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COMPARATIVE MEIOTIC CYTOLOGY AMONG DROSOPHILA SPECIES

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COMPARATIVE MEIOTIC CYTOLOGY AMONG *DROSOPHILA* SPECIES

By

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ABSTRACT

Physical connections established by homologous recombination are normally sufficient to establish proper co-orientation of chromosomes during prometaphase of female meiosis I. Nonexchange chromosomes can still segregate because they are connected by heterochromatic threads, which are thought to connect homologous chromosomes and ensure co-orientation in the absence of a chiasma. In *Drosophila*, the nonexchange chromosomes (such as the Muller F element, also called the "dot chromosome," which never undergoes recombination) move out on the spindle during prometaphase I, and can be found positioned between the spindle poles and the exchange chromosomes at the metaphase plate. By metaphase I arrest, these chromosomes congress to a single mass. A previous study (Gilliland *et al.* 2015b) found a visible difference in the prometaphase dot-dot chromosome separation of two different *Drosophila* species (*Drosophila melanogaster* and *Drosophila simulans).* The mean dot-dot distance in *D. melanogaster* females (11.3 μm) was nearly twice as large as in *D. simulans* (6.1 μm). This difference in dot chromosome distances between these two species could be a result of their heterochromatin content; *D. melanogaster* has a larger amount of heterochromatin and has a longer average dot-dot chromosome distance, while *D. simulans* has less heterochromatin and a smaller dot-dot distance. A speculative further interpretation is that if the heterochromatic repeats on a chromosome form the threads connecting these nonexchange homologs, then having a greater amount of those repeats may increase thread length and enable those homologs to move farther apart from each other before the tether pulls tight enough to prevent further movement. A second difference between these species is that while *D. melanogaster* has many common

polymorphic chromosome inversions, *D. simulans* is monomorphic with no common inversions (Lemeunier and Aulard 1992). As inversions block crossing over, increasing the abundance of inversions should make meioses with nonexchange chromosomes more common. Because other nonexchange chromosomes in *D. melanogaster* are positioned between the dots near the spindle poles and the exchange chromosomes at the metaphase plate during prometaphase, having the dots further out could provide more space for additional nonexchange chromosomes to move out on the spindle. If this additional space is beneficial, then the greater amount of space on the spindle provided by the longer dot-dot distance in *D. melanogaster* might help this species tolerate common inversions, leading to selection for increased dot-dot distances. We aimed to understand how these heterochromatic threads change chromosome positioning during *Drosophila* meiosis, or whether they might also have other evolutionary effects such as affecting the abundance of inversions across *Drosophila* species. We sampled 14 *Drosophila* species with and without common inversions and measured their average dot-dot distances during meiotic prometaphase to see if their distances correlate with either the abundance of inversions, the amount of heterochromatin or both. We did not find a strong correlation with either factor in these species, which suggests that neither inversions nor the amount of heterochromatin in the genome determine dot-dot distances. However, while doing this work, we noticed substantial variation in the size of the dot chromosomes among these species, and that the proportion of oocytes with chromosomes out on the spindle appeared strongly correlated with dot chromosome size. This suggests the variation in the time spent doing the prometaphase chromosome movements is proportional to the size of the dot chromosome in these species.

ACKNOWLEDGEMENTS

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CONTENTS

INTRODUCTION

The purpose of meiosis is the faithful passage of genetic information from one generation to another. When meiosis functions properly, the integrity of the genome is preserved in the next generation, and viable offspring are produced. Meiotic defects, however, can result in sterility (failure to produce offspring) or developmental defects in offspring, often leading to premature death (Handel and Schimenti 2010). In fact, aneuploidy is one of the leading known causes of human congenital birth defects and miscarriages. The accurate segregation of chromosomes during meiosis is essential to prevent these genetic defects and ensure fertility. In many organisms, proper segregation is ensured by crossing over which results in recombination and the formation of chiasmata. Chiasmata lock homologous chromosomes (homologs) together and constrain the centromeres to orient towards opposite poles of the meiotic spindle, thus ensuring the proper segregation of recombinant (chiasmate) chromosomes during meiosis I (Hughes *et al*. 2009). This process is also essential for exchange of genetic information between homologous chromosomes. However, some chromosomes spontaneously fail to undergo crossing over some fraction of the time, while others (such as the small dot chromosome in *D. melanogaster)* never undergo crossing over. But, despite the lack of chiasmata, these nonexchange homologs still segregate faithfully (Zhang and Hawley 1990). How this process of nonexchange chromosome segregation works is a major topic of research in the Gilliland lab.

There are several stages of meiosis but the lab is interested in prometaphase of female meiosis I in the fruit fly *Drosophila melanogaster*. This stage of meiosis is important in studying the mechanism of chromosome movements which includes

understanding how exchange and nonexchange chromosomes achieve accurate segregation. The series of events that occur in meiosis I of *Drosophila* oogenesis has recently been revised, leading to a better understanding of how chromosomes achieve proper co-orientation during prometaphase I in this model organism.

During prometaphase I, nonexchange chromosomes move out on to opposite sides of the spindle, which was noted in the first confocal studies of female meiosis (Theurkauf and Hawley 1992). These nonexchange chromosomes can be found positioned between the spindle poles and the exchange chromosomes at the metaphase plate. It was initially thought that the nonexchange chromosomes moved out towards opposite spindle poles at the start of prometaphase, and remained there until metaphase arrest. However, recent work has revised this model. Live imaging of female meiosis found that while the nonexchange chromosomes do move out on the spindle, they undergo dynamic movements on the meiotic spindle prior to their proper segregation, being capable of crossing the spindle and re-associating with their homologs to attempt reorientation (Fig. 1) and eventually congress at metaphase arrest (Hughes *et al*. 2009).

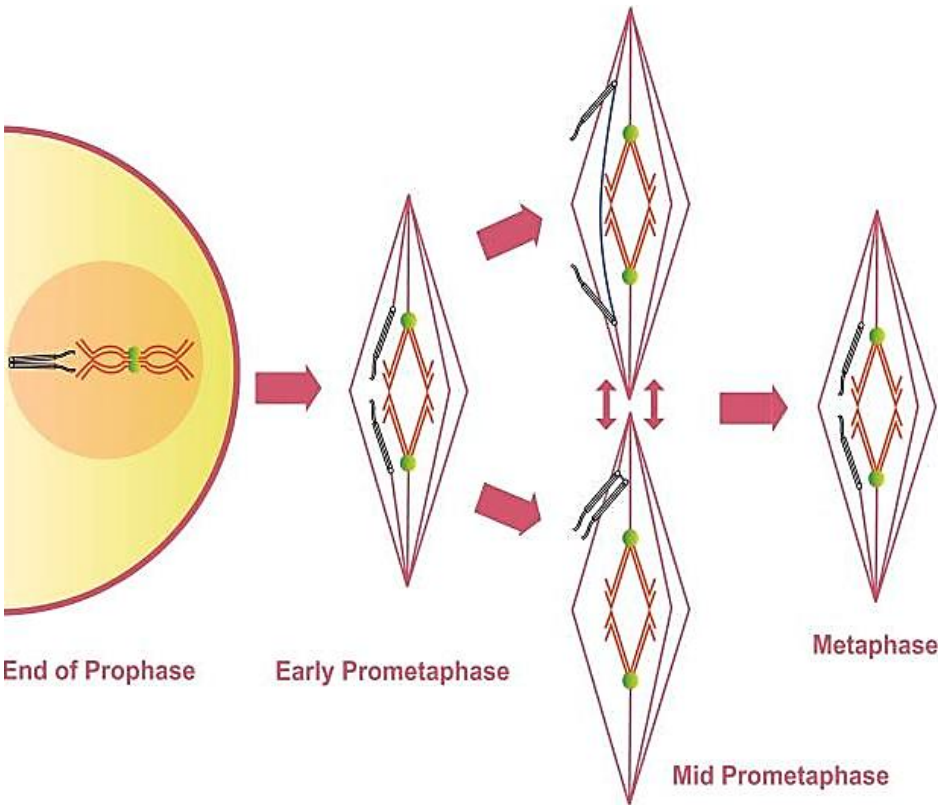


Figure 1. Revised model of *Drosophila* prometaphase. Chiasmate chromosomes (red) are locked at the spindle midzone with proper co-orientation due to the chiasmata holding them together while the centromeres (green) attached them to each spindle poles. Achiasmate chromosomes (black) are paired and co-oriented using heterochromatic pairing prior to germinal vesicle breakdown (GVBD). During mid prometaphase achiasmate chromosomes can move towards opposite poles or towards the same pole. (Diagram from Hughes *et al.* 2009).

The original confocal studies of meiosis in this species thought that metaphase arrest occurred with the nonexchange chromosomes positioned out on the spindle (Theurkauf and Hawley 1992). However, it was later shown that the symmetrical arrangement of the achiasmate chromosomes positioned between the poles and the spindle equator is actually a feature of mid-prometaphase, and by the end of prometaphase the achiasmate chromosomes congress to the metaphase plate prior to metaphase I arrest (Gilliland *et al.* 2009). In doing so, they join the chiasmate autosomes

and the chromosomes appear to form a single mass with a distinctive ‘lemon-shaped’ DNA morphology (Fig. 2).

