and were usually corrected upon repeated SSCP analysis. Since we were exploring the SSCP technique for the first time we were concerned that we might miss variation in the larger segments. In particular, slight (<1-mm) differences among alleles in three classes (segment 1, classes 1 and 2; segment 13, class 2) attracted our attention. While we could not obtain convincing repeatable differences, we nevertheless sequenced several suspected "variants" in those three classes. Because no DNA sequence differences were associated with the minor mobility variation we initially detected, we conclude that such slight mobility differences are best tested by sequencing but are unlikely to actually be new SSCP classes. Segment 2 shows striking variation in the number of bands (not their mobilities), although this variation is not repeatable within lines. It appears that some sample-to-sample variation can occasionally lead to the loss of one of the SSCP bands. These minor problems with the initial application of our SSCP protocol lead us to conclude that in most applications several representatives of each class should be independently reanalyzed and their DNA sequences determined.

On the other hand, comparison of the DNA sequences from our survey revealed only one case of heterogeneity within an SSCP allelic class (SSCP class 1 of segment 15). Of the 31 alleles of segment 15 with phenotype 1, 5 were sequenced. One of the 5 differs from the rest by an A-to-C substitution at position 900. A total of 24,360 bp can be compared for sequence identity within the various SSCP classes. Of these, only four showed this one difference.

Analysis of the results from the survey of SSCP-detectable variation among the 50 X chromosomes for the 20 segments in the $su(s)$ and $su(w^a)$ regions yielded an overall estimate of the average number of pairwise differences per nucleotide site, $\hat{\pi}^*$ (95% bootstrap confidence intervals): for $su(s)$, 0.0010 ($0.95–1.11 \times 10^{-3}$) for substitutional differences and 0.00020 ($1.6–2.5 \times 10^{-4}$) for insertion/deletion differences; for $su(w^a)$, 0.0021 ($1.9–2.4 \times 10^{-3}$) for substitutional differences and 0.00076 ($6.2–9.0 \times 10^{-4}$) for insertion/deletion differences. Since we expected the level of DNA sequence polymorphism in these two regions to be less than that previously reported for most of the of *D. melanogaster* genome, we estimated the largest values of $\theta(3N_e\mu)$ for which the probabilities of observing (in a sample of 50 alleles) $\leq$10 polymorphic nucleotide sites (out of 3213 bp) for $su(s)$ and $\leq$17 polymorphic nucleotide sites (out of 1955 bp) for $su(w^a)$ were <0.05. The 95% upper confidence values of θ are 0.0015 for $su(s)$ and 0.0036 for $su(w^a)$, well below 0.006, the typical value for loci in regions of normal crossing over (25).

The observed values of Tajima's D did not differ significantly from predictions of an equilibrium between genetic drift and selectively neutral mutation (23): for $su(s)$, 1.31 and -0.14, and for $su(w^a)$, -0.40 and 0.21 (for substitutional polymorphisms and insertion/deletion polymorphisms, respectively).

Patterns of linkage disequilibria among the polymorphic sites within and between the $su(s)$ and $su(w^a)$ regions are displayed at the bottom of Fig. 1. There is clearly more nonrandom association among sites within each region than between them. Despite this high incidence of linkage disequilibrium within regions, there are several pairs of sites at which all four gametic types were found, indicating some recombination.

DISCUSSION

SSCP analysis followed by stratified DNA sequencing is clearly efficient for surveying variation in regions of low polymorphism, as most of the variation was detected with much less effort than that needed for direct DNA sequencing of both strands. But there are questions about the nature and proportion of polymorphisms that can be reliably surveyed. To address these, we generally sequenced two or more members of each SSCP class in our survey. No insertion/deletion differences were found among the comparisons of members of the same class, but one substitutional polymorphism was observed within a single class. Since 24,360 bp were compared (within SSCP classes) and this difference occurred in the comparison of one with the other four sequenced members of that class, the estimate of the number of SSCP-undetectable substitutional differences per base pair between members of the same SSCP class is 2.0×10^{-4} [= (5/4)(4/24,360), corrected for bias], 14% of the overall estimate, $\hat{\pi}^*$ (1.44×10^{-3}). Bootstrap analysis indicates the actual proportion could be as high as 50%. The fact that 14 of 23 comparisons between SSCP classes (not involving any insertion/deletion differences) involve a single substitutional difference indicates that most individual nucleotide substitutions are detectable in this region.

su(s) and *su(w^a)* are located near the tip of the X chromosome of *D. melanogaster* at cytological locations 1B10–1C1 and 1E1–2, respectively. The amount of crossing over increases from an unmeasurable level in the yellow (*y*)–achaete-scute complex (*ASC*) region (1B1) to a normal level in cytological section 3. Recombinants between *y* and *su(s)* have been observed at the low rate of ≈0.0001 (R. A. Voelker, personal communication) and recombinants between *y* and *su(w^a)* occur at a somewhat higher rate, 0.0035 (M. Green, personal communication). We are interested in surveying DNA sequence polymorphism in these regions because previous studies have documented relatively reduced polymorphism in regions of no measurable crossing over, but quantitative analyses require nonzero rates (7, 13). Our goal at this point is to determine whether the polymorphism at these two loci, *su(s)* and *su(w^a)*, is low and increasing in the expected monotonic fashion. Our second goal is to demonstrate that SSCP can be used to reliably survey DNA sequence variation in such regions of low polymorphism.

Estimates of the average number of pairwise differences per nucleotide site in the *y–ASC* region range from 0.0004 to 0.0013 (see ref. 4 and references therein), while estimates of the average number of pairwise differences in regions of normal crossing over are ≈0.006 (25). The nearest locus centromere-proximal to *su(w^a)* that has been surveyed for DNA sequence variation in *D. melanogaster* is *Pgd*. The amount of crossing over near *Pgd* is expected to be reduced by approximately half compared with most of the euchromatic genome (7). The reported average number of pairwise differences in *Pgd* is 0.0024 (26). Notice also that the estimate for *su(s)* (0.0010) is significantly lower than that for *su(w^a)* (0.0021), based on the bootstrap confidence intervals, while both are bracketed by those for *y–ASC* and *Pgd*. Thus, the levels of polymorphism parallel the levels of crossing over per physical length.

The hitchhiking effect was proposed as an explanation for the association between crossing over per unit physical length and levels of DNA sequence polymorphism (1, 12). A second prediction of the hitchhiking effect is a skew in the frequency spectrum of segregating sites (1, 25). None of the calculated values of Tajima's *D* statistic based on the results in Fig. 2 deviated from the neutral-theory expectation of zero, reinforcing recent observations suggesting that this prediction of the hitchhiking model may not be widely observable. Some potentially relevant limitations of the present analysis of the hitchhiking-effect model include the interactions of multiple linked selected substitutions and the impact of population subdivision. While the background (deleterious)-selection model does not predict a skew in the frequency spectrum of segregating sites, the observed reductions in DNA sequence polymorphism are apparently too extreme to be explained by that model (14). Further studies of polymorphism and divergence in genomic regions of low crossing over should permit the quantitative testing of more realistic theoretical models.

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