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Non-crisscross inheritance in crosses between X-linked mutant strains of *Drosophila melanogaster*: treasuring exceptions.

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Introduction

According to Bridges (1916), the exceptional flies that emerged from crosses between an X-linked *vermilion-eyed* female and a *red-eyed* male of *Drosophila melanogaster* (then known by its junior synonym *D. ampelophila*) are those who failed to express the typical crisscross inheritance of daughters with red eyes (as their father) and sons with *vermilion* eyes (as their mother). Instead, the exceptional flies include matroclinous daughters with *vermilion* eyes as their mother and patroclinous sons with red eyes as their father. Trying to save the paradigm of sex chromosomes inheritance, Bridges proposed a then bizarre explanation for the origin of the exceptional flies as being a result of a non-disjunction of the two X chromosomes during anaphases of the first meiotic division. Six years later, Lilian Vaughan Morgan (1922), the wife of Thomas Hunt Morgan, was the first to report the presence of a non-crisscross inheritance of a sex-linked trait in a line where 100% of the offspring were exceptional. Morgan proposed the presence of an attached X chromosome as a possible explanation, which was confirmed through cytological analysis. The attached X chromosomes may originate from a Robertsonian translocation (centric fusion) involving the pericentromeric heterochromatin. As pointed out by Moore (1986) the *Drosophila* group of the Columbia University, under the leadership of T.H. Morgan, learned how to treasure exceptions to get deep understandings of an inexplicable phenomenon.

Whenever a basic genetics experiment is carried out using *Drosophila melanogaster* X-linked mutations, there is a great probability for such exceptional flies (matroclinous females and patroclinous males) to show up in samples from 1,000 to 2,000 indentified flies of the F_1 Generation. However, the X-linked mutant used as a probe to allow the detection of exceptional flies must be present in the parental female, otherwise the crisscross inheritance, typical of sex linkage, will not show up.

The Genetics discipline of the Biological Sciences course at the *Universidade de Sao Paulo* provides freshman students the first opportunity to carry out an experiment on the model organism *Drosophila melanogaster*. The experiment aims primarily to give students tools to discover the inheritance patterns (an important basic genetic concept) of four selected morphological mutations, which are intentionally changed from year to year. The ideal experimental design is that the crosses occur between two double-mutant strains, the male parental line bearing two of the X-linked mutations and the female one autosomal and one X-linked recessive mutation. Once the patterns are determined, students are asked to map the X-linked genes using the male offspring without the need of performing testcrosses. The students dedicate 105 min per week and the whole experiment (three generations) takes only about one and a half months to be completed. It begins with crosses between two homozygous double-mutant lines named unknown Alpha and unknown Beta, which constitutes the parental generation, and follows with the analysis of the next two generations (F_1 and F_2). The mutations present in the Alpha and Beta strains and the offspring (F_1 and F_2 generations) of their crossings are a mystery to be unveiled, *i.e*., a challenge to the students through heuristic learning. Flies must be sexed in the late pupal stage, based on presence/absence of sexual combs, and individualized in vials with usual banana-agar culture medium; males being selected from the Alpha line and females, from the Beta line. Upon emerging a couple of flies must be anesthetized with triethylamine fumes (Fuyama, 1977) by each student to have their mutations identified under stereomicroscope. This substance is recommended, because flies remain anesthetized for at least 45 min and students have enough time to identify them without any stress. We suggest the use of LED illuminator, which does not cause flies to die because of heating during a longer analysis.

Over more than one decade, while carrying out lab experiments with *Drosophila melanogaster* for teaching basic principles of Genetics, we have noticed that most of the times we were more worried about exploring the typical inheritance pattern of different mutations than to take time to explore the by-products of such crosses as those represented by the exceptional flies. Later on, treasuring exceptions that showed up in undergraduate basic Genetics experiments we have realized the extraordinary value of also exploring the by-products that may occur in the experiments. In most cases the exceptional flies detected in the F_1 generation proved, through additional experiments, to be originated from primary nondisjunction of X chromosomes of Beta line females. In this paper we will present a case we considered the most remarkable one.