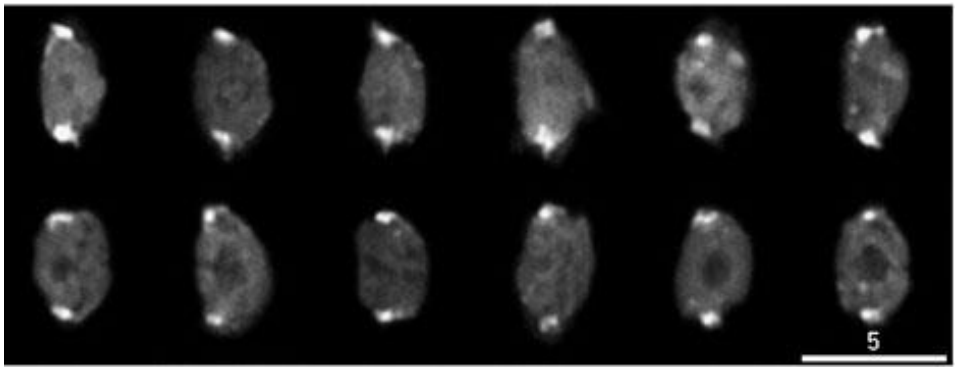


Figure 2. Oocyte nuclei in aged virgin females form a single compact DNA mass. A montage of representative oocyte nuclei from 4 dpe (days post eclosion) virgin females exhibiting the compact “lemon” configuration with no chromosomes out from the main mass. DAPI staining shows the bright spots at the tips of the karyosome are the heterochromatic dot chromosomes. (Diagram from Gilliland *et al*. 2009)

These observations have allowed the development of a classification system describing the stages from germinal vesicle breakdown (GVBD) to metaphase I arrest in *Drosophila* oocytes. Early prometaphase I is the period from GVBD to the completion of a bipolar spindle. Mid-prometaphase defines the period during which achiasmate homologs are clearly separated from the main mass and positioned between the center of the spindle and the poles. Late prometaphase I describes the stage in which the achiasmate chromosomes retract to the main mass in a fashion that results in their proper orientation. Finally, metaphase I arrest describe the stage at which all the chromosomes are clustered into a lemon shaped structure (Fig. 3), where the oocyte arrests until passage through the oviduct, fertilization, and entry into anaphase I (Hughes *et al.* 2009).

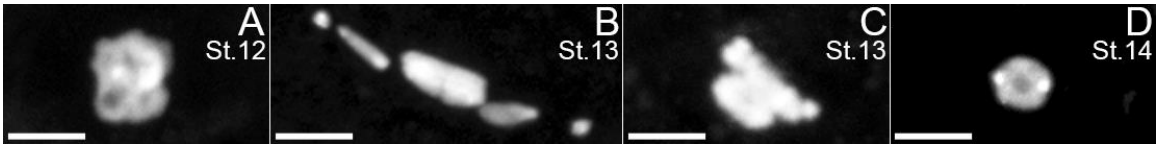


Figure 3. Nonexchange chromosomes (lined up between the dot and the exchange chromosomes) move out on the spindle during mid prometaphase and congress into a "lemon shaped" configuration by Metaphase arrest. Scale bar is 2 μm. St. represents the oogenesis stages of each cell. (W. Gilliland, unpub. Data)

Given that these nonexchange (achiasmate) chromosomes can move away from and congress back to the metaphase plate, the question arises as to how such movements are coordinated? Several labs have provided evidence for the existence of connections between nonexchange homologs during meiosis I. It was demonstrated that during anaphase I in crane fly spermatocytes that severing a trailing chromosome arm sometimes resulted in the severed fragment crossing the metaphase plate and then re-associating with its homolog on the opposite half of the spindle (LaFountain *et al*. 2002). This re-association suggests that homologs are connected in some way during meiosis I. Additionally, in mammalian tissue culture there were shown to exist threads that bind to the protein PICH (Plk-1 interacting checkpoint helicase), and what appears to be centromeric DNA connecting sister chromatids during mitosis in cultured cells. The PICH containing threads progressively increase in length during metaphase and disappear during anaphase (Baumann *et al.* 2007).

It is thought that this physical connection between achiasmate homologous chromosomes in *Drosophila* oocytes assists with their dynamic movements to facilitate the re-establishment of co-orientation. Recently, Hughes *et al*. (2009) demonstrated that during this oscillating chromosome movement in prometaphase, achiasmate homologs are connected by a thread that spans large distance. While chiasmata are normally

sufficient to establish coorientation, this discovery of the existence of threads connecting achiasmate homologous chromosomes (such as the small "dot" chromosome) provided a mechanism for their proper coorientation and accurate segregation (Fig. 4). These threads are made of heterochromatin (Hughes *et al*. 2009), and are thought to establish tension between homologs to facilitate coorientation of nonexchange chromosomes. However, the mechanism that establishes these tethers, what genes are required for their proper functioning, and how they are ultimately resolved remain largely unknown. One recent report found that topoisomerase II was required for the separation of the paired blocks of heterochromatin, suggesting that the maintenance and resolution of these connections must be regulated in part by normal chromatin maintenance pathways (Hughes and Hawley 2014).

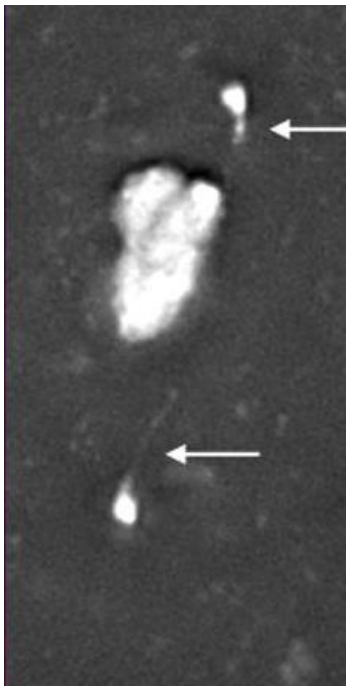


Figure 4. Heterochromatic thread connecting the dot chromosomes in a *D. melanogaster* oocyte. The exchange chromosomes are present in the large mass, while the small dots are out on the spindle. Arrows point to the threads, which are not visible across their entire length. (DNA/DAPI) (Diagram from Hughes *et al*. 2009)

Previous studies have also shown that heterochromatic homology is both necessary and sufficient to ensure proper segregation of achiasmate homologs (Hawley *et al*. 1992). It is currently unknown how these heterochromatic threads change chromosome positioning during *Drosophila* meiosis, or whether they might have other evolutionary effects, but the linkage of dot chromosomes by heterochromatic threads ensures connection to their homologs in the absence of chiasmata, and accurate segregation to avoid meiotic error. The dot chromosomes can move out on the spindle poles, remain connected until late prometaphase and rejoin the exchange chromosomes by metaphase arrest. However, oocytes carrying a monovalent *compound-4* chromosome (which has no homolog) have greatly reduced prometaphase chromosome movements, even when another chromosome is made nonexchange (Gilliland *et al.* 2015a). This suggests that the dot chromosomes might be critical for facilitating the prometaphase chromosome movements and congression.

Gilliland *et al.* (2015b) recently observed that two closely related sister species of *Drosophila* (*Drosophila melanogaster* and *Drosophila simulans)* have different dot-dot chromosome distances during prometaphase I. *D. melanogaster* showed a mean dot-dot distance of 11.3 µm, nearly twice as large as in *D. simulans* (6.1 µm). The authors speculated that natural differences between these species could explain the difference in their dot-dot distances. Since heterochromatic threads connecting nonexchange chromosomes are thought to be how they achieve proper coorientation (Hughes *et al*. 2009), this result suggested that threads play a role in chromosome positioning and provides evidence that the amount of heterochromatin on the dot chromosomes changes the distance between homologs. This difference in dot chromosome distances between

these two species could be a result of their heterochromatin content; *D. melanogaster* has a larger amount of heterochromatin (Ferree and Barbash 2009) and has a longer average dot-dot chromosome distance, while *D. simulans* has less heterochromatin and a smaller dot-dot distance. An increased amount of heterochromatin on the dot could cause longer threads; the large amount of heterochromatin in *D. melanogaster* could provide it with enough "rope" to pull its dot chromosomes farther apart, whereas *D. simulans*, which has a lower amount of heterochromatin, would have less "rope" and hence a shorter dot-dot distance. Another difference between these two closely related species is the amount of inversions they have in natural populations. Immediately after its origin, a new inversion becomes polymorphic within that species in which it has risen. If this inversion does not go extinct, it can persist to become common or fixed within the species. While natural populations of *D. melanogaster* harbor many common chromosomal inversions with an average of one inversion per fly, *D. simulans* is monomorphic with no common inversions and only one inversion per 200 flies (Lemeunier and Aulard 1992). As inversions block crossing over, increasing the abundance of inversions will make meiosis with nonexchange chromosomes more common (Gilliland *et al*. 2015b). In *D. melanogaster*, nonexchange chromosomes move out on the spindle during prometaphase I. While the significance of this movement is unknown, Gilliland *et al*. (2015a) speculated that it may be involved in how the oocytes achieve proper nonexchange chromosome coorientation and the metaphase arrested karyosome structure. Because nonexchange chromosomes in *D. melanogaster* are positioned between the dots near the spindle poles and the exchange chromosomes at the metaphase plate, having the dots further out would provide more space for additional nonexchange chromosomes to move

fully out on the spindle (Gilliland *et al*. 2015b). If this additional space helps it facilitate these movements, then the greater amount of space between the dots on the spindle provided by the larger dot-dot distance in *D. melanogaster* may allow that species to tolerate common inversions. Based on the pattern observed in these two species, it is unknown if the contrast in the dot-dot chromosome separation is correlated with the amount of heterochromatin or the abundance of inversions across *Drosophila* species. It could be that longer dot-dot threads evolved first, which allowed inversions to build up in the population, or maybe these inversions accumulated first, favoring the evolution of longer threads to accommodate their segregation. Either way, this model suggests that *Drosophila* species with common inversions like *D. melanogaster* would have a greater dot-dot distances than species that lack them and species with more heterochromatin would have a greater dot-dot distance than species with less. The goal of this project was to determine whether the differences observed between *D. melanogaster* and *D. simulans* are correlated with the amount of heterochromatin, the abundance of inversions, or both across *Drosophila* species.

