Lack of Degeneration of Loci on the Neo-*Y* Chromosome of *Drosophila americana americana*

B. Charlesworth, D. Charlesworth, J. Hnilicka, A. Yu and D. S. Guttman

Department of Ecology and Evolution, University of Chicago, Chicago, Illinois 60637-1573

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ABSTRACT

The extent of genetic degeneration of the neo-*Y* chromosome of *Drosophila americana americana* has been investigated. Three loci, coding for the enzymes enolase, phosphoglycerate kinase and alcohol dehydrogenase, have been localized to chromosome *4* of *D. a. americana*, which forms the neo-*Y* and neo-*X* chromosomes. Crosses between *D. a. americana* and *D. virilis* or *D. montana* showed that the loci coding for these enzymes carry active alleles on the neo-*Y* chromosome in all wild-derived strains of *americana* that were tested. Intercrosses between a genetically marked stock of *virilis* and strains of *americana* were carried out, creating F_3 males that were homozygous for sections of the neo-*Y* chromosome. The sex ratios in the F_3 generation of the intercrosses showed that no lethal alleles have accumulated on any of the neo-*Y* chromosomes tested. There was evidence for more minor reductions in fitness, but this seems to be mainly caused by deleterious alleles that are specific to each strain. A similar picture was provided by examination of the segregation ratios of two marker genes among the F_3 progeny. Overall, the data suggest that the neo-*Y* chromosome has undergone very little degeneration, certainly not to the extent of having lost the functions of vital genes. This is consistent with the recent origin of the neo-*Y* and neo-*X* chromosomes, and the slow rates at which the forces that cause *Y* chromosome degeneration are likely to work.

THE *Y* chromosomes of many animal and some plant species are genetically degenerate, with few active genes other than those required for functions specific to the heterogametic sex (MULLER 1918; WESTERGAARD 1958; WHITE 1973; BULL 1983; GRAVES 1995). In several groups, it is known that the loss of genetic activity on the *Y* chromosome has been accompanied by the evolution of dosage compensation, so that equal amounts of *X* chromosomal gene products are produced in males and females, despite the difference in ploidy level (MULLER 1932; LYON 1961; LUCCHESI 1993). It is clear from comparative evidence that *Y* chromosome degeneration has evolved independently in several different lineages (BULL 1983), suggesting that relatively simple evolutionary forces are involved.

The nature of these forces has been debated for many years. It is generally accepted that *Y* chromosomes were originally homologous to *X* chromosomes, and that they have lost genetic activity through the accumulation of deleterious mutations, whose spread at *X*-linked loci is prevented by selection. The absence of recombination between all or part of the *X* and *Y* chromosomes almost certainly plays a major role in promoting the accumulation of deleterious mutations and repetitive DNA sequences on the *Y* chromosome (MULLER 1918; FISHER 1935; NEI 1970; CHARLESWORTH 1978, 1991, 1996; LUCCHESI 1978; RICE 1987, 1994; JABLONKA and LAMB 1990). There is currently no agreement on the details of the mechanisms of such accumulation, and no tests of the various hypotheses with data from natural populations have been carried out.

As pointed out by LUCCHESI (1978), the neo-*X* and neo-*Y* chromosomes of certain members of the genus Drosophila, which are formed by centric fusions between an autosome and a basic *X* or *Y* chromosome, provide excellent material for studying intermediate stages of *Y* chromosome degeneration. The absence of crossing over in male Drosophila means that the homologue of an autosomal arm that becomes fused to the *X* chromosome cosegregates with the original *Y* in male meiosis (provided that disjunction is regular), and hence is inherited in exactly the same fashion as a regular *Y* chromosome, although it is not physically joined to it. An autosome that is fused to the *Y* chromosome is obviously inherited together with the *Y*, while its homologue cosegregates with the *X* chromosome. In both cases, the neo-*Y* chromosome has exactly the same nonrecombining and permanently heterozygous status as a regular *Y* chromosome, although it plays no role in sex determination. It is therefore exposed to the same evolutionary forces that are thought to lead to the degeneration of the original *Y* chromosome. Genetic investigations of species that have acquired their neo-*Y* chromosomes at widely different times may therefore shed light on these forces.

In species with an ancient neo-*Y* chromosome, such

Corresponding author: Brian Charlesworth, Department of Ecology and Evolution, University of Chicago, 1101 E. 57th St., Chicago, IL 60637-1573. E-mail: bcworth@pondside.uchicago.edu

as *D. pseudoobscura,* the neo-*Y* chromosome appears to have completely degenerated, and the homologous arm of the neo-*X* chromosome in males is fully dosage compensated (LUCCHESI 1978, 1993). A more recent event generating a neo-*Y* chromosome has occurred in the close relative of *D. pseudoobscura, D. miranda,* as a result of a fusion between the *Y* chromosome and autosomal element *C* of the basic Drosophila karyotype (DOBZHANSKY and TAN 1936; MULLER 1940). In this case, only some of the genes on the neo-*Y* have lost function, and only part of the homologous arm of the neo-*X* has responded by becoming dosage compensated (MACKNIGHT 1939; STROBEL *et al.* 1978; DAS *et al.* 1982; BONE and KURODA 1996; MARÍN *et al.* 1996; STEINEMANN *et al.* 1996).

An even more recently evolved system of this kind is represented by *D. americana americana,* a member of the *virilis* species group. In this taxon, autosomal element *B* (chromosome 4 of *D. virilis* and its relatives) has become fused to the *X* chromosome (Figure 1). This fusion is absent from the close relatives of *D. a. americana: D. americana texana, D. novamexicana, D. lummei* and *D. virilis* (PATTERSON and STONE 1952; THROCKMORTON 1982). *D. a. texana* and *D. a. americana* hybridize readily in the laboratory and in nature, and for this reason are usually regarded as subspecies (THROCKMORTON 1982). In contrast to *D. miranda,* there are no obvious cytological features of the neo-*Y* in *D. a. americana* that suggest degeneration (HUGHES 1939; our own observations), and no molecular evidence for dosage compensation of genes on the neo-*X* (BONE and KURODA 1996; MARÍN *et al.* 1996). In addition, crosses between wild-type male *D. a. americana* and female *D. virilis* homozygous for the recessive fourth chromosome mutations *px* and *cd* yield wild-type F_1 male offspring (STALKER 1940). This implies that there are active alleles of these two loci on the neo-*Y* of *D. a. americana.*

This lack of evidence for degeneration of the neo-*Y* in *D. a. americana* is consistent with the low level of differences between *D. a. americana* and its close relatives at the level of electrophoretic alleles (THROCKMORTON 1982; SPICER 1992) and DNA sequences (TOMINAGA and NARISE 1995; HILTON and HEY 1996; NURMINSKY *et al.* 1996). *D. a. americana* therefore offers a unique opportunity to use data on the molecular evolution and variation of genes on the neo-*Y* chromosome to study very early stages of *Y* chromosome degeneration along the lines suggested by CHARLESWORTH (1996). An essential first step is to establish the extent to which loss of gene activity may have already evolved on this chromosome, and this is the purpose of the research described in the present paper. Two methods were used. The first was to study the activity of allozyme loci on the neo-*Y*, by means of F_1 hybrids between male *D. a. americana* and females of related species with different electrophoretic alleles at the loci in question. The second was to examine the viability of flies that have been made homozygous for sections of the neo-*Y*, as a result of intercrosses with a marked strain of *D. virilis.* Both methods show that there is indeed little or no degeneration of loci on the neo-*Y*, at the level of complete loss of function of important genes.

MATERIALS AND METHODS

Species and strains studied: Stocks of several member of the *virilis* group of Drosophila were obtained from the National Drosophila Species Resource Center, Department of Biological Sciences, Bowling Green State University, Bowling Green, Ohio: *D. a. americana, a. texana, virilis, littoralis* and *montana.* In addition, one strain each of *D. lummei* and *D. novamexicana* were provided by Dr. JERRY COYNE. The following strains of *D. a. americana* were used. These were originally derived from flies trapped in the wild up to 55 years ago (PATTERSON *et al.* 1942; WARTERS 1944; HSU 1952): 0951 (from Anderson, Indiana), 0951.1 (Poplar, Montana), 0951.2 (Chinook, Montana), 0951.3 (Millersburg, Pennsylvania), 0951.4 (Keelers Bay, Lake Champlain, Vermont), 0951.6 (Chadron, Nebraska), 0951.7 (Oakdale, Nebraska), 0951.9 (Myrtle Beach, South Carolina). The first of these is referred to as strain *am-0,* and the others as *am-1, am-2* etc., in what follows.

A multiply marked strain of *D. virilis* (stock 1051.46, abbreviated to *vir-46*) was used in many of the crosses described below. It contains the following recessive markers for all the major autosomes: *b* (broken cross-vein, map location *2-188*), *tb* (tiny bristles, *3-104*), *gp-L2* (gap in L2 vein, *3-118*), *cd* (cardinal eye color, *4-32*), *pe* (peach eye color, *5-203*) (ALEXANDER 1976; GUBENKO and EVGEN'EV 1984). It was found that *pe* was difficult to classify except on a background homozygous for *cd,* which limited the information that could be obtained with this marker. Several wild-derived stocks of *D. virilis, littoralis* and *montana* were also surveyed by electrophoresis: the stock designations were abbreviated in the same way as for the stocks described above.

Stocks of *D. a. americana, a. texana, virilis,* and *lummei* were routinely maintained at 18° or 22° (the life cycle is 3–4 weeks at these temperatures), on standard cornmeal-soyameal-yeast-agar medium with propionic acid as a mold inhibitor, in either half-pint milk bottles or 3-inch vials. Stocks of *D. littoralis* and *D. montana* were maintained at 18° in vials with banana medium. All crosses between species were carried out at 18° in vials with banana medium. Virgin females and males were aged at least a week after collection, before matings were set up. Given the low success rate in obtaining F_1 hybrid offspring in crosses between pairs of different species (at best, a dozen or so flies emerged in crosses between *D. a. americana* and *virilis,* and most crosses between *D. a. americana* and *montana* failed to yield any offspring), the F_1 crosses were set up with at least 12 pairs of males and females, with the cotton plugs pushed halfway down the vials to increase the probability of matings. The mated flies were transferred twice at weekly intervals, and flies were collected from all transfers. Subsequent backcrosses involved single intercross males or females, who were provided with at least six mates from the stock to which the backcross was being made.