A special case of non-crisscross inheritance in a basic Genetics experiment

During part of the first semester (from late March through mid May) of 2008, a total of 122 enrolled students were assigned to groups of mostly four students to carry out the same identical experiment, which consisted of five individual crosses (per group of students) between *eosin forked* males and *ebony crossveinless* females. A total sample of 1,483 flies of F_1 generation, taken at random, was identified by all the students regarding their sexes and the presence/absence of one or more out of the four genetic markers. The sample consisted of 656 *crossveinless* males and 827 wild females, which clearly exhibited the crisscross inheritance regarding the *crossveinless* mutation. Additionally 12 exceptional imagines were identified: four *eosin forked* patroclinous males and eight

crossveinless matroclinous females. It is worthwhile to note that two of the males and six of the exceptional females were found in the same vial, *i.e.*, they were descendants of a single female. On the other hand, the remaining two exceptional females were also descendants of another single female and the remaining two exceptional males were descendants of two different females. The four patroclinous males were considered exceptional because they did not receive, as expected, the X chromosome of the mother but received it from the father instead, since it carried the recessive alleles for *eosin* eyes and *forked* bristles. So were the eight matroclinous female daughters, because they did not receive the X chromosome of the father, since it carried the dominant allele for complete wing veins and its influence would have prevailed. The crosses between *crossveinless* males and wild females of the F_1 generation were performed, consisting of 8 individual crosses per group of students. A total sample of 2,760 flies (half of each sex) of the F_2 generation was identified. Males were distributed in 16 phenotypes, while the females in only four.

The exceptional flies, except for one female who did not recover from the anesthetic, were used to carry out additional experiments, aiming to find out an explanation for the amazing condition, *i.e.*, the occurrence of non-crisscross inheritance regarding the X-linked *crossveinless* gene. The four males were individually crossed to virgin females from the enigmatic Beta strain (*crossveinless ebony*), exhibited normal sexual behavior, and copulated from 15 to 21 min. However, two out of the four of them, those descendants from two different females, proved to be sterile, because the females laid large amount of eggs but no larvae emerged.

The two females originated from a single parental female were individually transferred to new vials at least once a week until no sperm remained in the spermathecae and their offspring were analyzed. The results proved that they were the product of a primary nondisjunctional event on the meiosis of their mother, the more frequent numerical chromosomal aberration we have observed in similar experiments. The five females originated from another single parental Beta female were submitted to the same process. Virgin females were obtained by individualizing pupae of the offspring of one of the five females and subsequently crossed to *white-coffee* males, aiming to analyze further the non-crisscross inheritance. Amazingly, for many generations the offspring consisted of *crossveinless* or *crossveinless ebony* females and *white-coffee* males, *i.e.*, a non-crisscross strain was established.

As the *ebony* mutation causes approximately 20% of mortality in mutant flies in relation to wild type (Lindsley and Zimm, 1992), getting rid of it produces a stronger line. Several individual test crossings between *crossveinless* females and *ebony* males and the analysis of offspring allowed us to exclude the heterozygous ones, finally getting a non-crisscross lineage composed only by *crossveinless* females and *white-coffee* males.

Materials and Methods

Two strains of *Drosophila melanogaster*, the classical wild type Samarkand, and one non-crisscross isofemale line (*white-coffee* males and *crossveinless* females), isolated by the senior author and currently maintained in the *Laboratorio de Drosofilideos do Departamento de Genetica e Biologia Evolutiva do Instituto de Biociencias da Universidade de Sao Paulo*, were analyzed.

Aiming to find an explanation for the occurrence of a 100% non-crisscross lineage containing *crossveinless* females and *white-coffee* males, third instar larvae were cytologically analyzed. The larvae were taken at random from among those crawling up the walls of the vials, dissected under a stereomicroscope over a black background, and sexed through the analysis of the paired gonads in the posterior third of the body. Testes are much larger, oval-shaped, and more loosely attached to the opaque fat body than the ovaries (Demerec, 1950).

The pro-metaphase and/or metaphase mitotic chromosomes were prepared according to the technique detailed by Baimai (1977) with some modifications as is follows. The larva was dissected directly in 1% trisodium citrate solution (and not in normal saline solution), and the brain ganglia were transferred to a new drop of the same solution (without pretreatment with colchicine) where it remained for ~ 10 min and then transferred to a drop of fixative (3:1 ethanol-acetic acid solution) for 1 min on the left lateral half of a siliconized microscope slide. The brain ganglia were removed from the fixative solution to a drop of 25 µl of 60% acetic acid placed on the right lateral half the same slide and dissociated with the aid of a pair of minuten pins inserted into a shortened wooden chopstick. The latter drop containing the cell suspension is transferred to a clean microscope slide on a warming plate at about 45ºC with the aid of a 200 µl standard pipette tip in a 5-40 µl micropipette. The cell suspension is heat-dried onto the slide and stained with 10% Giemsa, as detailed by Baimai (1977). At first, the microscope slide was checked from scanning magnification through 40× objective of a light microscope, and then digital photomicrographs of the best plates were taken with a 100× objective of a Photomicroscope.