This hypothesis was tested by sampling 14 *Drosophila* species (including *D. melanogaster* and *D. simulans*) with and without common inversions, and measuring their average dot-dot chromosome distances during meiotic prometaphase to see if those distances correlate with either the abundance of inversions, the amount of heterochromatin or both. For example, *Drosophila virilis*, which has a large amount of heterochromatin (Bosco *et al*. 2007), (like *D. melanogaster*) but no common inversions (like *D. simulans*) was a species we were particularly interested in testing.

After testing all these species, we found very little support for either of our initial hypotheses. There was no significant correlation between the amount of genomic heterochromatin and dot-dot distances among all species. Similarly, there also appeared to be no strong correlation between the presence of common inversions and dot-dot length. However, while doing the work we observed substantial variation in the size of the dot chromosomes, with the largest *(D. similis)* having a cross-sectional area over 10 times larger than that of the smallest *(D. hydei)*. We also found that chromosome sizes were strongly correlated with the proportion of oocytes with chromosomes out on the spindle. Because these were fixed samples, we interpret this to mean that oocytes with larger dot chromosomes spend a longer amount of time undergoing the prometaphase chromosome movements.

REVIEW OF LITERATURE

Drosophila life cycle:

The life cycle of *Drosophila* starts with an adult female fly that undergoes fertilization to produce an embryo (Fig. 5). This embryo then goes through series of molting stages (1st instar larva to 3rd instar larva) within three days before eventually developing into a pupa in another 2 ½ -3 days. This pupa then fully develops into an adult fly in 3-4 days. This process of egg to egg-laying adult takes 10 days in total, making this short life cycle a key factor in its use for cytological experimentation.

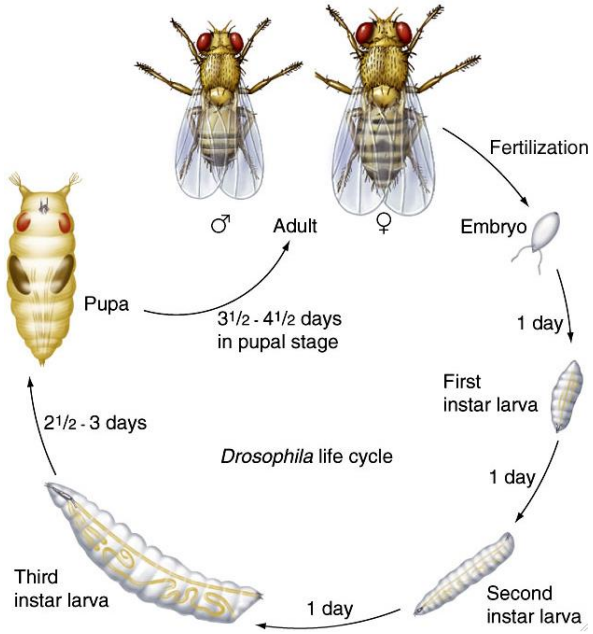


Figure 5. Life Cycle of *Drosophila*. The key stages of the life cycle of *Drosophila melanogaster*. After fertilization, there are three larval instar stages (molts), a pupa stage, eclosion and maturity to adulthood. [Figure from The McGraw-Hill Companies, Inc.]

Stocks of adult *Drosophila* flies of a specific genotype are kept in vials containing a mixed nutrient source where they can extract yeast for food. When the adult males and females of known genotypes are introduced together in larger bottles for mating, they produce a large amount of eggs within a few days (Greenspan 1997). Virgin females can

be collected because after females eclose from their pupal cases they require around 8 hours to finish metamorphosis before they are capable of mating. By removing all adults from the bottle, any newly eclosed adults that emerge within the next six hours are guaranteed to be virgins, which is essential for doing controlled matings.

Comparative Biology:

Drosophila are also easy and inexpensive to raise in the lab, which has made it a good model organism used by many genetic researchers. The *Drosophila* genus is estimated to have several thousand species (Singh 2015), some of which have had their genomes sequenced, making it a very important tool not only for studying evolution but for comparative biology among closely related species. The vast knowledge about this organism has created powerful genetic tools, including well-annotated genome sequences and balancer chromosomes. These balancer chromosomes suppress recombination with their homologues, allowing the maintenance of lethal and sterile mutants as balanced heterozygotes (Lattao *et al*. 2011).

A large number of closely related species have been studied, and researchers have noted the amount of heterochromatin and inversions in various species of *Drosophila.* This was possible not only because of the availability of genome sequence, but also due to the existence of a well-established phylogenetic tree (Fig. 6). Because closely related species share much of their evolutionary history, we typically expect that they resemble one another morphologically more so than distantly related species (Sokal R.R 1992).

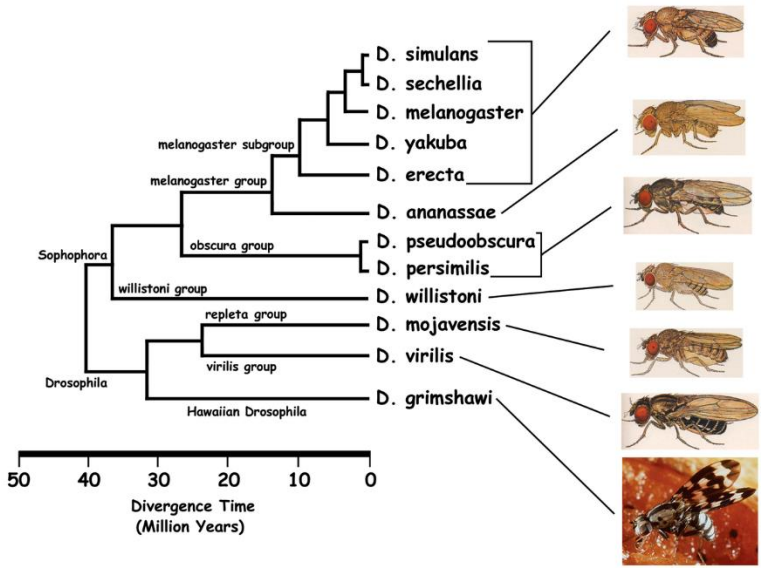


Figure 6. Consensus Phylogenic tree of *Drosophila* Species. This tree includes the *Sophophora* group represented by *melanogaster*, *obscura* and *willistoni* and the *Drosophila* group represented by the *virilis*, *repleta* and *Hawaiian* groups. *(Figure from Seetharam and Stuart, 2013)*

Drosophila Genome

Drosophila have a X-Y sex determination system, meaning that (like humans) the *XY* males are the heterogametic sex. These species have 4 to 6 pairs of homologous chromosomes (4 in *D. melanogaster),* which can be identified by their size and shape. A *D. melanogaster* female has two copies of chromosome *1* (more commonly called the *X* chromosome), *2*, *3*, and *4* while a male has one *X* chromosome, one *Y* chromosome, and two each of chromosome *2*, *3*, and *4* (Fig. 7). The *X* and *Y* chromosomes are involved in sex determination, and are thus called the sex chromosomes. Chromosomes *2*, *3*, and *4* are called autosomes. Although much of this species development is conserved, and there are similar patterns of gene expression that flies and humans share, sex determination is one area where the two have distinct differences. While the presence of a *Y* sex

chromosome makes a human male, the *Y* chromosome does not determine sex in flies even though it does contain genes necessary for male fertility (forming sperm in adults). In flies, the sex is determined by the ratio of *X* chromosomes to autosome sets. Normally, flies have either one or two *X* chromosomes and two sets of autosomes. If there is but one *X* chromosome in a diploid cell (1X: 2A), the fly is male. If there are two *X* chromosomes in a diploid cell (2X: 2A), the fly is female (Bridges 1921). The smallest chromosome is known as the "dot" chromosome (or, in the principle model organism *D. melanogaster*, the fourth chromosome), which is ~2 Mbp in length. It is an obligately nonexchange chromosome, which means that it never undergoes recombination.

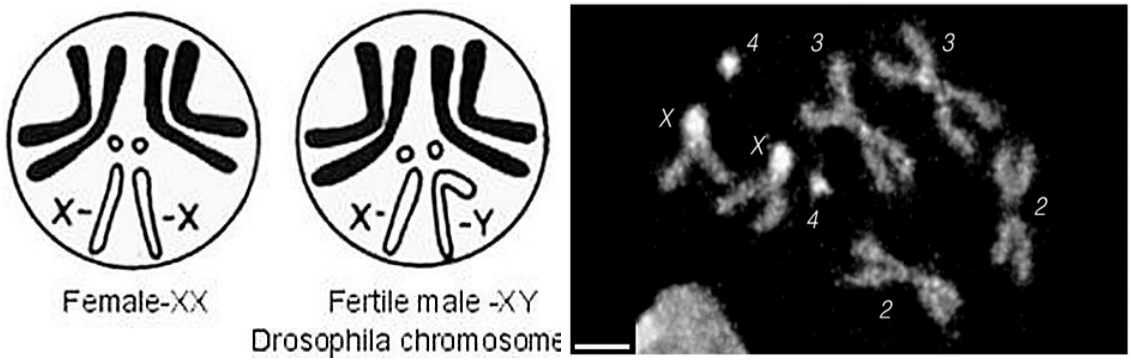
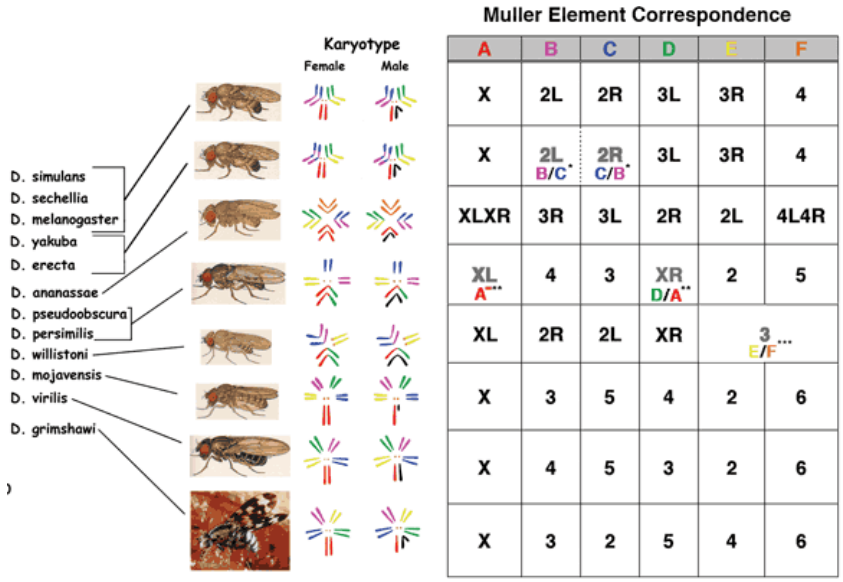