Cytological methods: The *D. a. americana* stocks were checked by examination of acetic-orcein squashes of colcemid-treated mitotic chromosomes, using a modification of the protocol of ASHBURNER (1989). At least 10 larvae from each strain were examined. In all cases except strain *am-2,* all larvae showed the karyotype expected for *americana:* a pair of dot chromosomes, one pair of metacentric and one pair of acrocentric autosomes, a pair of metacentric *X* chromosomes in females, and a metacentric *X,* an acrocentric autosome and

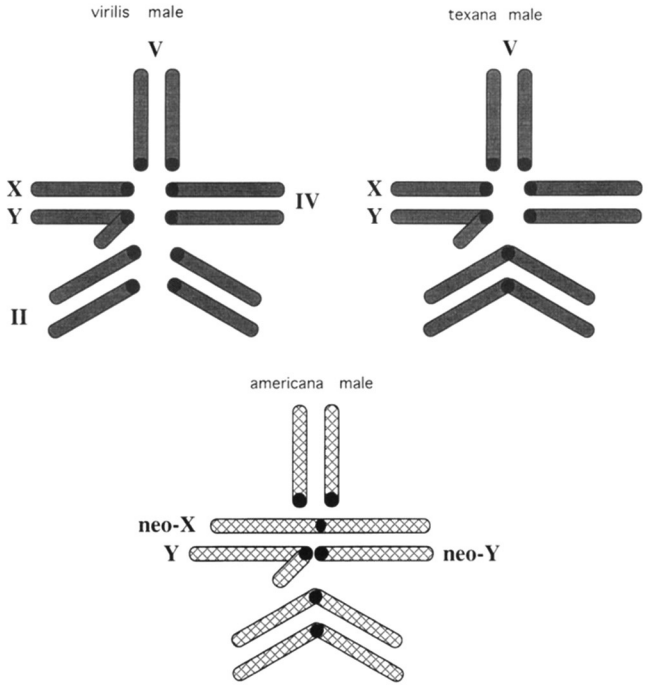

FIGURE 1.—The mitotic metaphase karyotypes of *D. virilis*, *D. americana texana* and *D. a. americana* males. The roman numerals indicate the numbering of the chromosomes in *D. virilis*: *II*, Muller's element *E*: *III*, element *D*; *IV*, element *B*; *V*, element *C* (THROCKMORTON 1982). The *Y* chromosome is depicted as a metacentric with a long and a short arm, although in reality it is acrocentric.

the acrocentric neo-*Y* in males (Figure 1). *am-2* appeared to be segregating for this karyotype and for the karyotype of *D. a. texana* (which lacks the *X*-autosome fusion). Since it may have been contaminated by *texana*, this strain was therefore not used any further.

For *in situ* hybridization localization of the *adh* locus in *D. a. americana*, polytene chromosome preparations were made from third instar larval salivary glands and were hybridized to a biotinylated probe using a modification of the procedures of MONTGOMERY *et al.* (1987) and SNIEGOWSKI and CHARLESWORTH (1994). A 1.4-kb probe for the *D. virilis Adh-1* gene (NURMINSKY *et al.* 1996) was prepared by PCR for this purpose.

Electrophoretic methods: Electrophoresis was done on cellulose acetate plates according to standard protocols (HEBERT and BEATON 1989) with a run time of 20 min. Tables 1 and 2 show the buffer systems and electrophoretic conditions used for the different enzymes.

Samples of adult flies of the species and strains to be studied were ground in different grinding buffers, according to the enzyme to be stained. For *pgk*, *got*, *eno*, and *α-gpdh*, the grinding buffer was 10 mg DTT per 10 ml 0.1 M Tris-HCl pH 8.0, while for *adh* and *mdh*, the pH values were 8.3 and 8.6, respectively. Samples were ground in ice-cold buffer (25 μl per fly) in 0.5 ml Eppendorf tubes, cooled on ice, and centrifuged in a microcentrifuge in a cold room for 5 min. They were then either frozen at −20° for running later or loaded onto the cellulose acetate plates. *Pgk*, *eno* and *got* could be scored only with freshly ground samples, while the other enzymes worked well with frozen material. The position at which the samples were loaded on the plates was important for getting clear results. Only larval proteins were loaded in the position at the end of the plates, while all other systems were stained on plates loaded in the centre position. To get adequate staining intensity, it was often necessary to repeat the loading multiple times (two to five) onto the same plate.

After running, the plates were stained for the enzymes listed in Table 2. *Adh*, *got* and *mdh* were stained according to RICHARDSON *et al.* (1986), and so was *α-gpdh*, except that the stain buffer was pH 7.6. The dehydrogenases were stained in agar overlays poured over the plates at the end of the run. The overlay contained 5 ml stain solution and 5 ml of freshly made 2% agar solution cooled to about 60° before mixing with stain.

For *eno* and *pgk*, modifications were made to the method described in note 10a(vi) (p. 223) of RICHARDSON *et al.* (1986). These two enzymes were run on Iso-Flur cellulose acetate plates (Helena Labs). A stain-soaked filter paper square was placed on top of a plate and rolled with the broad end of a glass Pasteur pipette to ensure good contact between the paper and the plate. The plate, with its filter paper of stain, was wrapped in Saran wrap to prevent drying out and incubated for 25 min at room temperature. It was finally flooded with a mixture of 0.25% MTT and 0.5% PMS for 15 sec, and rinsed under a hot tap to develop the stained plate as a dark background with pale bands at the sites of enzyme activity. To get good contrast between the background and the pale bands, it was necessary to optimize the concentrations of stain ingredients. For *eno*, we used a stock solution of 20 ml 0.1 M Tris-HCl pH 7.6 containing 60 mg 3-phosphoglycerate, disodium salt, 30 mg ADP, and 100 μl of 1 M $MgCl_2$. One-ml aliquots of this buffer were frozen at −20°, and 2.5 mg NADH, 2500 units of phosphoglycerate mutase (Sigma P9665), 900 units of lactate dehydrogenase (Sigma L2395), and 160 units of pyruvate kinase (Boehringer 128-155) were added to stain one plate. For *eno*, 230 μl of 2-2-diphosphoglycerate solution (10 mg/ml H_2O) were added as a cofactor for *pgm*, which improves the appearance of the bands. For *pgk*, aliquots of the following buffer were frozen: 20 ml 0.05 M Tris-HCl pH 8.0, containing 60 mg 3-phosphoglycerate, 120 mg ATP, 300 μl of 1 M $MgCl_2$, and 200 μl of 0.5 M EDTA. Thirty milligrams of NADH and 1200 units of glyceraldehyde-3-phosphate dehydrogenase (Boehringer 105-686) were added to 1 ml of this solution.

Larval serum proteins were also stained using Coomassie Blue, as there is a locus controlling one of these on 2L of *D. melanogaster* (ROBERTS and EVANS-ROBERTS 1979). Many bands

TABLE 1

Buffer systems used for the different enzyme stains

Buffer	Stock (amounts per liter)	Dilution
Tris/glycine, pH 8.5	Tris base 30 g, glycine 144 g	1/10
Tris/EDTA, pH 7.8	Tris base 1.82 g, boric acid 0.34 g, Na_2 EDTA.$2H_2O$ 1.86 g, $MgCl_2$ 0.95 g	—
Citrate/morpholine, pH 7.2	Citric acid monohydrate 40.2 g, N-(3-aminopropyl)-morpholine (Sigma A-9028) to pH 7.2 (~75 ml)	1/4

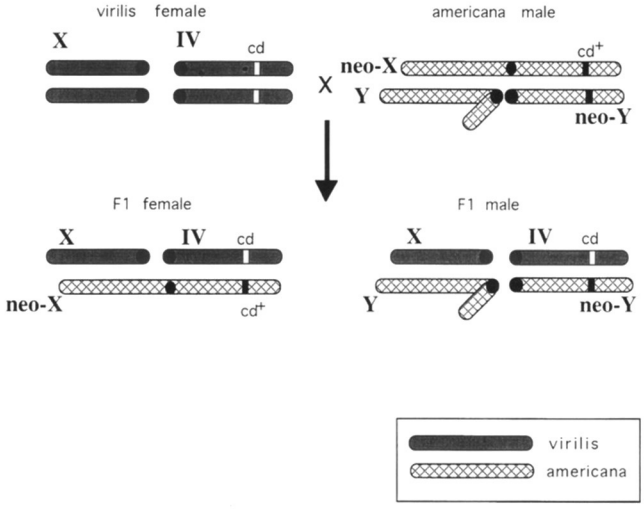

FIGURE 2.—The F_1 progeny of a cross between female *D. virilis* and male *D. a. americana*. The *virilis* strain is shown as homozygous for the fourth chromosome mutation *cd*, whereas the *americana* strain carries cd^+. For any chromosome *4* locus at which *virilis* and *americana* differ in allelic state, the F_1 males will show the phenotype of the heterozygote, if the locus is active on the neo-*Y*, and the phenotype of the homozygote for the *virilis* allele if the locus is inactive.