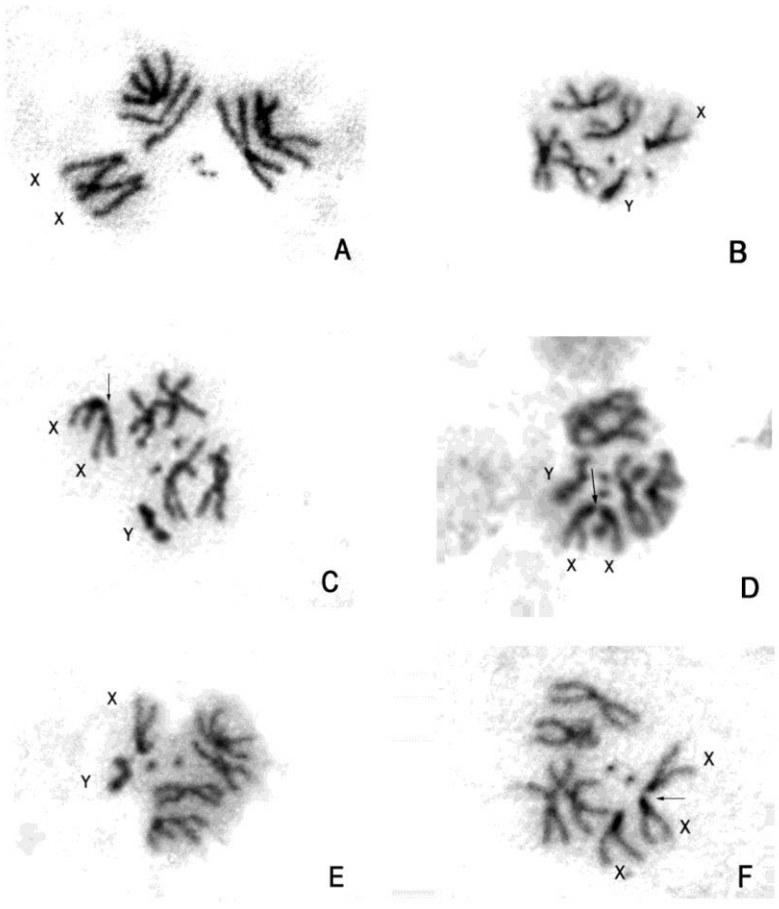

Figure 1. A) pro-metaphase mitotic plate of a wild type female from the Samarkand strain, showing two unattached X chromosomes and three pairs of autosomes (two V-shaped pairs and one dot-shaped pair). B) metaphase mitotic plate of a wild type male from the Samarkand strain, showing one X chromosome and one Y chromosome. C, D) two metaphase mitotic plates of *crossveinless* females XXY, the arrow points to the region of the attachment in the X chromosomes. E) pro-metaphase mitotic plate of a *white-coffee* male, depicting a normal karyotype with one X chromosome and one Y chromosome, like the wild type male. F) pro-metaphase mitotic plate of a wild trisomic female XXX (two attached and one unattached X, forming a trivalent mostly paired through their pericentric heterochromatin); the arrow points to the region of attachment of two out of the three X chromosomes.

Results and Discussion

Mitotic plates of normal female (Figure 1A) and male (Figure 1B), both with a diploid number 2n = 8, were obtained from a classical wild type strain (Samarkand) and photomicrographed for comparison purposes as shown in Figure 1. Analyses of the photomicrographs of the most

frequent type of mitotic plates of the non-crisscross line confirmed the hypothesis of attached X chromosomes in the *crossveinless* female flies, which therefore have a diploid number 2n = 9, consisting of three pairs of autosomes and three sexual chromosomes (XXY); the attached chromosomes X being V-shaped, longer than each of the also V-shaped chromosomes 2 and 3 and usually unpaired to the almost entirely heterochromatic Y (Figures 1E, F). The *white-coffee* males have a normal diploid number 2n= 8 (XY) as depicted in Figure 1C. Rare females also bearing the same diploid number 2n = 9 were also detected; however, they are devoid of a Y chromosome but are trisomic for the X chromosomes instead, two of them being attached (Figure 1D). The karyotypes we have found, except for the triplo-X, are similar to those obtained by Lilian Morgan in 1922.