Figure 7. *Drosophila melanogaster* Genome. Left -The *X/Y* sex chromosomes (white), chromosomes *2* and *3* (in black) and the small dot chromosomes located in the center. (Burian *et al*. 2000), Right- DAPI-stained chromosomes of an Oregon–R *Drosophila* female larval neuroblast cell, showing eight chromosomes. Scale bar is 2 μm (Image from Gilliland *et al*. 2016)

Dot Chromosome:

The dot chromosome is small, mostly heterochromatic, and is exceptional among the autosomes in that it does not undergo recombination in females under standard laboratory conditions (Sandler and Szauter 1978). It is homologous among many

Drosophila species, but because autosomes within a species are numbered from largest to smallest, it is not always the 4th chromosome in all species. While present in most species, some (e.g. *D. willistoni*) have lost the dot chromosome due to chromosome fusion events (Fig. 8), and the functional importance of this chromosome is not known.



Muller Element Correspondence

A	B	C	D	E	F
X	2L	2R	3L	3R	4
X	2L B/C*	2R C/B*	3L	3R	4
XLXR	3R	3L	2R	2L	4L4R
XL A***	4	3	XR D/A**	2	5
XL	2R	2L	XR	3 ... E/F	
X	3	5	4	2	6
X	4	5	3	2	6
X	3	2	5	4	6

Figure 8. Muller Element Arm Synteny Table. In *D. willistoni* there is a fusion of the Muller F element into the distal end of the E element. Thus, there are no free dot chromosomes in this species. *(Figure from Drosophila Fly Base 2.0)*

Comparative genomics in *Drosophila* began when linkage maps of morphological traits were used to establish the homologies of six chromosomal arms in closely related species (Donald 1936; Sturtevant and Tan 1937). These early studies established the idea that genes are conserved on the same chromosome arm among species, but one difficulty encountered with early comparative genome analyses was that chromosomal arm nomenclature varied among species. Muller (1940) overcame this problem by using the banding patterns of *D. melanogaster* salivary gland chromosomes to identify six blocks of the genome, and then compared other species of flies to see where those blocks were located. Muller assigned a letter to each of the chromosome arm on the basis of the *D.*

melanogaster genome (chromosomal arm equals Muller element: X=A, 2L=B, 2R=C, 3L=D, 3R=E, 4=F). While there were multiple arrangements and changes in chromosome number, the dot chromosome (element F) could be identified in many species. The conservation of the banding patterns within these Muller elements has made it easier to infer chromosomal rearrangements in *Drosophila*.

Drosophila **Female Meiosis I:**

Meiosis is a form of eukaryotic cell division that forms gametes (eggs or sperm). During meiosis, diploid cells replicate their chromosomes once and divide twice, producing four haploid daughter cells. This segregation shuffles the genome content, generating genetic diversity in the progeny. Diploid organisms have two copies of each kind of chromosome, called homologs, with a single copy of each type of chromosome being received in the gamete from each parent. Before meiosis I, DNA replication first occurs during premeiotic S phase and results in the formation of two identical DNA molecules, called sister chromatids. During prophase I, homologs pair with each other, which is necessary to ensure the proper segregation of homologs. These pairings are usually maintained by crossing over, which establishes chiasmata that lock the homologs together until they are resolved during anaphase I. The nuclear envelope surrounding these homologs breaks down at the start of prometaphase I, and microtubule spindle formation begins. The spindle attaches to the kinetochores (protein motors located at the centromeres) and bipolar tension properly co-orients the homologs (Pinsky and Biggins 2005), before the oocyte arrests at metaphase I. (Fig. 9)

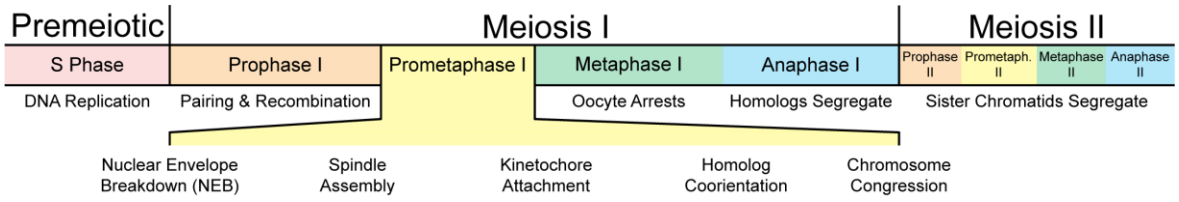


Figure 9. **Key Events of Meiosis**. DNA replication occurs in premeiotic S phase. Crossing over occurs in prophase I, while a series of events occurs in prometaphase I to coorient homologous chromosomes before the oocyte arrests at metaphase I

***Drosophila* Oogenesis:**

Drosophila oogenesis occurs in the ovary, a bundle of ~16 ovarioles, each of which is an assembly line factory for egg production (Becalska and Gavis 2009). In fruit flies, oogenesis begins with the formation of a 16-cell cyst of interconnected germ stem cells. Only one of the cyst cells becomes the oocyte, with the remaining 15 cells becoming polyploid nurse cells. The nurse cells/oocytes are surrounded by somatic follicle cells, constituting an egg chamber. This egg chamber progresses through 14 morphologically distinct stages across the ovariole (Fig. 10). At the end of stage 10, the nurse cells begin to contract and 'dump' their contents into the oocyte before undergoing apoptosis. The follicle cells that migrate to enclose the oocyte act as an eggshell to protect the mature egg. This model makes it easy to compare oocytes at different stages of meiosis within a single ovariole. This entire process of oogenesis, from stem cell to mature eggs, usually takes around 72 hours.

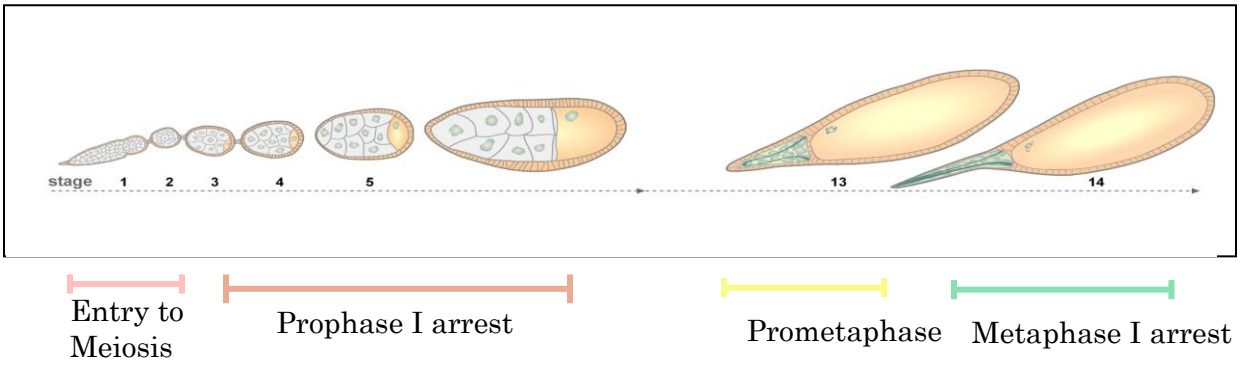


Figure 10. **Stages of Development: Oogenesis in *Drosophila*** showing how the different meiotic stages (not all shown) correspond with oocyte development. The germarium is to the left, with egg chambers becoming more mature moving to the right. The nuclear envelope breaks down at the stage 12/13 transition. (Xiang *et al*. 2007)

Dot chromosomes in Prometaphase I:

The dot chromosomes appear to play a role in the prometaphase chromosome movements. A previous study (Gilliland *et al.* 2015a) found suppressed prometaphase movement in flies with a monovalent *Compound-4* chromosome, which is a single chromosome that carries the same number of genes normally found on both homologs, with no pairing partner. The *C(4)* chromosome is capable of movement when a normal *4* is also present, but on its own does so infrequently, even when the *X* is made nonexchange, or when a heterologous *Compound-X* chromosome is available for pairing. These results suggest that a normal function of the dot chromosomes seen in many *Drosophila* species may be facilitating or organizing proper chromosome movement on the prometaphase I spindle.

Heterochromatin:

Heterochromatin is largely noncoding repetitive (satellite) DNA that is tightly compacted for most of the cell cycle. Heterochromatin has been called 'junk DNA,' but is actually important for normal chromosome function. Heterochromatin is required for nonexchange chromosome segregation and ensures proper coorientation of its homologs (Hawley *et al.* 1993), and blocks of heterochromatin become physically paired in *D. melanogaster* prophase (Dernburg *et al.* 1996). These blocks of heterochromatin also form the threads that connect these nonexchange chromosomes during the prometaphase phase stage of meiosis I (Hughes *et al.* 2009). It was also shown that a deletion that removed some of the *4* heterochromatin decreased the mean dot-dot separation in fixed images (Gilliland *et al.* 2015a). These results suggest that the amount of heterochromatin influences thread length, and may determine how nonexchange homologs move during prometaphase before they are pulled back together during congression at metaphase arrest.