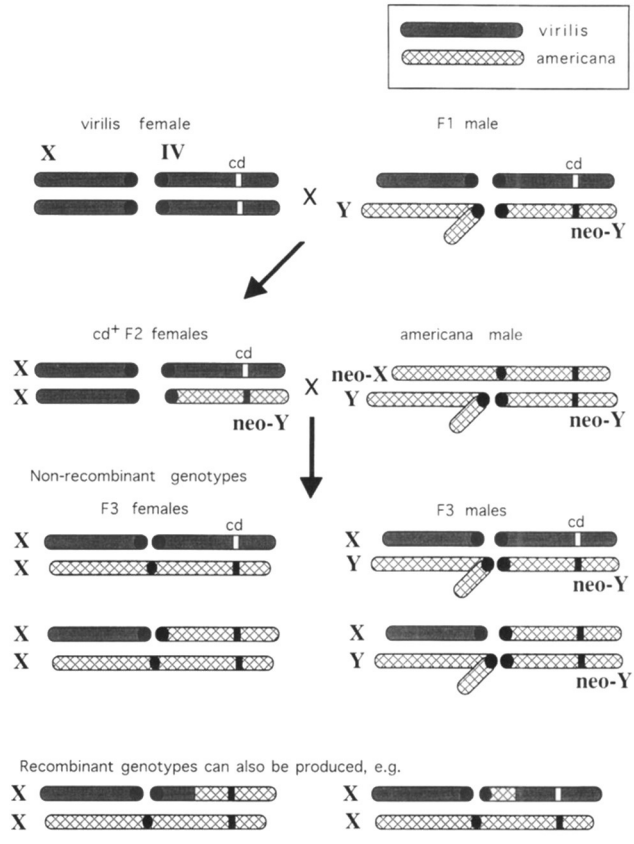

FIGURE 3.—The breeding program for making segments of the *americana* neo-*Y* chromosome homozygous. F_1 males as shown in Figure 2 are backcrossed to *virilis* females. If cd^+ female F_2 progeny are mated with *americana* males, the F_3 male progeny will be homozygous for portions of the neo-*Y* chromosome from *americana*. The extent of this homozygosity for a given F_3 male depends on the number and location of crossovers between the *virilis* and *americana* fourth chromosomes, in the F_2 female germ cell from which its maternal fourth chromosome was derived. F_3 females will always be heterozygous for a neo-*X* chromosome, derived from their F_3 *americana* fathers.

were seen, and there were no clear species differences. It therefore seemed unlikely that loci on the *virilis* group fourth chromosome could be identified, and no further work was done on these proteins.

Tests for degeneration of enzyme loci on the *americana* neo-*Y* chromosome: To test for degeneration of the enzyme loci *pgk* and *eno*, crosses were made between females of *D. virilis* and males of *D. a. americana* from a strain with a different allozyme mobility for a fourth chromosomal allozyme locus. The reciprocal crosses were also made. If the locus is active in the fourth chromosome of *americana* males, then F_1 male progeny in which this chromosome comes from an *americana* male should show both the *americana* and the *virilis* bands, as in the males from the reciprocal F_1 and in F_1 females from both reciprocal crosses (see Figure 2). If the locus is inactive, F_1 male progeny with an *americana* male parent will show only the phenotype of the *virilis* parent. Several strains of *americana* were tested, as it is possible that active neo-*Y* chromosome alleles at these loci might be present in some, but not all, of the strains. *Vir* and *am* parental strain samples, and F_1 male and female progeny were run on the same gel. The samples were coded so that the person scoring the gel was unaware of the identities of the samples.

Construction of flies homozygous for sections of the *D. americana* neo-*Y*: The ability to intercross the marked strain of *D. virilis* with *D. a. americana* allows the design of a crossing scheme to produce flies that are homozygous for sections of their neo-*Y* chromosome. This scheme was suggested to us by Dr. ALLEN ORR, and is shown in Figure 3, starting with F_1 males from the cross shown in Figure 2. The *virilis* females used in the crosses were always from the multiply marked *vir-46* strain, whereas the *americana* flies were derived from a number of different stocks. The crosses were coded so that the counter of the F_3 and F_4 progeny was unaware of their identity.

The sex ratios in the F_3 generation can be used as an index of the extent of neo-*Y* degeneration. The cd/cd^+ females produced in the F_2 generation are heterozygous for a *vir-46* chromosome *4* and an *americana* neo-*Y* chromosome. Crossing over can occur at this stage, so that fourth chromosomes transmitted to their F_3 progeny contain an unknown fraction of *virilis* and *americana*-derived material. But the paternally derived genomes in the F_3 males all carry a complete *americana* neo-*Y*, whereas the paternally derived genomes in the F_3 females will carry the *americana* neo-*X*. This means that half of the F_3 male zygotes will be homozygous for sections of the neo-*Y*, whereas there is no possibility of this in F_3 females. A deficiency of surviving males relative to females in the F_3 generation of this set of crosses is therefore consistent with deleterious effects of homozygosity for alleles carried on the neo-*Y*.

There are two potential problems in interpreting any such deficiency of males. First, male offspring in crosses between Drosophila species, and in their subsequent backcrosses, are sometimes more inviable than female offspring, a manifestation of Haldane's Rule (HALDANE 1922; COYNE and ORR 1989; WU and PALOPOLI 1994). Deficiencies of males are often observed in the F_1 progenies of crosses between *virilis* and *americana* (PATTERSON *et al.* 1942, Table 8). Second, the stocks of *americana* used in these experiments have been maintained for many years in laboratory culture, with very small effective population sizes. Recessive or partially recessive deleterious

alleles can thus accumulate relatively rapidly on the neo-*Y* chromosome by mutation and genetic drift, through several population genetic mechanisms (CHARLESWORTH 1996). There is direct evidence for accumulation of deleterious mutations on nonrecombining chromosomes in small laboratory populations (CROW and SIMMONS 1983; CHARLESWORTH and CHARLESWORTH 1985; ALBORNOZ and DOMÍNGUEZ 1994; RICE 1994). Homozygosity for parts of the neo-*Y* chromosome that are derived from the same stock will expose the effects of these accumulated mutations, in addition to any deleterious mutations that may have been present when the chromosomes were originally collected from the wild.

To deal with such potential causes of deficiencies of males in the F_3 generation, the following procedures were adopted. First, a set of "control" F_2 and subsequent backcrosses were set up, using F_2 females that were homozygous for *cd* instead of heterozygous for *cd* and wild type. In this way, both fourth chromosomes transmitted to the progeny are entirely of *virilis* origin, and so any deficiency of males in F_3 has nothing to do with homozygosity for the *americana* neo-*Y*, but instead may reflect the operation of Haldane's Rule effects. Comparisons of the sex ratios in the "experimentals" (with cd/cd^+ F_2 mothers) and controls will test for a greater deficiency of male in the experimentals, which would provide evidence for direct effects of homozygosity for the *americana* neo-*Y*.

The problem of exposure of deleterious alleles that are present only on neo-*Y* chromosomes from the same stock can be overcome by intercrossing F_2 females to *americana* males derived from a different stock from the progenitor stock used to generate the F_1 individuals. Comparison of the sex ratios of "inbred" F_3 crosses, in which both *americana* chromosomes come from the same stock, with those of "outbred" stocks in which they are of different origin, will reveal effects of deleterious alleles of common origin.

The effects of homozygosity for sections of the *D. a. americana* neo-*Y* on the segregation of marker alleles: An alternative method for detecting deleterious recessive effects of neo-*Y* chromosome alleles is to follow the segregation of a marker gene for which *virilis*- and *americana*-derived alleles (illustrated by *cd* and cd^+ in Figure 4) can be distinguished. Since crossing over can occur in the F_2 females, the marker does not permit the whole neo-*Y* to be followed, but the two classes of F_3 male progeny will be unequal in frequency if strongly deleterious alleles are linked to the allele carried on the neo-*Y*. This test is simplest to apply to a marker with codominant alleles, and enolase is suitable for this purpose (Table 2 and Figure 3).

A similar analysis can be done using the recessive fourth chromosome mutation *cd*, which is present in the *vir-46* stock. Since the cd/cd^+ F_3 flies are phenotypically identical, this requires progeny testing of F_3 individuals by testcrosses of single males or females to *vir-46*, and scoring the resulting progeny for segregation of *cd*. cd^+/cd^+ parents will clearly fail to produce any phenotypically *cardinal* offspring, whereas cd/cd^+ are expected to produce a 1:1 ratio of *cardinal* to wild-type eye color. Low F_3 male fertility hampered this analysis, because many testcrosses failed completely, and others did not produce enough offspring to diagnose the parental genotype with high confidence.

The following convention for inferring parental genotypes was adopted. F_4 progenies with less than four individuals were discarded, regardless of phenotype. The parents of F_4 progenies with less than seven individuals that failed to segregate *cardinal* were classified as "doubtful", and the parents of progenies of size seven or more that failed to segregate *cardinal* were classified as cd^+/cd^+. On the assumption of 1:1 segregation, the probability that a family of size seven with a cd/cd^+ parent fails to segregate is only 0.008, so the error produced by this procedure is low unless the proportion of doubtful progenies is high.

There is, of course, the possibility that the segregation ratios for *cd* in the F_4 crosses depart substantially from Mendelian expectation, due to viability disturbances. This would introduce some errors into the above analyses, if there were a deficiency of cd/cd offspring. This was investigated by maximum likelihood estimation of the frequency of cd/cd offspring, π, using data from all segregating progenies. The likelihoods were calculated by conditioning on the probability of detecting a segregating progeny, by the standard procedure of dividing the binomial probabilities for a sample of size n by $1 - (1 - \pi)^n$ (KEMPTHORNE 1957, p. 193). The value of π that maximizes the resulting log-likelihood function was found by Newton-Raphson iteration. There was no evidence for a substantial deficiency of cd/cd offspring (data not shown).

RESULTS

Electrophoretic phenotypes of the taxa studied, and location of the loci: Table 2 summarizes the results of an electrophoretic survey of the taxa, using the strains described above. Differences in the electrophoretic patterns were seen between *D. a. americana* and *D. virilis* for enolase (*eno*), phosphoglycerate kinase (*pgk*), and glutamic-oxaloacetic transaminase (*got*), and the latter two enzymes also showed variation between different strains of *D. virilis*. For the other enzymes, all strains of both these species showed identical mobilities. There were, however, differences between *D. a. americana* and *D. montana* for malic dehydrogenase (*mdh*) and alcohol dehydrogenase (*adh*).

To map the allozyme loci, a cross was made between males of the multiply marked strain of *D. virilis, vir-46,* and females of a strain (either of *D. virilis* itself, or of *D. a. americana*) having a different mobility for a given enzyme. F_1 flies were backcrossed to *vir-46* and the phenotypes scored in the backcross progeny. The detailed results are shown in Table 3, and a summary of the results of the mapping experiments is given in Table 4.

In Table 3, genotype 1 denotes progeny heterozygous for the allozyme and wild type for the marker (*i.e.*, the same phenotype as the F_1 flies), while genotype 2 denotes flies with the phenotype of the *vir-46* strain at both the allozyme and marker loci. The recombinant progeny genotypes 3 and 4 are, respectively, those heterozygous for the allozyme and homozygous for the *vir-46* marker, and the reciprocal type with the allozyme phenotype of *vir-46* and wild type at the marker locus. To test for linkage, chi-squared tests were done for independence of the segregation of each marker and the allozymes. The numbers of flies scored differ for the different markers because there were several failures of the electrophoretic scoring, which is inevitable with these extremely difficult staining methods.

