selection for groups of genes within inversions: 1) in \textit{D. melanogaster} for changes in response to global warming (Balanya \textit{et al}., 2006) and body size (Kennington \textit{et al}., 2007); 2) in butterflies for wing color (Joran \textit{et al}., 2011), and 3) in humans for an increase in offspring numbers (Stefansson \textit{et al}., 2005). Yet, the genes have not been identified in the inversions of these four examples. Hence, students might also discuss the two alleles of an odorant-binding protein gene (\textit{Gp-9}) that is located in a large inversion that determines the social structure of fire ants (Krieger and Ross, 2002; Wang \textit{et al}., 2013). For example, the two variants of the \textit{Gp-9} gene determine if ant colonies have one queen or many queens, because of the killing of queens by workers if the queens have the wrong \textit{Gp-9} genotype.


\textbf{An attempt to identify new recessive sex-linked visible mutations in \textit{Drosophila melanogaster}.}

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The first attached-X chromosome in \textit{Drosophila melanogaster}, where two X chromosomes are attached to a single centromere, was isolated by Lillian V. Morgan in 1921 (Morgan, 1922). This compound stock, which is now called C(1)RM (the two X chromosomes are attached in reverse), and the C(1)DX attached X chromosome (the two X chromosomes are attached in tandem), which was isolated by H. J. Muller in 1943 (Muller, 1943), have been used to isolate new visible mutations on
the X chromosome by the following cross (see Auerbach, 1962, p. 39; Woodruff et al., 1979; Norris and Woodruff, 1992; for reviews of this topic). In this cross, \( y \) is a yellow-body mutation, \( f \) is a forked (small) bristle mutation, and + is the symbol for wild type (for descriptions of the compound autosome stocks and mutants, see Lindsley and Zimm, 1992).

\[
\text{C(1)DX, } y f / Y \text{ females } \times \text{ single } + / Y \text{ male}
\]

In this experiment, we mated single wild-type males, which were scanned for preexisting visible morphological aberrations, with three C(1)DX, \( y f / Y \) females, and screened F1 patroclinous males (receiving their X chromosome from their fathers) for new altered phenotypes. In particular, we looked for changes in 20 known X-linked mutations that alter wings (miniature, cut, rudimentary, notch, crossveinless, fused, scalloped, and cut); bristles (forked, bobbed, and singed); eye color (white, vermilion, carnation, garnet, prune, ruby, and carmine); eye shape (lozenge); and body color (yellow) (see Lindsley and Zimm, 1992). Any male progeny with altered phenotypes were mated again to C(1)DX, \( y f / Y \) females to confirm that the presumptive X-linked mutants bred true (the F2 male progeny have the same new phenotype as the F1 males). Rare dominant autosomal mutations would appear in both male and female F1 and F2 progeny. Any mutants that bred true were mated with wild-type (Canton-S) females to determine if the mutations were recessive (progeny females would be wild type) or dominant (progeny females would be mutant). Rare triplo-X females were also observed at a low frequency in the above cross. The triplo-X females are easy to identify, because they are wild type for \( y \) and \( f \), have small deformed wings, are weak (move slowly), develop slowly (eclose from pupae in older vials), and are sterile (Lindsley and Grell, 1992).

We screened 10,603 F1 males for new recessive visible mutations. A total of 27 presumptive visible mutants were tested to see if they bred true by mating them to C(1)DX, \( y f / Y \) females. These presumptive mutants included abnormal wings, abdomens, heads, legs, and antennae, or dark eyes. Of these presumptive mutants, eight were sterile and 18 did not breed true. One white-eyed male (from a cross with Canton-S males) did breed true. This white-eyed male was observed with 13 red-eyed males. This gives a rate for X-linked visible mutations of 0.0094 percent (1/10,603, yet, see below). Woodruff, et al. (1979) recovered no mutants out of 30,748 males, and Norris and Woodruff (1992) recovered seven mutants out of 23,092 males in similar crosses. We also recovered 149 triplo-X females; 81 of these triplo-X females were mated and all were sterile, as expected.

Additional crosses determined that the new white-eyed mutation was X linked and recessive (see above). To determine if the white-eyed mutation was an allele of the X-linked white locus of \( D. \) melanogaster, Canton-S females were mated to \( w^{1118} \) males, and F1 heterozygous + / \( w^{1118} \) females were mated with the new white-eyed males. From these crosses we observed 122 wild-type F2 females and 126 white-eyed F2 females. Hence, the new white-eyed mutation was an allele of the white locus, and we named it \( w^{bg} \) (white bowling green).