Inversions:

An inversion is a chromosome rearrangement in which a segment of a chromosome is reversed end to end. Chromosomal inversions have been pervasive during the evolution of *Drosophila* (Guillen and Ruiz 2012), and there is a growing recognition that chromosome inversions affect rates of adaptation, speciation, and the evolution of sex chromosomes (Hoffmann and Rieseberg 2008). One consequence of inversions is that they can prevent recombination with a normal-sequence homolog, as recombination

within an inversion can lead to large deletions and duplications of material. Inversions can be polymorphic, with different inversion types segregating within natural populations, or fixed, such as when two related species are each monomorphic for a different inversion type. Common polymorphic inversions that are present at high frequencies in multiple populations of the same species must have persisted within the species for quite some time. Conversely, a rare inversion found only within one population is likely to have arisen very recently. Species can differ in the abundance of inversions; for example, *D. melanogaster* is a polymorphic species with many common inversions, while its sister species *D. simulans* is monomorphic without common inversions (Lemeunier and Aulard, 1992). In a population sample, *D. melanogaster* carries about 1 inversion per fly, in contrast to about 1 inversion per 200 flies in *D. simulans*. The evolutionary cause of this species difference is unknown.

While recombination can lead to lethality in the progeny of inversion heterozygotes, in *Drosophila* these chromosomes do not actually undergo crossing over. Instead there is an increase in the number of nonexchange chromosomes that have to segregate by the nonexchange segregation pathway (Gong *et al.* 2005). This suggests that, since inversions block crossing over, a population with abundant inversions like *D. melanogaster* should have nonexchange chromosomes out on the spindle more frequently during meiosis than a species without abundant inversions.

Species Differences in the Dot Chromosome.

Recent observations have discovered that the chromosomes from closely related species of *Drosophila* have notably different behaviors. Examination of prometaphase

oocytes found a visible difference in the dot-dot chromosome separation of two species of *Drosophila* (Gilliland *et al.* 2015b). This experiment found that the mean dot-dot distances in pure-strain *D. melanogaster* females (11.3 μm) was nearly twice as large as in *D. simulans* (6.1 μm) (Figure 11).

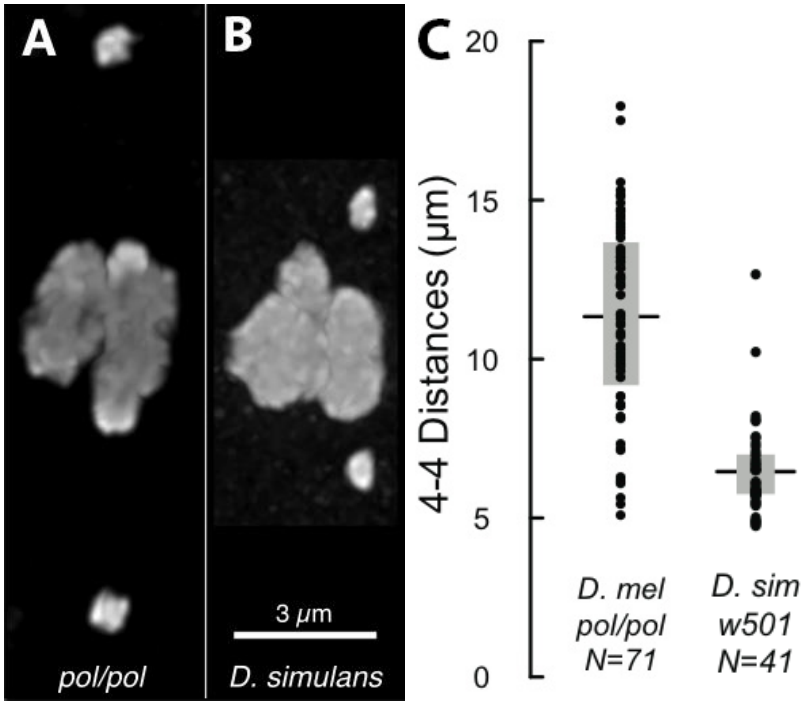


Figure 11. Dot-Dot distance measurements. **A-** Longer dot-dot chromosome distance in *D. melanogaster*. **B** –smaller dot-dot chromosome distance in *D. simulans.* **C**- The average dot-dot distances of each species, which are 11.3 μm in *D. melanogaster* and 6.1 μm in *D. simulans.* (Image from Gilliland *et al.* 2015b).

This difference is significant, because the dot chromosome in both species contains the majority of a particular satellite heterochromatin repeat sequence, AATAT. This repeat is more abundant in *D. melanogaster,* making up 3.1% of the genome versus 1.9% in *D. simulans* (Lohe and Brutlag 1987). One hypothesis is that the *D. melanogaster* dot may move out farther because more heterochromatin is available to build the tether.

However, the functional reason for this prometaphase chromosome movement is unknown. One possible reason is that having the dots out further out might provide more space for nonexchange chromosomes to move out fully onto the spindle. If this additional space is beneficial (such as reducing the time needed to complete prometaphase, or avoiding deleterious entanglements among multiple nonexchange chromosomes), then the greater amount of space on the spindle provided by the longer dot-dot tethers in *D. melanogaster* may help this species to tolerate common inversions (Gilliland *et al.* 2015b). Since *D. simulans* lacks common inversions, it would not require as much space for its nonexchange chromosomes to move out on the spindle, hence the shorter dot-dot chromosome separation. The cause and effect relationship in this model is unknown; it could be that the longer heterochromatic thread evolved first, which allowed inversions to accumulate in the population. Alternatively, the species could have accumulated inversions for other reasons, which then favored the evolution of longer heterochromatic threads. This project aimed to test whether the extent of dot chromosome separation is associated with the abundance of inversions, the amount of heterochromatin, or both among different *Drosophila* species.

AIM OF STUDY

Our lab identified a visible difference between *D. melanogaster* and *D. simulans* in the meiotic behavior of the small dot chromosome, and proposed that this difference may be correlated with the presence or absence of inversions between these species or, alternatively, the amount of heterochromatin in the genome (Gilliland *et al*. 2015b). My thesis proposal seeks to test this hypothesis by measuring the separation between the dot chromosomes during female meiosis prometaphase in two sets of *Drosophila* species: ones with common inversions (*like D. melanogaster*) and ones without common inversions (like *D. simulans*), and then compare the dot-dot distances to inversion type and amount of heterochromatin across species.

Question to be answered: Is the dot chromosome separation correlated with either the abundance of inversions, the amount of heterochromatin, or both across species of *Drosophila*.

HYPOTHESES - Species with more heterochromatin are expected to have longer dot – dot separation than species with less. Alternatively, species with more inversions should have longer dot-dot separation than those with less.

METHODOLOGY

Drosophila Stocks used

We performed a literature search to identify pairs of closely related species that, like *D. melanogaster* and *D. simulans*, differ in whether they have cosmopolitan inversions in natural populations. Stocks of different *Drosophila* species were obtained from the UC San Diego Drosophila Species Stock center. Ideally, species were chosen in closely related pairs with and without inversions that were distributed across the phylogeny, to avoid the confounding effects of common ancestry. In addition to *D. melanogaster* and *D. simulans* data from the previous study, we identified 6 species with common inversions and 6 species without common inversions. A species phylogeny with divergence times was built by entering taxa names in the TimeTree database (Kumar *et al.* 2017). One species (*Drosophila similis*) was not listed in their database. As this species is in the *dunni* subgroup which is closely related to *D. cardini* group, the divergence time between *D. dunni* and *D. cardini* should be identical, and was used to construct the tree in lieu of *D. similis* (Fig. 12).