For *pgk*, the backcross flies scored were derived from crosses between two strains of *D. virilis* (the marker strain *vir-46* and the wild-type strain *vir-49*), and all progeny scored were female. The backcross was as fol-

TABLE 2

Enzyme loci studied and their buffer systems

Enzyme	Buffers used and electrophoretic conditions	Alleles in the strains	
		F	*S*
Enolase (*eno*)	Tris/glycine, 200 V	*vir-46*	*am-0, 1, 3, 4, 6, 7, 9, mont* (8 strains); *litt* (3 strains)
Phosphoglycerate kinase (*pgk*)	Tris/glycine, 200 V	*vir-46* plus 7 other strains: *am-1, 2, 3, 4, 6, 7, 9; tex* (2 strains); *mont* (12 strains); *litt* (4 strains)	*vir-49; mont-21*
Alcohol dehydrogenase (*adh*)	Tris/EDTA, 200 V	*vir-46* plus 9 other strains: *am-0, 1, 2, 3, 4, 6, 7, 9; tex* (2 strains); *lummei; litt-0*	*mont-22*
Glutamic-oxaloacetic transaminase (*got*)	Tris/EDTA, 200 V	*vir* (2 strains)	*vir-46* plus 8 other strains: *am-1, 2, 6, 7, 9; tex* (2 strains); *litt* (4 strains)
Malic dehydrogenase-2 (*mdh*)	Citrate/morpholine,[a] 100 V	*vir-46* plus 9 other strains: *am-0, 1, 2, 3, 4, 6, 7, 9*	*mont* (12 strains), *litt-0*
α-glycerophosphate dehydrogenase (*α-gpdh*)	Citrate-morpholine, 110 V	No differences among species or strains	

[a] Both the soaking and running buffers for runs to be stained for *mdh* had 2.5 mg NAD, 100 μl of 1.5 M $MgCl_2$, and 75 μl of 0.5 M EDTA added (per 100 ml of buffer).

lows: *vir-46* female × F_1 male from (*vir-46* female × *vir-49* male). Table 3 shows that there is evidence for linkage between *pgk* and the fourth chromosome marker *cd*, consistent with *pgk* being on chromosome *4*. The *pgk* alleles also segregate independently of chromosomes 3 and 5. Note that only *cd/cd* flies can be scored for the fifth chromosome marker, so that recombination between chromosome *4* and *5* generates classes 2 and 4 only. Sex linkage is also ruled out because, if *pgk* were sex linked, the F_1 males would carry the *X* chromosome

TABLE 3

Results of tests of linkage of the allozyme loci to visible markers of *D. virilis*

	Genotype				
	Nonrecombinant		Recombinant		
Chromosome[a]	1	2	3	4	χ^2
			Phosphoglycerate kinase		
2 (*b*)	6	7	1	1	8.04**
3 (*tb, gp-L2*)	4	4	3	4	0.08
4 (*cd*)	7	8	0	0	15.0***
6 (*pe*)	—	4	—	4	—
			Enolase[b]		
2 (*b*)	11	7	14	10	0.89
3 (*tb*)	11	7	13	10	0.06
3 (*gp-L2*)	9	10	15	7	2.01
4 (*cd*)	17	14	7	3	7.32**
			Glutamic-oxaloacetic transaminase		
2 (*b*)	9	8	13	12	1.57
3 (*tb*)	13	10	9	10	0.02
3 (*gp-L2*)	14	9	8	11	0.32
4 (*cd*)	7	15	15	5	0.24
5 (*pe*)	15	13	0	2	22.9***

Details of the crosses and markers are given in the text. $^{**}P < 0.01$; $^{***}P < 0.001$.

[a] Chromosome marker is in parentheses.

[b] See text for the method for testing for linkage of the enolase locus with chromosome 5.

TABLE 4

Locations of the five enzyme loci in *D. virilis/americana*

Locus	Chromosome
Enolase	*4* (Muller's element *B*)
Phosphoglycerate kinase	*4*
Alcohol dehydrogenase	*4*
Malic dehydrogenase	Not known
Glutamic-oxaloacetic transaminase	*5* (Muller's element *C*)

from their maternal parent, *vir-46*, so that no backcross progeny heterozygous for the allozyme could appear. There is also a significant chi-squared value for chromosome *2*, for which most progeny were of nonrecombinant genotypes. This may be due to inviability of recombinant genotypes.

For *eno*, the backcross that yielded progeny was as follows: *vir-46* male × F_1 female from (*am-7* female × *vir-46* male). The progeny scored were all female and wild type for the fifth chromosome marker *pe*. With linkage to chromosome *5*, the wild-type progeny would be heterozygous for *eno*, so the fact that the *eno* segregation was 17:25, not significantly different from 1:1 ($\chi^2 = 1.52$, 1 d.f., $P > 0.05$), shows that the locus for *eno* is not on chromosome *5*. Table 3 shows that linkage to chromosomes *2* and *3* was not supported, while linkage to *cd* was significant, indicating linkage to either chromosome *4*, or to the *X* chromosome. The frequency of recombination between *cd* and *eno* in this cross is 0.24 (SE = 0.07). Crosses of the type *vir-46* female × F_1 male from (*vir-46* female × *am* male) were also done to test for degeneration of the locus on the male-transmitted fourth chromosome of *americana* (see below). These progeny are also informative about possible linkage. If the *eno* locus were on the *X* chromosome, none of the backcross male progeny would receive the allele from *americana*, and so hybrid allozyme patterns would not be seen. As there were many males in the progenies with this pattern, we can rule out *X*-linkage for this locus. Other data supporting these conclusions are presented in Table 7, and are discussed in the sections below on the segregation of *eno* and *cd* alleles in the F_3 generation of intercrosses between *virilis* and *americana*.

For *got*, we carried out the backcross *vir-46* female × F_1 male from (*vir-46* female × *vir-8* male). Progeny of both sexes were scored, and both sexes segregated both allozyme types, which rules out linkage to the *X* chromosome. Table 3 shows that the locus for this enzyme shows linkage only to chromosome *5*. The two apparent recombinant progeny were presumably the result of errors in scoring. As there are two *got* loci in *D. melanogaster*, on chromosome arms 2L and 2R (LINDSLEY and ZIMM 1992), the locus scored in *americana* might have been expected to be on either the neo-*Y* chromosome (homologous with 2L), or chromosome *5* (homologous with 2R).

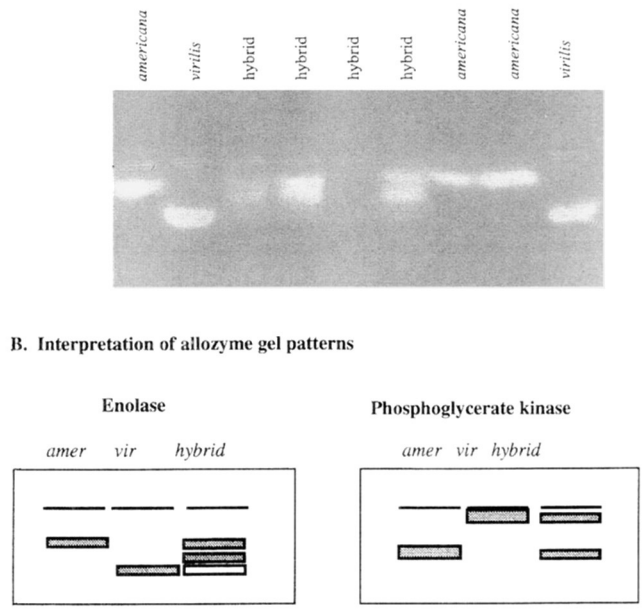

FIGURE 4.—Phosphoglycerate kinase and enolase gels, showing *D. virilis*, *D. a. americana*, and F_1 hybrid individuals. (Top) Photograph of an enolase gel. (Bottom) Diagrammatic interpretation of the gel patterns for both enzymes. Phosphoglycerate kinase gels were hard to photograph, so that no photograph is shown.

The *mdh* and *adh* loci could not be mapped genetically, as no allozyme differences were found between stocks that in our hands produced fertile hybrids with *vir-46*. The probe for *adh* was hybridized to a male from strain 0 of *D. a. americana* (see MATERIALS AND METHODS). As expected from its location in *D. virilis* (NURMINSKY *et al.* 1996), the probe hybridized to band 49B, at the base of chromosome *4*. Since no sequence for Drosophila *mdh* has been published, it was not possible to localize the gene for this enzyme. There are two *mdh* loci in *D. melanogaster*, one on element *B* (2L) and the other on element *E* (3R) (LINDSLEY and ZIMM 1992), so that it is possible that we are not studying the neo-*Y* locus in *americana*.

Tests for degeneration of the enzyme loci on the *americana* neo-*Y* chromosome: None of the F_1 crosses scored for enzyme phenotypes (see MATERIALS AND METHODS) showed any evidence for degeneration: *americana* bands were present in all males. Some representative results are shown in Figure 4. It is also possible to perform a similar test in backcross generations, using the visible marker *cd* to indicate which flies have the hybrid genetic constitution for the fourth chromosome, and this test was done for a number of *americana* strains. With one exception, the two tests gave concordant results, which are summarized in Table 5. The exception was a backcross involving F_1 males with *am-7* male parents; the five backcross males scored for *eno* showed no sign of the *americana* (*S*) allele, whereas four F_1 males that were subsequently scored all had the hybrid (*F/S*)

TABLE 5

Tests of degeneration of enzyme loci on the neo-*Y* of *D. a. americana*

Strains tested	No. of males tested for each locus			
	eno	*pgk*	*adh*	*mdh*
0	2	3	—	—
1	3	4	4	—
3	1	3	—	—
4	3	6	—	—
6	3	5	3	3
7	4	5	—	—
9	3	4	—	—

—, not tested.

phenotype. Other backcross results for this strain, shown in Table 7, confirm the latter finding. We have no explanation for this discrepancy, other than an error in scoring.