The technique described by Baimai (1977) results in clear cut mitotic pro-metaphase and/or metaphase plates and, most importantly, it yields sharp differentiation between the euchromatic and heterochromatic regions of the chromosome complement. The photomicrographs show that the attachment of the X chromosomes occurred through their centromeric regions. Figures 1A and 1B depict the chromosomes of Samarkand wild type female and male, respectively. All the analyzed female larvae had a diploid number 2n = 9 and were trisomic for the sexual chromosomes, most of them had two X chromosomes attached to each other in addition to the Y chromosome, as shown in Figures 1C and 1D. The *white-coffee* male has a similar karyotype to the wild type male, showing two pairs of large V-shaped chromosomes, one pair of dot-shaped, and one rod-shaped X chromosome, in addition to one J-shaped Y chromosome, as shown in Figure 1E. Few female individuals, however, had three X chromosomes as shown in Figure 1F that shows the presence of two X chromosomes fused through their centromeric region (largest V-shaped chromosome) and one free, rod-shaped X chromosome.

It should be pointed out that usually attached X strains are not stable, and this instability was first reported by Anderson (1925) soon after Lilian Morgan (1922) had published her findings. He concluded that instability, in his case, whose attachment was induced by X-Ray irradiation, is associated with a detachment rate of X chromosomes, where an attached X adult female is eventually able to produce two types of ova, with one X or one Y chromosome. Anderson (1925) found a proportion of about 1 crisscross inheritance offspring per 1300 non-crisscross descendants in his lineage.

However, in our non-crisscross strain, which probably originated from a spontaneous rearrangement of parental female X chromosomes, we have observed a higher frequency of about 1 crisscross offspring per 300 non-crisscross descendants. We propose that the higher rate we have observed is due to the occurrence of the trisomic females (XXX) that emerge from the puparia as fertile females. Such a hypothesis was suggested because we have found triplo-X third instar larvae as shown in Figure 1C, which will account for the occurrence of fertile wild females, and a higher frequency of regular, *i.e.*, crisscross, offspring in the lineage. The analyses of the different phenotypes that eventually were identified in the lineage are detailed in Table 1. Based on the genetic analysis of the unstable strain along successive generations, we suspect that the three X chromosomes, that paired as an almost perfect trivalent during meiotic metaphase stage, being completely homologues, will have a higher probability of detachment of the attached pair during the subsequent anaphase stage than in a trivalent formed in the XXY females, where pairing occurs more loosely between the two attached X and the slightly, mostly heterochromatic, partially homologous Y chromosome. So, to be maintained, the attached X chromosome strain must be continuously scanned in order to detect and eliminate the wild female flies that promote the instability driving the strain back to the stable XY/XX condition.

Therefore, we believe that the wild and *white-coffee* females, in addition to the *crossveinless* males, were descendants of the fertile trisomic wild type females (XXX). The wild type males were

the result of a recombination event between the *crossveinless* and *white-coffee* loci. However, the wild females, who first appear in any given generation, are most probably the fertile trisomic ones that have escaped dying during the pupal stage, where the lethal effect of the triplo-X condition mainly occurs, as pointed out by Brehme (1937).

Table 1. Phenotypic classes and respective number of individuals sampled from many generations (pooled together) of the non-crisscross strain, composed of *crossveinless* females and *white-coffee* males. A total of 2,103 individuals, 1,000 females and 1,103 males, were sampled. *Crossveinless* females and *white-coffee* males were the most frequent classes, and are the product of a non-crisscross inheritance. The rarer classes found in the offspring were wild females and males, *white-coffee* females and *crossveinless* males.

females			males		
+	*cv*	w^{cf}	+	*cv*	w^{cf}
9	988	3	1	4	1,098

+ = wild type; *cv* = *crossveinless*; w^{cf} = *white-coffee*

Conclusion

The unusual X-linked inheritance pattern exhibit in 100% of the offspring of a lineage of *Drosophila melanogaster*, called non-crisscross inheritance and composed of *crossveinless* females and *white-coffee* males, was a result of the occurrence of a compound (attached) X chromosome. The genetic results are similar to those obtained by Lilian V. Morgan in 1922 and were as well confirmed by the cytological analysis that proved the existence of an attached X in the female matroclinous offspring. We afford the opinion that the instability of the analyzed strain starts whenever one fertile wild type triplo-X female escapes from dying during the critical pupal stage.

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Simple high school laboratory exercise on mate attraction and reproductive isolation in *Drosophila*.

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Species are often defined as groups that fail to successfully interbreed with other groups. Behaviors that keep groups from interbreeding help maintain biodiversity on our planet, and students