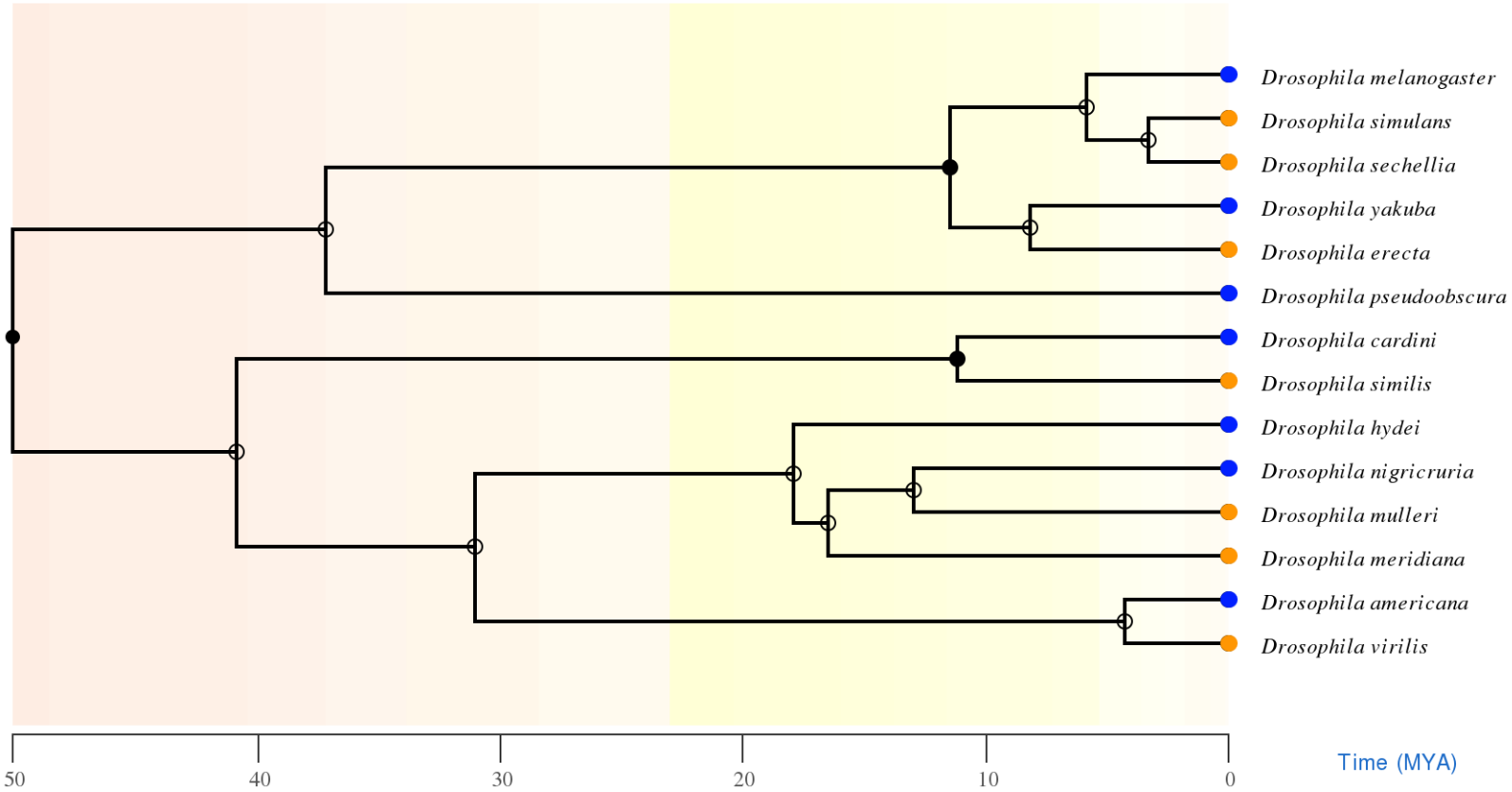


Figure 12. TIMETREE view showing the phylogeny of the 14 D*rosophila* species used for this project. Blue dots - *Drosophila* species with common inversions. **Orange dots** - *Drosophila* species without common inversions. *(*Kumar *et al. 2017)*

Drosophila Growth Media

Fly stocks for each species were raised on the growth media recommended by the Drosophila species stock center. Food recipes used were:

Bloomington Formula: Based on the standard recipe used by the Bloomington stock center. Ingredients include Genesee scientific mix which contains Yellow cornmeal, Agar, Corn Syrup solids, Inactive Nutritional Yeast and Soy Flour mixed in water (0.175 g mix / mL). Propionic acid and Tegosept were added to recipe as antifungal agents. This food was used for *D. americana* (reared at room temperature) as well as *D. melanogaster, D. simulans, D. virilis, D. pseudoobscura, D. sechellia, D. yakuba* and *D. erecta* all reared at 25°C.

Banana food: Based on Drosophila stock center recipe. Ingredients include Agar (10.3 g), yeast (20.63 g), Blended banana (103.13 g), Karo® syrup (71.25 g), Liquid Malt extract (22.5 g), 100% ethanol (22.5 ml) and Methylparaben (1.678 g) mixed in 1L of deionized water. Bottles sprinkled with yeast are papered to create a healthy pupation surface. This food was used for *D. cardini,* and *D. meridiana,* all reared at room temperature.

Banana- Opuntia food: Based on Drosophila stock center recipe. Ingredients and volume are the same as in Banana food, plus a supplement of Powdered Opuntia cactus (1.36g) mixed in 1L of deionized water. Bottles sprinkled with yeast are papered to create a healthy pupation surface. This food was used for *D. hydei* (reared at 25°C) as well as *D. mulleri, D. similis,* and *D. nigricruria,* all reared at room temperature.

Dot-Dot distance preps

Drosophila species stock in vials were transferred to new bottles containing fresh medium and allowed to mate (~10 females and 5 males per bottle). After a few weeks,

bottles were cleared of adults and virgin females were collected 6 hours later. We wanted to dissect females when many oocytes were in prometaphase, which in practice was shortly before females began to lay fertilized eggs. For most species, females were aged in yeasted vials with males for 42 hrs. after collection, and so were 42-48 hours post eclosion at the point of dissection; the exceptions were *D. meridiana*, *D. americana*, and *D. nigricruria,* which were dissected after 3.5 days. To standardize prep conditions, a timer was started as the vial was anesthetized with CO_2, followed by hand-dissection of ovaries as quickly as possible in room temperature 1x Robb's media + 1% BSA (Matthies *et al.*, 2000), and ovaries were transferred to a second well of media after extraction. For each prep, ten females of a species were dissected, and the ovaries were incubated in Robb's until the timer reaches 7 min, then buffer plus ovaries was pipetted into a 1.5 mL Eppendorf tube and allowed to settle. At 8 min., the Robb's was aspirated, and 1.3 mL of room temperature fixative (a 1:1 mix of 16% EM grade Paraformaldehyde (Ted Pella) with William's Hypotonic Oocyte Preservation and Stabilization Solution (Gillies *et al.* 2013), combined immediately before use) was applied. After fixation at room temperature for 5 min, oocytes were washed briefly in PBST (PBS + 0.1% Triton-X 100), ovarioles rapidly pipetted with a p1000 pipette to separate individual oocytes and then washed 3x in PBST for 15 min each, stained in PBST plus DAPI for 6 min, washed again in PBST (3x quickly followed by 2x 15 min) then mounted on slides in SlowFade Gold (Invitrogen) and sealed at the edges with nail polish.

Imaging and Quantification

To ensure oocytes were not missed or double counted, microscope slides were photographed on a dissecting microscope and a print of the photo was used as a map to mark oocytes. Oocytes were marked at low magnification (10X) using the LAS AF software (www.leica.com) "mark and find" panel. All confocal images of oocyte chromosomes with the dot out on the spindle were collected with the 63X objective on the DePaul Leica TCS SPE II confocal microscope, and the images were deconvolved using Huygens Essential.

Estimation of dot-dot distances was done by combining XY distances (measured with the LAS AF line tool) with Z distances (determined by multiplying the number of confocal sections between the centers of the dot light cones by the section thickness in orthogonal projections) using the Pythagorean theorem (distance = sqrt ($xy^2 + z^2$)) in Excel. Measurement was restricted to oocytes that had at least one dot chromosome out on the spindle, and the other locatable. Since chromosomes do not have completely sharp edges, a chromosome was classified as "out" if there was at least a 50% dip in background-subtracted fluorescent intensity, measured on the dot and the space between the dot and the adjacent chromosome using the line ROI tool. Oocytes with both dot chromosomes on the same side of the spindle, or with additional nonexchange chromosomes or other abnormal configurations, were not included in the analysis, as those distances may be affected by the configuration. Our target was to obtain useable measurements from 30 oocytes with at least one dot chromosome out on the spindle for each species. Comparisons of measurements of different species was done using pairwise *t*-tests to assess significance within both groups of common inversions and without

common inversion.

To calculate the dot chromosome sizes, figures where at least one dot chromosome was out on the spindle were selected and the sizes of the dots was measured using LAS AF software "free-hand tool" to estimate the pixel area of at least 20 dot chromosomes of each species.

To calculate the proportion of oocytes with chromosomes out on the spindle for each species, we divided the total number of oocytes of each species with their dot chromosome out on the spindle by the number of prometaphase oocytes sampled. (Number of oocytes with Dot out/ number of oocytes sampled).

Heterochromatin divergence of *Drosophila* species.

In our literature search to identify species to use in this study, our primary consideration was the presence or absence of common inversions. Therefore, we did not exclude species that had not had their heterochromatin measured yet (reasoning that if the pattern we found seemed promising, those species could be measured at a later date), and as a result, we had heterochromatin abundance for only 9 of our 14 species. Heterochromatin measurements were obtained from a study (Bosco *et al*. 2007) that estimated genome sizes for 12 *Drosophila* species. (Table 1)

Data analysis – *t*-tests were used to estimate the significance of differences between species with common inversions and species without common inversions based on the dot-dot length. The Pearson Correlation coefficient was calculated to determine the correlation between the proportion of chromosomes out on the spindle and chromosome

size, and regression analyses were done to assess significance. All calculations and plots were done in R (http://cran.r-project.org).

RESULTS

The species studied, and the data collected for this project, are summarized in Table 1.

Table 1: Data for each species includes Heterochromatin percentage (Bosco *et al*. 2007), whether the species has common inversions, mean dot-dot distance, number of oocytes with dot out on the spindle, total number of oocytes sampled, proportion of oocytes with 1+ dots out on the spindle, and mean dot chromosome size.

Species Name	% HC	Common Inversions	Mean dot-dot distance (μm)	Oocytes with 1+ dot out	Total oocytes sampled	Proportion with dot out on the spindle	Mean dot chromosome size (μm^2)
D. virilis	43	No	7.17	38	280	0.15	0.29
D. americana	25	Yes	6.47	38	192	0.20	0.30
D. sechellia	24	No	7.81	33	185	0.18	0.39
D. yakuba	21	Yes	7.48	31	92	0.34	0.70
D. melanogaster	20	Yes	11.33	71	147	0.48	0.85
D. hydei	17	Yes	4.74	31	330	0.09	0.15
D. simulans	16	No	6.15	35	80	0.44	0.64
D. pseudoobscura	10	Yes	5.67	35	220	0.16	0.34
D. erecta	7	No	5.93	30	269	0.11	0.26
D. cardini	-	Yes	8.40	34	120	0.28	0.72
D. meridiana	-	No	5.44	34	209	0.16	0.29
D. mulleri	-	No	5.37	37	160	0.23	0.39
D. nigricruria	-	Yes	5.93	34	240	0.14	0.22
D. similis	-	No	9.83	33	70	0.47	2.01

No relationship between polymorphic inversion abundance and dot-dot distances

We first asked whether polymorphic inversions correlated with the dot-dot chromosome distance across *Drosophila* species. For each species, we measured the dot-dot lengths for oocytes with dots out on the spindle, and grouped them by whether inversions are abundant in natural populations (Fig. 13). We found that there was no

significant difference in dot-dot distances between species with common inversions and those without common inversions (*t* test, p > 0.079).