Adh and *mdh* were tested using *D. montana* as the female parents in crosses with *D. a. americana,* since there were no mobility differences among the *virilis* and *americana* strains (see above). These crosses rarely produced viable F_1 male progeny, so that information on only a very limited number of strains of *americana* was obtained, despite the fact that 121 crosses between *americana* and *montana* were set up.

The results for all four enzymes were consistent: apart from the exception mentioned above, the F_1 and backcross males with *americana* neo-*Y* chromosomes always showed the phenotype expected if the neo-*Y* carries active alleles at the loci that code for them.

Effects of homozygosity for sections of the *D. a. americana* neo-*Y* on backcross sex ratios: As described in MATERIALS AND METHODS, the sex ratios of the F_3 progenies produced by the breeding design shown in Figure 4 shed light on the the extent of degeneration of the neo-*Y* chromosome, with a deficiency of males indicating deleterious effects of homozygosity for sections of the neo-*Y*. The sex ratio data for the controls and experimental F_3 progenies, for both inbred and outbred crosses, are shown in Table 6 (sex ratio is defined here as the proportion of males). These derive from over 500 backcrosses of single F_2 females to *americana* males, many of which were unsuccessful due to sterility and/or inviability.

The largest data set is for inbred matings involving the *americana* strain *am-0*. It will be seen that there is a significant deficiency of males ($\chi^2 = 38.5$, 1 d.f., $P < 0.001$) among the "experimental" crosses (derived from cd/cd^+ F_2 females) and an apparently smaller deficiency ($\chi^2 = 6.76$, 1 d.f., $P < 0.01$) among the "controls" (derived from cd/cd F_2 females). A 2 × 2 contingency test shows that there is no significant difference between these crosses with respect to sex ratio, however ($\chi^2 = 2.67$, 1 d.f., $P > 0.10$). This is consistent with the possibility that the deficiency of males is at least partly due to Haldane's Rule effects (see MATERIALS AND METHODS).

A smaller amount of data is available from eight outbred matings involving F_2 females with an *am-0* grandparent (the F_2 male parents came from the stocks *am-1, am-6* and *am-9*). The sex ratio of the pooled data for experimental outbred crosses was significantly higher than that of the experimental inbred crosses ($\chi^2 = 9.33$, 1 d.f., $P < 0.01$). This suggests that the deficiency of males in the experimental crosses is at least partly due to homozygosity for partially or completely recessive deleterious neo-*Y* alleles that are unique to the *am-0* stock. The difference in sex ratio between the inbred and outbred control crosses is not significant, as expected under this interpretation.

The only other inbred experimental cross to yield a significant deficiency of males is that for *am-9* ($\chi^2 = 11.7$, 1 d.f., $P < 0.001$). This failure to detect a biased sex ratio is not simply due to small numbers of individuals in the other inbred experimental crosses. The 2 × 2 contingency comparison of the inbred experimental crosses of *am-0* and *am-3* shows that these are highly significantly different ($\chi^2 = 11.4$, 1 d.f., $P < 0.001$), suggesting that *am-3* indeed has a less biased sex ratio than *am-0*. There is less convincing evidence for a difference between *am-6* and *am-0* ($\chi^2 = 4.9$, 1 d.f., $P < 0.05$), and no significant difference between *am-7* and *am-0*. There is a general, but not uniform, tendency for the control crosses to have higher sex ratios than their experimental counterparts, but these differences are never significant in any individual case.

The pooled data for all experimental and control inbred crosses give sex ratios of 0.458 and 0.463, respectively (corresponding to 5740 and 1821 total individuals), and the pooled data for the experimental and control outbred crosses give sex ratios of 0.473 and 0.483, respectively (1321 and 1548 total individuals). There is therefore a relatively small overall bias in the F_3 progeny sex ratio, with some suggestion that both Haldane's Rule and inbreeding effects contribute to the deficiency of males. But the deviations from a 1:1 sex ratio, even for the *am-0* inbred experimental crosses (which show the largest female bias), are relatively small, which is inconsistent with very severe reductions in fitness for homozygous sections of the *americana* neo-*Y*. For example, if there were a recessive lethal allele at one locus on all neo-*Y* chromosomes in a given stock, the crossing scheme in Figure 3 shows that one-half the F_3 males would be homozygous for the lethal, and hence would die, reducing the sex ratio to 0.33, if females and males have intrinsically the same viability. If there were several loosely-linked lethals, the sex ratio would be even lower. Since Haldane's Rule effects cause inviability of males rather than females (PATTERSON *et al.* 1942), this assumption is, if anything, conservative. The sex ratio data therefore conclusively rule out the

TABLE 6

Sex ratios in the F_3 progenies of the *americana-virilis* backcrosses

Experimental crosses				Control crosses			
Cross	Females	Males	Sex ratio	Cross	Females	Males	Sex ratio
			Inbred crosses				
am-0 (36)	1173	891	0.432 ± 0.011	*am-0* (27)	617	529	0.461 ± 0.020
am-3 (28)	749	717	0.489 ± 0.013	*am-3* (6)	50	44	0.468 ± 0.051
am-6 (7)	230	220	0.489 ± 0.024	*am-6* (9)	92	90	0.495 ± 0.037
am-7 (12)	164	133	0.448 ± 0.029	*am-7* (17)	179	136	0.432 ± 0.028
am-9 (29)	797	666	0.455 ± 0.013	*am-9* (1)	40	44	0.524 ± 0.054
			Outbred crosses				
am-3 (3)	162	127	0.439 ± 0.029				
am-0 (8)	147	162	0.523 ± 0.028	*am-0* (6)	240	216	0.474 ± 0.023
am-6 (19)	387	336	0.467 ± 0.018	*am-6* (24)	560	532	0.487 ± 0.015

The numbers in parentheses are the numbers of crosses that were scored. The number after ± indicates the standard error, estimated assuming binomial sampling of genotype frequencies.

possibility that recessive lethals have accumulated on the neo-*Y* chromosomes.

The effects of homozygosity for sections of the *D. a. americana* neo-*Y* on the segregation of enolase alleles: The result of scoring F_3 male progeny for *eno* genotypes are shown in Table 7 (only progeny whose electrophoretic phenotypes were unequivocal are shown in the table; very few ambiguous individuals were found). Both inbred and outbred experimental crosses (see previous section) were scored; the control crosses are uninformative and were therefore not scored. In the case of inbred crosses with *am-0*, F_3 female progeny were also scored. These provide a partial control for segregation disturbances due to interactions between genes derived from the two species that reduce viability: it is possible that homozygosity for *am*-derived alleles on chromosome *4* could confer lower viability than heterozygosity for *am-* and *vir*-derived alleles, as reported by ORR and COYNE (1989) for the *X* chromosome in backcrosses between *virilis* and *americana*. This phenomenon would cause a reduced frequency of *S/S* females compared with the expectation of 1:1 *F/S* to *S/S*.

TABLE 7

Segregation of enolase in the F_3 progenies of the *americana-virilis* backcrosses

Cross	Sex	Electrophoretic phenotype[a] *F/S*	*S/S*
am-0 (inbred)	Males	27	16
am-0 (outbred)	Males	6	16
am-0 (inbred)	Females	18	15
am-3 (inbred)	Males	7	17
am-6 (inbred)	Males	21	12
am-6 (outbred)	Males	15	14
am-7 (inbred)	Males	5	22
am-9 (inbred)	Males	9	8

[a] *F* is the *virilis* allele and *S* is the *americana* allele.

There is highly significant heterogeneity in the segregation ratios among male progeny of the inbred crosses ($\chi^2 = 32.5$, 4 d.f., $P < 0.001$). A 2 × 2 contingency test shows that there is a significant difference in segregation ratio between the inbred and outbred *am-0* crosses ($\chi^2 = 6.37$, 1 d.f., $P < 0.02$). This suggests that the deficiency of homozygotes for the *americana* allele in the inbred *am-0* crosses reflects inbreeding effects, rather than exposure of deleterious alleles common to all neo-*Y* chromosomes. This conclusion is strengthened by the absence of any deficiency of *S/S* among the inbred crosses involving *am-3*, *am-7* and *am-9*. Indeed, in the case of *am-7* there is a highly significant deficiency of *F/S* ($\chi^2 = 10.7$, 1 d.f., $P < 0.01$). This is presumably due to unfavorable viability interactions between the predominantly *americana* genetic background and *virilis*-derived alleles on the fourth chromosome with the *F* allele. In addition, the 1:1 ratio of *F/S* to *S/S* in the F_3 female progeny of *am-0* inbred crosses seems to exclude the possibility that hybrid inviability effects of the type reported by ORR and COYNE (1989) are the cause of the deficiency of *S/S* among the corresponding males.

These results also confirm that *eno* is not on the true *X* chromosome, since *X*-linkage implies that all F_3 individuals must inherit an *F* allele, contrary to what is observed.

The effects of homozygosity for sections of the *D. a. americana* neo-*Y* on the segregation of *cardinal* alleles: The results for this case are shown in Table 8. As with *eno*, the *am-0* inbred F_3 crosses produce a deficiency of males that are homozygous for the *americana* allele. If the small number of doubtful individuals is ignored, we have $\chi^2 = 7.86$, 1 d.f., $P < 0.01$. There is no such deficiency among males in the outbred crosses with *am-*

TABLE 8

Segregation of *cardinal* in the F_3 progenies of the *americana-virilis* backcrosses

Cross	Sex	Inferred F_3 genotype		
		cd/cd^+	cd^+/cd^+	Doubtful[a]
am-0 (inbred)	Males	52	27	4
am-0 (outbred)	Males	3	11	5
am-0 (inbred)	Females	40	46	2
am-3 (inbred)	Males	5	23	13
am-6 (inbred)	Males	11	6	0
am-6 (outbred)	Males	11	13	2
am-9 (inbred)	Males	17	16	4

[a] This indicates that fewer than seven progeny were produced, and no *cd/cd* individuals were observed.

0, and there is a highly significant difference between the outbred and inbred crosses on a 2×2 contingency test ($\chi^2 = 9.70$, 1 d.f., $P < 0.01$). The inbred crosses for the other genotypes do not yield any significant deficiencies of cd^+/cd^+ males, and the *am-3* crosses give a highly significant deficiency of cd/cd^+ ($\chi^2 = 11.9$, 1 d.f., $P < 0.001$), similar to what was observed for *am-7* with enolase. Unfortunately, the *am-7* testcrosses had a very high failure rate, so that it was not possible to obtain meaningful data on this genotype.