To rule out the possibility that \( w^{bg} \) was not a new mutation of the white locus, but was a contaminant from a laboratory \( w^{1118} \) stock, the only white-eye mutant in the laboratory during this experiment, we designed primers that included the first exon of the white locus and ran a PCR analysis of part of the white gene in single males of Canton-S, \( w^{1118} \), which is the result of a 9,000 base-pair deletion that includes the first exon of the white locus (Platts et al., 2009), and \( w^{bg} \), which is assumed to have occurred on the Canton-S X chromosome. The PCR primers were: White.Ex1.F (5' - GTC CGC TAT CTC TTT CGC CA - 3') and White.In1.R (5' - ACG CCG CAG ACA ATT TGA TG - 3'). These primers were designed to amplify exon 1 (~385 base pairs) of the white locus. PCR amplifications were conducted in 20 μl volumes containing 4 μl extracted DNA, 2.0 mM MgCl2, 0.5 μM of each primer, 150 μM of each dNTP, 1× Promega GoTaq Buffer, and 1 unit of Promega GoTaq
DNA Polymerase. Samples were amplified using a MJ Research Thermal Cycler with the thermal profile of 35 cycles at 94°C for 30 sec, 61°C for 30 sec, and 72°C for 30 sec. PCR products were visualized under UV light by 1% agarose gel electrophoresis with ethidium bromide staining.

If \( w^{bg} \) were a contaminant from the \( w^{1118} \) stock, then we would expect to recover no PCR bands from the DNA of \( w^{1118} \) and \( w^{bg} \) males, but would get a band of 385 base pairs from the Canton-S male. If \( w^{bg} \) were a new mutation of the white locus, we would expect to recover no PCR band from the \( w^{1118} \) male, but would observe similar sized (385 base pairs) PCR bands from the Canton-S and \( w^{bg} \) males. The results of the PCR analyses are shown below (Figure 1). These PCR results do not support the hypothesis that \( w^{bg} \) is a new mutation of the white locus. In fact, it seems to be a \( w^{1118} \) contamination from our \( w^{1118} \) laboratory stock, since a band was not amplified for \( w^{bg} \) or \( w^{1118} \). Hence, we isolated no new X-linked visible mutations in this experiment (0/10,603).

The lack of recovery of visible mutants among 10,603 males screened in this study was not expected, since we had looked for mutations in at least 20 X-linked genes (see above), giving us over 200,000 gene tests (20 \( \times \) 10,603) for visible mutations. If the average mutation rate for a gene in \( D. melanogaster \) is about one in 100,000 (Drake et al., 1998), then we should have expected to recover two new mutants.

The deficit of visible mutants in this study, and similar studies (Woodruff et al., 1979), is one reason for the new motivation to measure mutation rates as changes in base pairs from genomic DNA sequences. For example, Haag-Liautard et al. (2007) have observed a base-pair mutation rate per generation for \( D. melanogaster \) of \( 8.4 \times 10^{-9} \). Since \( D. melanogaster \) has about 279,000,000 base pairs in their diploid genome (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/genome/browse/), there would be about two new base-pair mutations per fly each generation \((2.79 \times 10^{8} \times 8.4 \times 10^{-9} = 2.37)\). For humans, there are about 75 new base-pair mutations per human each generation \((6.47 \times 10^{9} \text{ bases times } 11.6 \times 10^{-9} \text{ base-pair mutation rate} = 75.05)\) (Campbell and Eichler, 2013; http://www.ncbi.nlm.nih.gov/genome/browse).

A class discussion of the results of this teaching exercise could include:

1) This experiment and others suggest that new visible mutants of \( D. melanogaster \) are very rare. Yet, early in T.H. Morgan’s laboratory at Columbia University in New York City a number of new sex-linked visible mutants were isolated (Kohler, 1994). How was this possible? Robert Kohler (1994) suggests that there was a burst of mutational activity in the Morgan laboratory due to the movement and insertion of transposable DNA elements (this idea was suggested to Kohler by Edward Lewis, Nobel Prize \textit{Drosophila} geneticist from Cal Tech). One might ask students to go to

![Figure 1.](image-url)
FlyBase [http://flybase.bio.indiana.edu] and search for the molecular basis of some of the early sex-linked mutations recovered in Morgan’s laboratory. For example, the sex-linked mutants white, forked, vermilion, prune, miniature, and garnet were caused by insertions of transposable DNA elements.

One possible reason for the burst of transposable DNA activity in Morgan’s laboratory was that they often screened the progeny of crosses between strains, which is known to activate some transposable DNA elements (Woodruff, Slatko, and Thompson, 1983; Crow and Dove, 1988). In addition, recombination in males was observed in Morgan’s laboratory (Muller, 1916; Bridges and Morgan, 1919). These recombination events in males are not the usual exchanges that occur in meiosis of females, but are now known to be caused by transposable DNA elements (Engels, 1989).

2) Students might wonder how one determines the base-pair mutation rate. A review of the techniques is given in Campbell and Eichler (2013, page 576, Box 1). The current method is to sequence the genomes of parents and their offspring and look for differences in base pairs (see Kong et al., 2012, where the base-pair mutation rate is estimated as $12 \times 10^{-9}$ per nucleotide per generation).

3) How does the rate of new visible mutants compare to the frequency of preexisting recessive visible mutations from nature or laboratory stocks? Students might read Woodruff and Onasch (2009) where 10 recessive visible mutants out of 40 tested wild lines of D. melanogaster were recovered by inbreeding experiments. This abundance of hidden genetic variation is one reason some consider new mutations to be a weak force in evolution (Hedrick, 2011; Relethford, 2012), although not all agree (see a discussion of the role of new mutations in evolution in Nei, 2013).