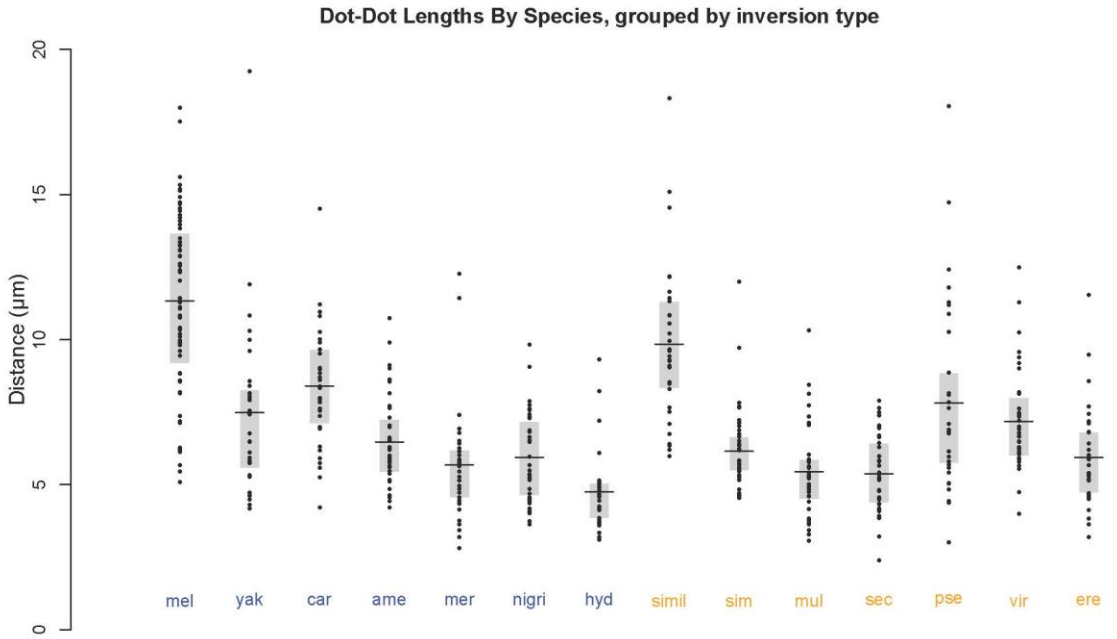


Figure 13: Dot-dot lengths by species. The mean dot-dot distance (horizontal lines) and the inner quartile range (boxes) are indicated, along with oocyte measurements for all 14 *Drosophila* species (*t* test, P-value > 0.079). All figures had at least one dot chromosome out on the spindle. Blue and Orange fonts represent species with and without common inversions, respectively. This analysis found that closely related sister species like *D. virilis* and *D. americana* failed to support our initial hypothesis that species with common inversions (*blue*) will have a greater dot-dot distance that those without common inversions (*orange*).

No strong correlation between heterochromatin abundance and dot-dot distances

Because recent work has identified heterochromatin tethers connecting nonexchange chromosomes (Hughes *et al*. 2009), we also asked whether the amount of heterochromatin in each species might be a key factor in determining the varying dot-dot distance observed initially in closely related species *D. melanogaster* and *D. simulans*

(Gilliland *et al*. 2015b). For each species, we compared their dot-dot length (Fig. 14) to their published amount of genomic heterochromatin (Table 1). We found no strong correlation between heterochromatin amount and dot-dot length among all species. (regression analysis, p = 0.48)

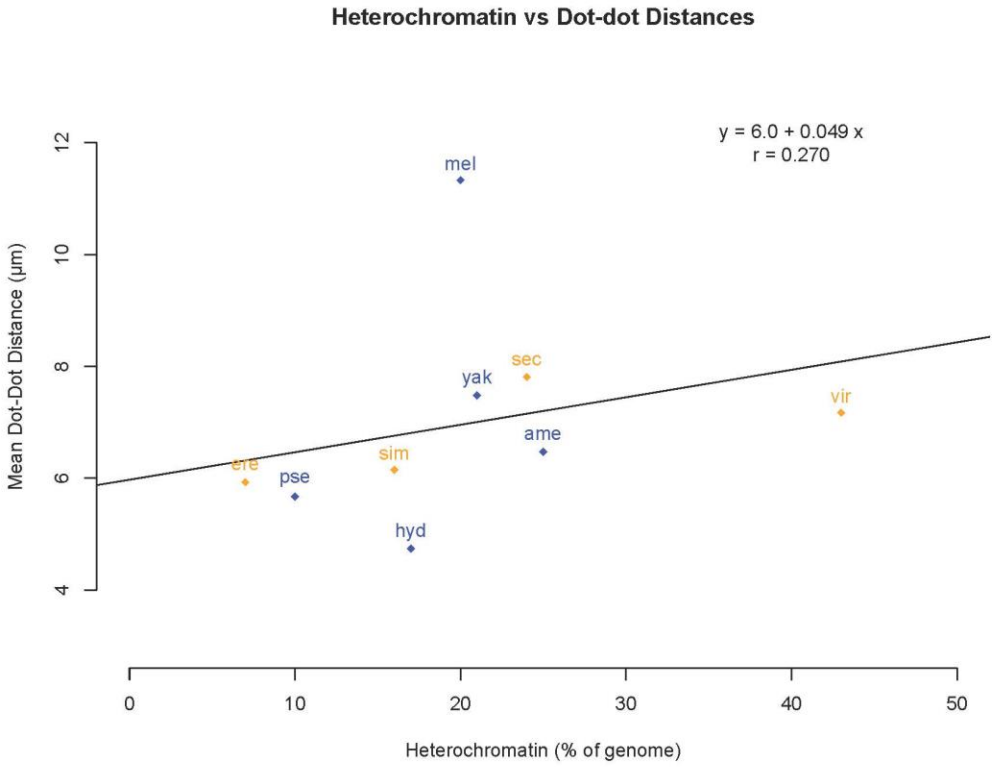


Figure 14: Dot-dot lengths vs percent heterochromatin across species. Comparisons of *dot-dot* length to amount of heterochromatin for the 9 *Drosophila* species in our sample with published heterochromatin content. (R=0.270). Blue and Orange fonts represent species with common inversions and without common inversions, respectively.

Quantifying dot chromosome sizes

While doing this work, we made the novel observation that the dot chromosomes among these species varied greatly in size (Fig. 15). To quantify this, we estimated chromosome sizes by measuring the pixel area of each dot chromosome out on the

spindle for all species sampled (Table 1). This found over 10-fold variation in dot chromosome sizes, from the smallest *(D. hydei)* at 0.15 µm to the largest *(D. similis)* at 2.01 µm.

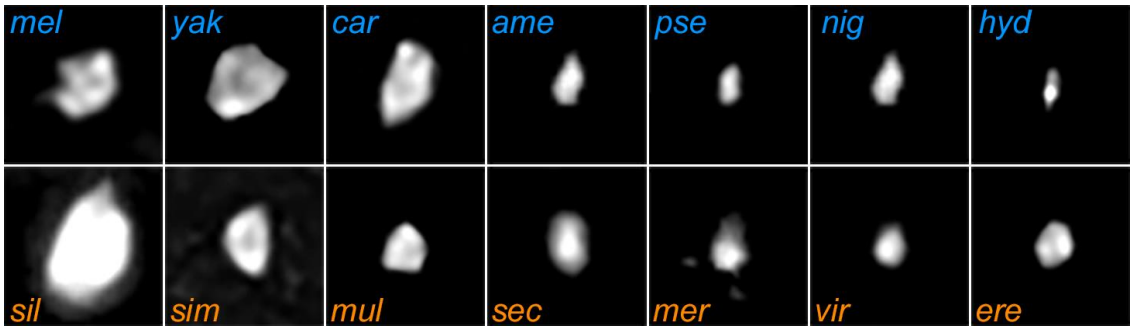


Figure 15: Image of a single dot chromosome size of each of the 14 *Drosophila* species. The ovaries from two-day-old virgin females were fixed and stained with DAPI, and oocytes were imaged to measure their dot-dot distances. The difference in chromosome sizes is clearly visible across species. Blue and Orange fonts represent species with common inversions and without common inversions, respectively.

Proportion of chromosomes out on the spindle

In addition to our observation of varying chromosomes sizes, we also noticed that some species had a much higher proportion of oocytes with chromosomes positioned out on the spindle during prometaphase I than others (Fig. 16). Based on the total number of oocytes examined per species, we were able to calculate the proportion of oocytes with at least one dot chromosome positioned out on the spindle during this stage of meiosis by dividing the number of oocytes with dot chromosome out by the total number of oocytes

in prometaphase that were sampled (Table 1). This found ~5-fold variation between species for the abundance of chromosomes out on the spindle.