The high frequency of doubtful genotypes in the case of *am-3* raises the question of what effect excluding these from the analysis may have on the estimate of the frequency of cd^+/cd^+ F_3 males. This can be investigated by determining the smallest value of θ, the frequency of cd^+/cd^+ individuals among F_3 males, that is compatible at the 5% probability level with the observed numbers of nonsegregating progenies. With n progeny from a cross with a given F_3 male, the probability of no segregation is simply $P = 1 - \theta + (1 - \pi)^n$, where π is the probability of *cd/cd* offspring. (π can safely be equated with 0.5, the Mendelian expectation; see MATERIALS AND METHODS). Taking the sum of the natural logarithms of P for all nonsegregating progenies for the genotype of males in question and equating it to *ln* 0.05 gives an equation that can be solved numerically for θ by Newton-Raphson iteration. In the case of *am-3*, there are 13 doubtful crosses, with a minimum θ estimate of 0.79, *i.e.*, there are likely to have been at least 10 cd^+/cd^+ parents. This greatly strengthens the evidence for an excess of cd^+/cd^+ F_3 males with *am-3*.

F_3 backcross females from inbred crosses with *am-0* neo-*Y* chromosomes were also studied for the segregation of *cd*. As can be seen from Table 8, there is no evidence for a deficiency of cd^+/cd^+ individuals, consistent with what was found for enolase.

These data also provide the opportunity for further examining the linkage between *cd* and *eno*, since many of the F_3 males could be typed for both loci. Pooling the results across all crosses, 16 out of 37 jointly typed F_3 males carried recombinant neo-*Y* chromosomes. If these data are added to the backcross data of Table 3, the overall estimate of recombination frequency between *cd* and *eno* is $26/78 = 0.33 \pm 0.05$. This should be viewed with some caution, as *americana* is polymorphic for a common medial inversion complex *a* + *b* on chromosome *4* (WARTERS 1944; HSU 1952), and several of the stocks we used are either homozygous for this arrangement or segregate for it (own observations). Different stocks may thus give different estimates of recombination frequencies. Strain *am-0* is homosequential with *virilis* (HSU 1952; own observations), so that the F_3 backcross results from this strain are representative of the situation in the absence of inversion heterozygosity on chromosome *4*. Six recombinants out of 22 progeny were observed in this case, a recombination frequency of 0.22, consistent with the results in Table 3. It thus seems clear that *cd* and *eno* are only loosely linked; given that *cd* is located 32 cM from the tip of chromosome *4* (ALEXANDER 1976), this suggests that *eno* may be proximal to *cd*. This remains to be confirmed by *in situ* hybridization or more detailed genetic mapping.

DISCUSSION

The results presented above are consistent with the idea that the neo-*Y* chromosome (the nonfused copy of the fourth chromosome) of *D. americana americana* shows little signs of degeneration, consistent with the old findings of STALKER (1940) on the loci *cd* and *px*, and with recent molecular data indicating a lack of dosage compensation of the neo-*X* in this species (BONE and KURODA 1996; MARÍN *et al.* 1996). (All of the *americana* strains listed in Table 5 gave wild-type F_1 males when males were crossed to *vir-46*, indicating that *cd* is active on the neo-*Y* chromosome of each strain.) Our data show that the enzyme loci *pgk* and *eno* are located on chromosome *4* of *virilis* and *americana*, and have active alleles on the neo-*Y* in all the *americana* strains that we have tested (Table 5). *Adh* is also on this chromosome and has active neo-*Y* alleles in the two strains of *americana* that we were able to test. If *mdh* is on the neo-*Y*, which has not been established, it must have an active allele in the single strain tested. Thus, we now have five active loci that are definitely on the neo-*Y*, and one whose location is uncertain. The localizations of *pgk*, *eno* and *adh* also provide further evidence to support the concept of a high degree of conservation of chromosome arm content in the genus Drosophila (MULLER 1940; PATTERSON and STONE 1952; WHITING *et al.* 1989; SEGARRA *et al.* 1996).

In addition, the experiments on sex ratios in the F_3 generations of intercrosses between *virilis* and *americana* show that there is generally only a small reduction in viability of males that have been made homozygous for sections of the neo-*Y* chromosome (Table 6). There is certainly no indication that lethal alleles have accumu-

lated on this chromosome, since the sex ratios are far from the value of 33% expected with a single recessive lethal allele (see above). According to PATTERSON and STONE (1952, p. 498), STURTEVANT also carried out tests of the homozygous viability of the *americana* neo-*Y* chromosome and found evidence for lethality in some cases but not others, but no details appear to have been published. Nevertheless, there is evidence from the inbred crosses involving *am-0* and *am-6* that indicates some degree of reduction in fitness associated with homozygosity for portions of the neo-*Y*. This question is explored in detail in the APPENDIX. The segregation data in particular seem to suggest that there are one or more fairly strongly deleterious, recessive or partially recessive genes between *eno* and *cd* that contribute to a substantial loss in fitness of homozygotes for the *am-0* and *am-6* neo-*Y* chromosomes.

But these effects are only detected when the maternal and paternal alleles come from the same strain, suggesting that inviability of the *americana* neo-*Y* homozygotes is mainly due to deleterious alleles that are strain-specific, rather than to a process of degeneration that is common to all neo-*Y* chromosomes of the species. This conclusion should be qualified, however, by noting that in some cases there are significant excesses of *americana* marker homozygotes, both for *eno* and *cd* (Tables 7 and 8). This indicates that incompatibilities between *virilis* and *americana* alleles may cause males that carry more of the *americana* genome with respect to chromosome *4* to survive better on the predominantly *americana* background of the F_3 intercrosses than males with a smaller proportion of *americana* alleles. Selection against genotypes homozygous for the *americana* allele at the marker locus, due to linked deleterious neo-*Y* alleles, might thus be obscured by these interspecies incompatibilities. The sex ratio data of Table 6 suggest, however, that these effects cannot be very large, unless females are affected by the incompatibilities to approximately the same extent as males, since otherwise a highly female-biased sex ratio in the F_3 generation would result. The 1:1 segregations of the marker genotypes among *am-0* F_3 females are inconsistent with a large effect of hybrid incompatibility on the relative viabilities of the fourth chromosome genotypes of females (Tables 7 and 8). Similarly, the biased sex ratio of the inbred control *am-0* cross in Table 6, where homozygosity for the neo-*Y* cannot affect the sex ratio, suggests that F_3 males are more affected by incompatibilities than females, in accordance with Haldane's Rule.

The lack of evidence for recessive lethal effects of the *americana* neo-*Y* chromosomes raises the question of whether this is expected, given that chromosomes of Drosophila extracted from natural populations are frequently homozygous lethal, and that lethal mutations accumulate on chromosomes that are maintained heterozygous by balancers (SIMMONS and CROW 1977; CROW and SIMMONS 1983). No balancer extraction data are available on the frequencies of lethal chromosomes in members of the *virilis* group, but in the *obscura* group (whose autosomes are of similar size to those of *D. virilis*), the frequency of recessive lethal chromosomes in a sample is ~0.2 for a typical autosome (SIMMONS and CROW 1977; SPERLICH *et al.* 1977). Since lethals seem predominantly to be selected against in the heterozygous state in Drosophila populations (SIMMONS and CROW 1977; CROW and SIMMONS 1983), the frequency of recessive lethal neo-*Y* chromosomes (without any degeneration) in a natural population is unlikely to exceed 0.2. A caveat concerning this conclusion is that the *odh* region on chromosome *2* of *americana* is associated with an unusually high frequency of lethals in chromosomes from natural populations (FEDER 1984). This is probably an exceptional situation, however, and is unlikely to represent the state of the neo-*Y* chromosome. Given the length of time that the strains used here have been maintained in the laboratory, it is likely that their neo-*Y* chromosomes have largely become identical by descent, so that the five different strains can be treated as representing five independently sampled neo-*Y* chromosomes. With a frequency of lethal chromosomes of 0.20, there is a probability of 0.33 that five independent chromosomes sampled from the wild would all be nonlethal.

Furthermore, the mutation rate for the *X* chromosomes in male *D. melanogaster* has been estimated as 0.0025 per chromosome per generation (CROW and SIMMONS 1983). In the absence of other data, this provides an estimate of the mutation rate for the *americana* neo-*Y*, which is of similar size to the *melanogaster X* chromosome. In 600 generations, which is approximately the time that the oldest strain (*am-0*) has been kept in the laboratory (WARTERS 1944), we would therefore expect a mean number of new lethal mutations per strain of 1.5, if selection is completely absent, and each strain has become identical by descent. The probability that a given strain lacks a lethal is $\exp(-1.5) = 0.22$. The probability that all five strains failed to accumulate any lethals is only 0.0005 on this basis. But this ignores selection against the heterozygous effects of lethals. With a mean selection coefficient of 0.03 against a heterozygous lethal that has arisen by spontaneous mutation (SIMMONS and CROW 1977) and an effective number of breeding males per strain of 20 each generation, use of the standard diffusion equation formula (CROW and KIMURA 1970, p. 426) implies that the fixation probability of a neo-*Y* lethal is only 0.013. The expected rate of substitution of lethals per generation is $20 \times 0.0025 \times 0.013 = 0.00065$ (KIMURA and OHTA 1971, p. 13), yielding an expected number of accumulated lethals of $600 \times 0.00065 = 0.39$ for a strain maintained for 600 generations. There is a probability of 0.08 that no lethals become fixed in five such strains. Overall, therefore, there is a probability of $0.33 \times 0.08 = 0.03$ that

five strains that have been maintained for 600 generations lack lethals on the neo-*Y* chromosomes, so that the observation does not raise serious concern, especially as the parameters used in the calculations are based on results from other Drosophila species, and are thus of somewhat uncertain relevance to *americana*.

This evidence for lack of degeneration of the *americana* neo-*Y* chromosome raises the question of why this should be the case. One simple answer is that there simply has not been enough evolutionary time since its origin for the various population genetic processes that cause degeneration to produce effects that can be observed at the level with which we are working. CHARLESWORTH (1996) has argued that the three major forces that are likely to cause *Y* chromosome degeneration (Muller's ratchet, selective sweeps, and background selection) will operate very slowly in populations with effective population sizes in the hundreds of thousands or millions, as seems to be the case in the Drosophila species that have been extensively surveyed for DNA sequence variation (KREITMAN 1983; MORIYAMA and POWELL 1996), and appears also to be true for *americana* and *texana* (HILTON and HEY 1996). The evidence from cytology and hybridization data (PATTERSON and STONE 1952, Chapter 10; THROCKMORTON 1982), protein electrophoresis (THROCKMORTON 1982; SPICER 1992), and DNA sequence comparisons (TOMINAGA and NARISE 1995; HILTON and HEY 1996; NURMINSKY *et al.* 1996) suggests that *americana* and *texana* are extremely closely related. Indeed, the lack of divergence between *texana* and *americana* observed for the *per* locus by HILTON and HEY (1996) suggests that the two species or subspecies have either only diverged very recently, or are subject to a good deal of gene flow connecting their local populations. The time since the centric fusion evolved may thus be effectively zero on the scale of molecular sequence divergence. In contrast, molecular data indicate a time of divergence of 2 Mya for *D. miranda* and *D. pseudoobscura* (NORMAN and DOANE 1990; RUSSO *et al.* 1995). The neo-*Y* of *miranda* has evolved a long way toward complete degeneration, with dosage compensation extending over many parts of the neo-*X* (MACKNIGHT 1939; STROBEL *et al.* 1978; DAS *et al.* 1982; KRISHNAN *et al.* 1991; STEINEMANN *et al.* 1993; BONE and KURODA 1996; MARÍN *et al.* 1996; STEINEMANN *et al.* 1996). The time available for this level of degeneration is clearly much greater than for the *americana* neo-*Y*.

PATTERSON and STONE (1952, p. 498) also discussed the question of the lack of degeneration of the *americana* neo-*Y* and suggested that recombination in males between the neo-*Y* and its homologue, or introgression of the nonfused chromosome *4* from *texana*, could prevent its degeneration. While there is no direct evidence on the frequency of male recombination in *americana*, it appears to be a very low frequency phenomenon in both *virilis* and *littoralis* (ALEXANDER 1976; COYNE 1988; LUMME and LANKINEN 1988). The generally achiasmate meiosis of higher Diptera (GETHMANN 1988) makes it seem unlikely that male recombination could be an important factor in *americana*.

It is more difficult to assess the possible role of hybridization between *americana* and *texana* in introducing unfused fourth chromosomes into the former, and thereby inhibiting the process of degeneration. There is evidence for such hybridization in nature, in populations at or near the zone of contact between the two species across the middle of the USA (STONE and PATTERSON 1947; CARSON and BLIGHT 1952; THROCKMORTON 1982). The very large geographic range of *americana* across the northern part of the USA east of the Rocky Mountains (THROCKMORTON 1982) makes it seem unlikely that unfused *texana* fourth chromosomes could penetrate into all populations of *americana*, particularly those in the West, and there are no reports of this in the literature, but further population surveys of mitotic karyotypes are needed to be sure of this. As stated in the MATERIALS AND METHODS, we found that strain *am-2* segregated for the *americana* and *texana* karyotypes. We do not believe that this is because this stock is derived from wild flies of hybrid origin. According to the National Drosophila Species Resource Center listing, it was originally designated as strain 17961.9 and was used by STONE (1949) for a test of the effect of the *X-4* centric fusion on female fertility in crosses between *americana* and *texana*. This implies that the karyotype of this stock was *americana* at the time of Stone's tests and has subsequently changed, either by detachment of the fusion or by contamination with *texana*.

The operation of these causes of nondegeneration of the *americana* neo-*Y* can be tested for as follows. A nonrecombining chromosome arm in Drosophila is expected to show an extremely low level of genetic variation, because of the effects of selective sweeps or background selection (KAPLAN *et al.* 1989; CHARLESWORTH *et al.* 1993; STEPHAN 1995). For example, assume that the mutation rate for nonlethal deleterious alleles for the neo-*Y* is one-half the rate estimated for the second chromosome of *D. melanogaster*, in accordance with the relative sizes of the two chromosomes. Using the conservative value of 0.15 for this latter rate (CROW and SIMMONS 1983; KEIGHTLEY 1994), and assuming that the harmonic mean selection coefficient against heterozygous deleterious mutations is 0.02 (CROW and SIMMONS 1983; CHARLESWORTH and HUGHES 1997), the expected value of the neutral nucleotide site diversity under the background selection model relative to the classical neutral value is $\exp(-0.075/0.02) = 0.02$ (CHARLESWORTH *et al.* 1993). From this cause alone, therefore, there should be a very low level of silent variation on the *americana* neo-*Y*. If a substantial level of silent genetic variation is found for loci on the neo-*Y*, comparable to that for loci on other chromosomes, this would indicate that the postulated processes have not been at work,

either because of gene flow between *texana* and *americana* or because of a very recent origin of the centric fusion. We are currently investigating this question.

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LITERATURE CITED

ALBORNOZ, J., and A. DOMÍNGUEZ, 1994 Inversion polymorphism and accumulation of lethals in selected lines of *Drosophila melanogaster*. Heredity **73:** 92–97.

ALEXANDER, M. L., 1976 The genetics of *Drosophila virilis*, pp. 1365–1427 in *The Genetics and Biology of Drosophila*, Vol. 1c, edited by M. ASHBURNER and E. NOVITSKI. Academic Press, New York.

ASHBURNER, M., 1989 *Drosophila. A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

BONE, J. R., and M. KURODA, 1996 Dosage compensation regulatory proteins and the evolution of sex chromosomes in Drosophila. Genetics **144:** 705–713.

BULL, J. J., 1983 *Evolution of Sex Determining Mechanisms.* Benjamin Cummings, Menlo Park, CA.

CARSON, H. L., and W. C. BLIGHT, 1952 Sex chromosome polymorphism in a population of *D. americana*. Genetics **77:** 572.

CHARLESWORTH, B., 1978 Model for evolution of *Y* chromosomes and dosage compensation. Proc. Natl. Acad. Sci. USA **75:** 5618–5622.

CHARLESWORTH, B., 1991 The evolution of sex chromosomes. Science **251:** 1030–1033.

CHARLESWORTH, B., 1996 The evolution of chromosomal sex determination and dosage compensation. Curr. Biol. **6:** 149–162.

CHARLESWORTH, B., and D. CHARLESWORTH, 1985 Genetic variation in recombination in Drosophila. I. Responses to selection and preliminary genetic analysis. Heredity **54:** 71–84.

CHARLESWORTH, B., and K. A. HUGHES, 1997 The maintenance of genetic variation in life-history traits, in *Evolutionary Genetics from Molecules to Morphology*, edited by R. S. SINGH and C. B. KRIMBAS, Cambridge University Press, Cambridge. (in press).

CHARLESWORTH, B., M. T. MORGAN and D. CHARLESWORTH, 1993 The effect of deleterious mutations on neutral molecular variation. Genetics **134:** 1289–1303.

COYNE, J. A., 1988 Do males of *Drosophila littoralis* have free recombination? Hereditas **109:** 281–282.

COYNE, J. A., and ORR, H. A., 1989 Two rules of speciation, pp. 180–207 in *Speciation and Its Consequences*, edited by J. A. ENDLER and D. OTTE. Sinauer, Sunderland, MA.

CROW, J. F., and M. KIMURA, 1970 *An Introduction to Population Genetics Theory.* Harper and Row, New York.

CROW, J. F., and M. J. SIMMONS, 1983 The mutation load in Drosophila, pp. 1–35 in *The Genetics and Biology of Drosophila*, Vol. 3c, edited by M. ASHBURNER, H. L. CARSON and J. N. THOMPSON. Academic Press, London.

DAS, M., D. MUTSUDDI, A. K. DUTTAGUPTA and A. S. MUKHERJEE, 1982 Segmental heterogeneity in replication and transcription of the X_2 chromosomes of *Drosophila miranda* and conservativeness in the evolution of dosage compensation. Chromosoma **87:** 373–388.

DOBZHANSKY, T. and C. C. TAN, 1936 Studies on hybrid sterility. III. A comparison of the gene order in two species, *Drosophila pseudoobscura* and *Drosophila miranda*. Z. Ind. Abstamm. Vererb. **72:** 88–114.

FEDER, J. H., 1984 *Experimental Analysis of Second Chromosome Lethals in Natural Populations of Drosophila americana.* Ph.D. dissertation, University of Chicago, Chicago, IL.

FISHER, R. A., 1935 The sheltering of lethals. Am. Nat. **69:** 446–455.

GETHMANN, R. C., 1988 Crossing over in males of higher Diptera (Brachycera). J. Hered. **79:** 344–350.

GRAVES, J. A. M., 1995 The origin and function of the mammalian *Y* chromosome and *Y*-borne genes—an evolving understanding. Bioessays **17:** 311–320.

GUBENKO, I. S., and M. B. EVGEN'EV, 1984 Cytological and linkage maps of *Drosophila virilis* chromosomes. Genetica **65:** 127–139.

HALDANE, J. B. S., 1922 Sex-ratio and unisexual sterility in hybrid animals. J. Genet. **12:** 101–109.

HEBERT, P. D. N., and M. J. BEATON, 1989 *A Practical Handbook of Cellulose Acetate Electrophoresis.* Helena Laboratories, Beaumont, TX.

HILTON, H., and J. HEY, 1996 DNA sequence variation at the *Period* locus reveals the history of species and speciation events in the *Drosophila virilis* group. Genetics **144:** 1015–1025.

HSU, T. C., 1952 Chromosomal variation and evolution in the virilis group of Drosophila. Univ. Tex. Pub. **5204:** 35–72.

HUGHES, R. D., 1939 An analysis of the chromosomes of the two subspecies *Drosophila virilis virilis* and *Drosophila virilis americana*. Genetics **24:** 811–834.

JABLONKA, E., and M. J. LAMB, 1990 The evolution of heteromorphic sex chromosomes. Biol. Rev. **65:** 249–276.

KAPLAN, N. L., R. R. HUDSON and C. H. LANGLEY, 1989 The "hitchhiking" effect revisited. Genetics **123:** 887–899.

KEIGHTLEY, P. D., 1994 The distribution of mutation effects on viability in *Drosophila melanogaster*. Genetics **138:** 1–8.

KEMPTHORNE, O., 1957 *An Introduction to Genetic Statistics.* John Wiley, New York.

KIMURA, M., and T. OHTA, 1971 *Theoretical Aspects of Population Genetics.* Princeton University Press, Princeton, NJ.

KREITMAN, M., 1983 Nucleotide polymorphism at the alcohol dehydrogenase locus of *Drosophila melanogaster*. Nature **304:** 412–417.

KRISHNAN, R., K. D. SWANSON and R. GANGULY, 1991 Dosage compensation of a retina-specific gene in *Drosophila miranda*. Chromosoma **100:** 125–133.

LINDSLEY, D. L., and G. G. ZIMM, 1992 *The Genome of Drosophila melanogaster.* Academic Press, San Diego.

LUCCHESI, J. C., 1978 Gene dosage compensation and the evolution of sex chromosomes. Science **202:** 711–716.

LUCCHESI, J. C., 1993 How widespread is dosage compensation? Sem. Dev. Biol. **4:** 107–116.

LUMME, J., and P. LANKINEN, 1988 Comments to Jerry A. Coyne: do males of *Drosophila littoralis* have free recombination? Hereditas **109:** 283.

LYON, M. F., 1961 Gene action in the *X*-chromosome of the mouse (*Mus musculus* L.). Nature **190:** 372–373.

MACKNIGHT, R. H., 1939 The sex-determining mechanism of *Drosophila miranda*. Genetics **24:** 180–201.

MARÍN, I., G. I. FRANKE, G. I. BRADSHAW and B. S. BAKER, 1996 The dosage compensation system of Drosophila is co-opted by newly evolved *X* chromosomes. Nature **383:** 160–163.

MONTGOMERY, E. A., B. CHARLESWORTH and C. H. LANGLEY, 1987 A test for the role of natural selection in the stabilization of transposable element copy number in a population of *Drosophila melanogaster*. Genet. Res. **49:** 31–41.

MORIYAMA, E. N., and J. R. POWELL, 1996 Intraspecific nuclear DNA variation in Drosophila. Mol. Biol. Evol. **13:** 261–277.

MULLER, H. J., 1918 Genetic variability, twin hybrids and constant hybrids in a case of balanced lethal factors. Genetics **3:** 422–499.

MULLER, H. J., 1932 Further studies on the nature and causes of gene mutations. Proc. 6th Int. Cong. Genet. **1:** 213–255.

MULLER, H. J., 1940 Bearing of the Drosophila work on systematics, pp. 185–268 in *The New Systematics*, edited by J. S. HUXLEY. Oxford University Press, Oxford.

NEI, M., 1970 Accumulation of nonfunctional genes on sheltered chromosomes. Am. Nat. **104:** 311–322.

NORMAN, R. A., and W. W. DOANE, 1990 Dosage compensation and dietary glucose repression of larval amylase activity in *Drosophila miranda*. Biochem. Genet. **28:** 601–613.

NURMINSKY, D. I., E. N. MORIYAMA, E. R. LOZOVSKAYA and D. L. HARTL, 1996 Molecular phylogeny and genome evolution in the *Drosophila virilis* group: duplications of the *Alcohol dehydrogenase* gene. Mol. Biol. Evol. **13:** 132–149.

ORR, H. A., and J. A. COYNE, 1989 The genetics of postzygotic isolation in the *Drosophila virilis* group. Genetics **121:** 527–537.

PATTERSON, J. T., and W. S. STONE, 1952 *Evolution in the Genus Drosophila.* MacMillan, New York.

PATTERSON, J. T., W. S. STONE and A. B. GRIFFEN, 1942 Genetic and cytological studies of the *virilis* species group. Univ. Tex. Publ. **4228:** 162–200.

RICE, W. R., 1987 The accumulation of sexually antagonistic genes as a selective agent promoting the evolution of reduced recombination between primitive sex chromosomes. Evolution **41:** 911–914.

RICE, W. R., 1994 Degeneration of a nonrecombining chromosome. Science **263:** 230–232.

RICHARDSON, B. J., P. R. BAVERSTOCK and M. ADAMS, 1986 *Allozyme Electrophoresis.* Academic Press, New York.

ROBERTS, D. R., and S. EVANS-ROBERTS, 1979 The genetic and cytogenetic localization of the three structural genes coding for the major protein of Drosophila larval serum. Genetics **93:** 663–679.

RUSSO, C. A. M., N. TAKEZAKI and M. NEI, 1995 Molecular phylogeny and divergence times of Drosophilid species. Mol. Biol. Evol. **12:** 391–404.

SEGARRA, C., G. RIBÓ and M. AGUADÉ, 1996 Differentiation of Muller's chromosomal elements *D* and *E* in the obscura group of Drosophila. Genetics **114:** 139–146.

SIMMONS, M. J., and J. F. CROW, 1977 Mutations affecting fitness in Drosophila populations. Ann. Rev. Genet. **11:** 49–78.

SNIEGOWSKI, P. D., and B. CHARLESWORTH, 1994 Transposable element numbers in cosmopolitan inversions from a natural population of *Drosophila melanogaster.* Genetics **137:** 815–827.

SPERLICH, D., H. FEUERBACH-MRAVIAG, P. LANGE, A. MICHAELIDIS and A. PENTZOS-DUPONTE, 1977 Genetic load and viability distributions in central and marginal populations of *Drosophila subobscura.* Genetics **86:** 835–848.

SPICER, G. S., 1992 Reevaluation of the phylogeny of the *Drosophila virilis* species group (Diptera: Drosophilidae). Ann. Entom. Soc. Amer. **85:** 11–25.

STALKER, H. D., 1940 Chromosome homologies of two sub-species of *Drosophila virilis.* Proc. Natl. Acad. Sci. USA **26:** 575–578.

STEINEMANN, M., S. STEINEMANN and F. LOTTSPEICH, 1993 How *Y* chromosomes become genetically inert. Proc. Natl. Acad. Sci. USA **90:** 5737–5741.

STEINEMANN, M., S. STEINEMANN and B. M. TURNER, 1996 Evolution of dosage compensation. Chr. Res. **141:** 413–429.

STEPHAN, W., 1995 An improved method for estimating the rate of fixation of favorable mutations based on DNA polymorphism data. Mol. Biol. Evol. **12:** 959–962.

STONE, W. S., 1949 The survival of chromosomal variation in evolution. Univ. Tex. Pub. **4920:** 18–21.

STONE, W. S., and J. T. PATTERSON, 1947 The species relationships in the *virilis* group. Tex. Univ. Pub. **4720:** 157–160.

STROBEL, E., C. PELLING and N. ARNHEIM, 1978 Incomplete dosage compensation in an evolving Drosophila sex chromosome. Proc. Natl. Acad. Sci. USA **75:** 931–935.

THROCKMORTON, L. H., 1982 The virilis species group, pp. 227–296 in *The Genetics and Biology of Drosophila,* Vol. 3b, edited by M. ASHBURNER, H. L. CARSON and J. N. THOMPSON. Academic Press, London.

TOMINAGA, H., and S. NARISE, 1995 Sequence evolution of the *Gpdh* gene in the *Drosophila virilis* group. Genetica **96:** 293–302.

WARTERS, M., 1944 Chromosomal aberrations in wild populations of Drosophila. Univ. Tex. Publ. **4445:** 129–174.

WESTERGAARD, M., 1958 The mechanism of sex determination in flowering plants. Adv. Genet. **9:** 217–281.

WHITE, M. J. D., 1973 *Animal Cytology and Evolution.* Cambridge University Press, Cambridge.

WHITING, J. H., M. D. PLILEY, J. L. FARMER and D. E. JEFFERY, 1989 In situ hybridization analysis of chromosomal homologies in *Drosophila melanogaster* and *Drosophila virilis.* Genetics **122:** 99–109.

WU, C.-I., and M. F. PALOPOLI, 1994 Genetics of postmating isolation in animals. Annu. Rev. Genet. **28:** 283–308.

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APPENDIX

We here present an analysis of the conclusions from the sex ratio and segregation analyses for the markers *eno* and *cd*, for inbred crosses involving strains *am-0* and *am-6*. The largest deviation from a 1:1 sex ratio is *am-0*; here, the ratio of males to females in the inbred F_3 cross is 0.76 ± 0.01. Since half the F_3 male progeny are homozygous for a given neo-*Y* allele, approximately additive action of the neo-*Y* genes affecting viability would imply that the sum of the reduction in viability caused by each locus is 0.48 ± 0.02.

In this cross, the ratio of *americana* homozygotes to heterozyotes for the marker loci is 0.59 ± 0.08 for *eno* and 0.52 ± 0.06 for *cd*; these are not significantly different. If a single locus is responsible for the reduction in viability associated with homozygosity for the *americana* allele, with recombination frequency r with respect to the marker in question, this ratio estimates $\{1 - s(1 - r)\}/(1 - rs)$, where s is the homozygous reduction in viability caused by this locus. The parameters s and r cannot, therefore, be estimated separately. A minimum estimate of s is given by setting r to zero, in which case we have $s = 0.41$ for *eno* and 0.48 for *cd*. The sum of these is clearly much greater than the sum of locus effects suggested by the sex ratio data.

A possibly more satisfactory alternative model for the marker-associated viability effects is to assume that the same locus is responsible for the deviations for both *eno* and *cd*, and is located about halfway between the two genes. The recombination frequency between *eno* and *cd* from all available data was estimated as 0.33, so that r would be ~ 0.16 on this model. Pooling the ratios for *americana* homozygotes to heterozyotes for *eno* and *cd*, gives $s = 0.61 \pm 0.06$ in this case, which is not significantly different from the estimate of total locus effects suggested by the sex ratio data. An even higher estimate of s would be obtained if the probable distortion of the segregation ratio of the markers due to incompatibility effects were allowed for, but the data are too limited to permit an accurate estimate of this. A similar effect is also seen for the *am-6* strain; the estimate of s on the assumption of a single locus halfway between *eno* and *cd* is 0.58 ± 0.10 for the inbred *am-6* cross.