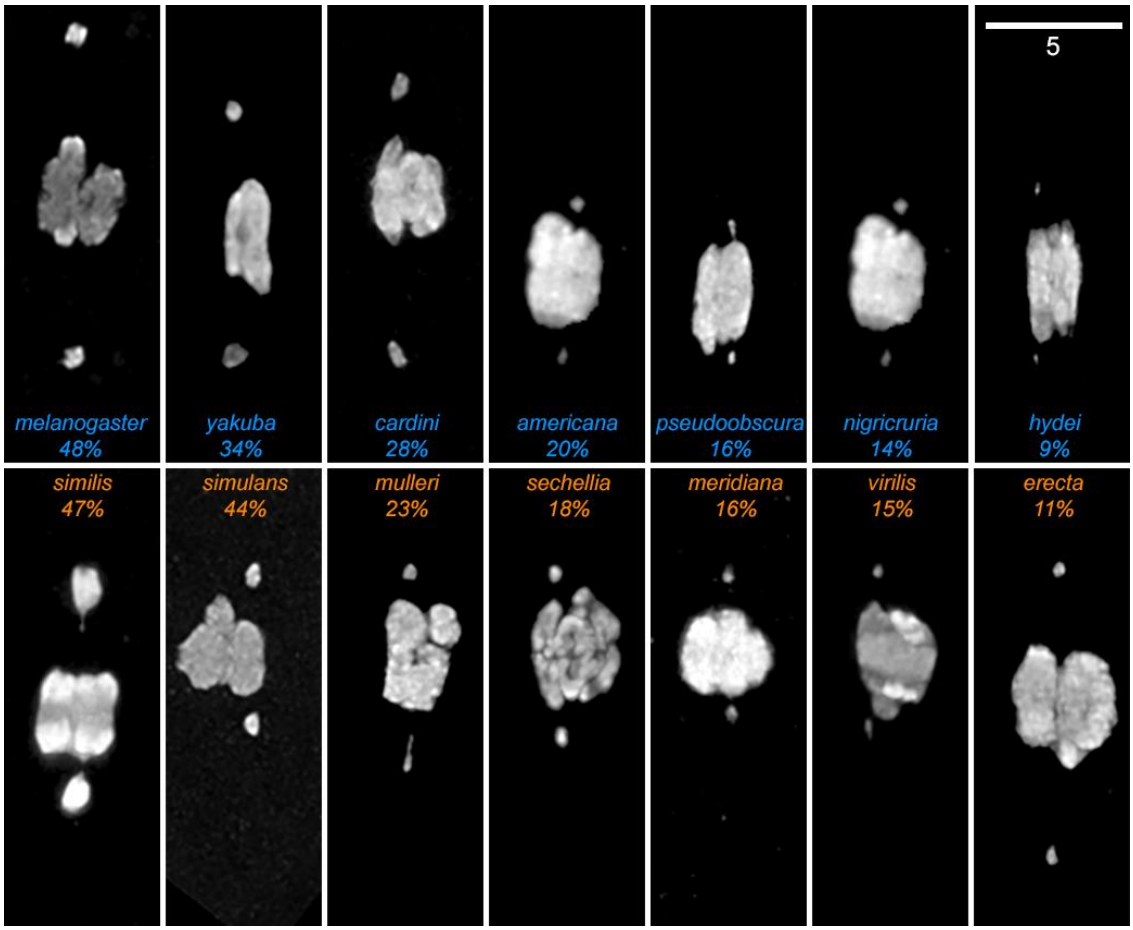


Figure 16: Dot chromosome sizes of 14 *Drosophila* species with their percentage of oocytes with chromosomes out on the spindle during prometaphase I. As the size of the chromosomes decreases the proportion of them out on the spindle also decreases across all species.

Interestingly, there is a correlation between the dot chromosome size and the proportion of chromosomes out on the spindle across all species. (Fig. 17)

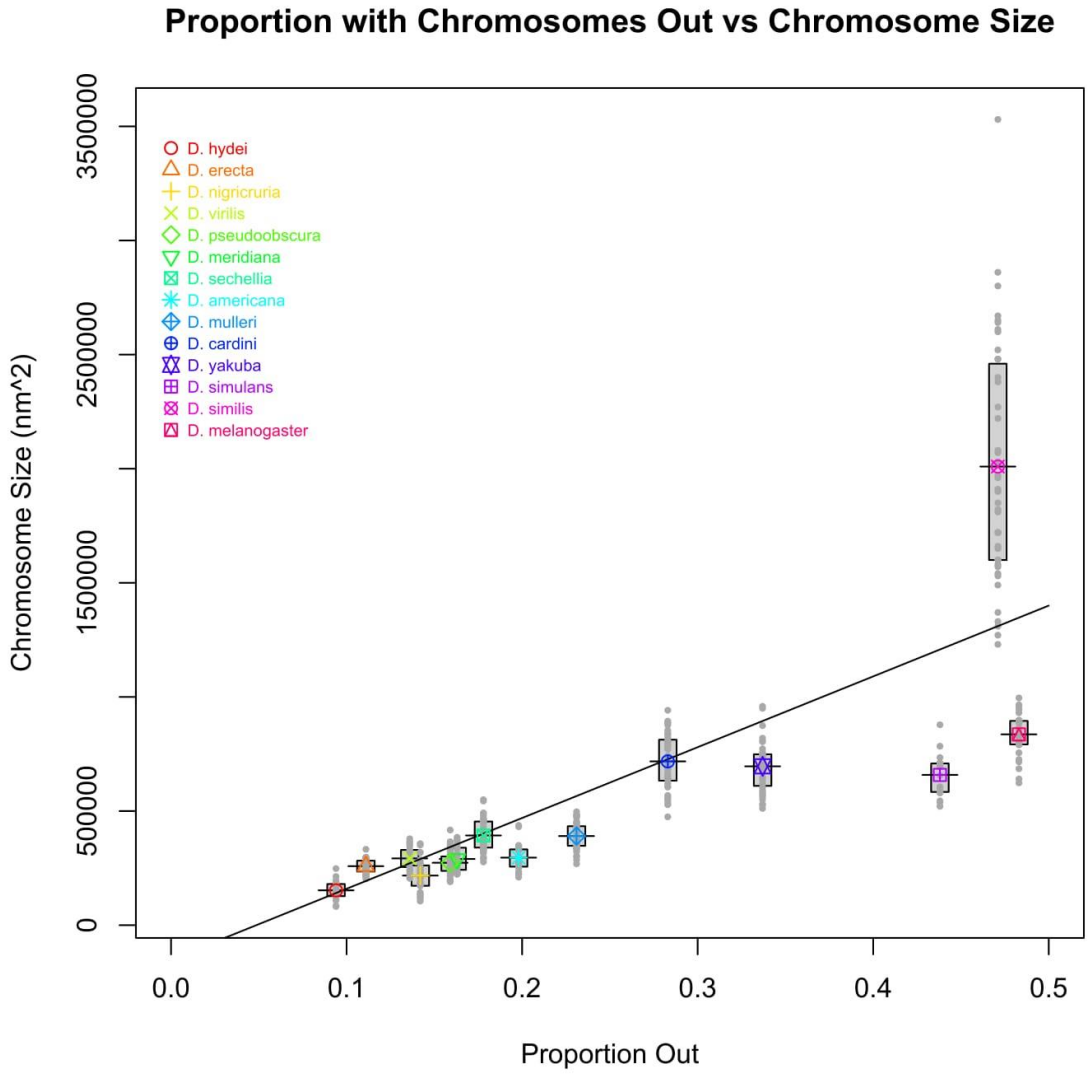


Figure 17: The proportion of chromosomes out on the spindle is correlated with dot chromosome sizes of 14 *Drosophila* during Prometaphase I. The correlation co-efficient is 0.79. The outlier here is *Drosophila similis* which has the biggest dot chromosomes among sampled species.

***Drosophila similis* large dot chromosome size**

As seen in Figure 17, *D. similis* has a much larger dot chromosome than other species. This dot chromosome looks comparable in size to the *X* chromosome in *D. melanogaster* (Fig. 18). Since some *Drosophila* species (like *D. willistoni)* no longer have a free dot chromosome due to a chromosomal fusion event, it is possible that the same might have happened in *D. similis*. We plan to perform a brain squash to determine if this has occurred.

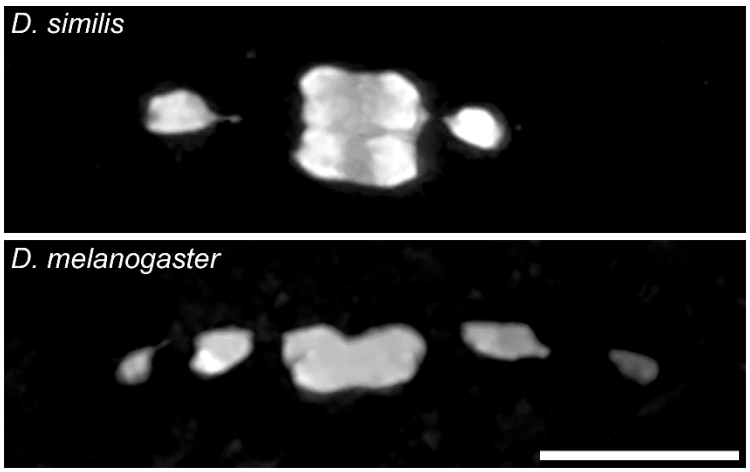


Figure 18: The bottom panel is a *D. melanogaster* oocyte from a female heterozygous for the balancer FM7; this forces the *X* chromosomes to be nonexchange. The dot chromosomes are furthest to the left and right, then the *X* chromosomes (the single bright heterochromatin mass identifies the normal *X* on the left, while FM7 is on the right) are between those and the exchange autosomes at the metaphase plate. Note the size of the *D. similis* "dot" chromosome (upper panel) is very close to the *D. melanogaster X* chromosome. (Scale bar: 5 µm)

If we exclude *D. similis* and recalculate the correlation between chromosome size and chromosomes out on the spindle, the correlation coefficient increases to 0.93 (Fig. 19).

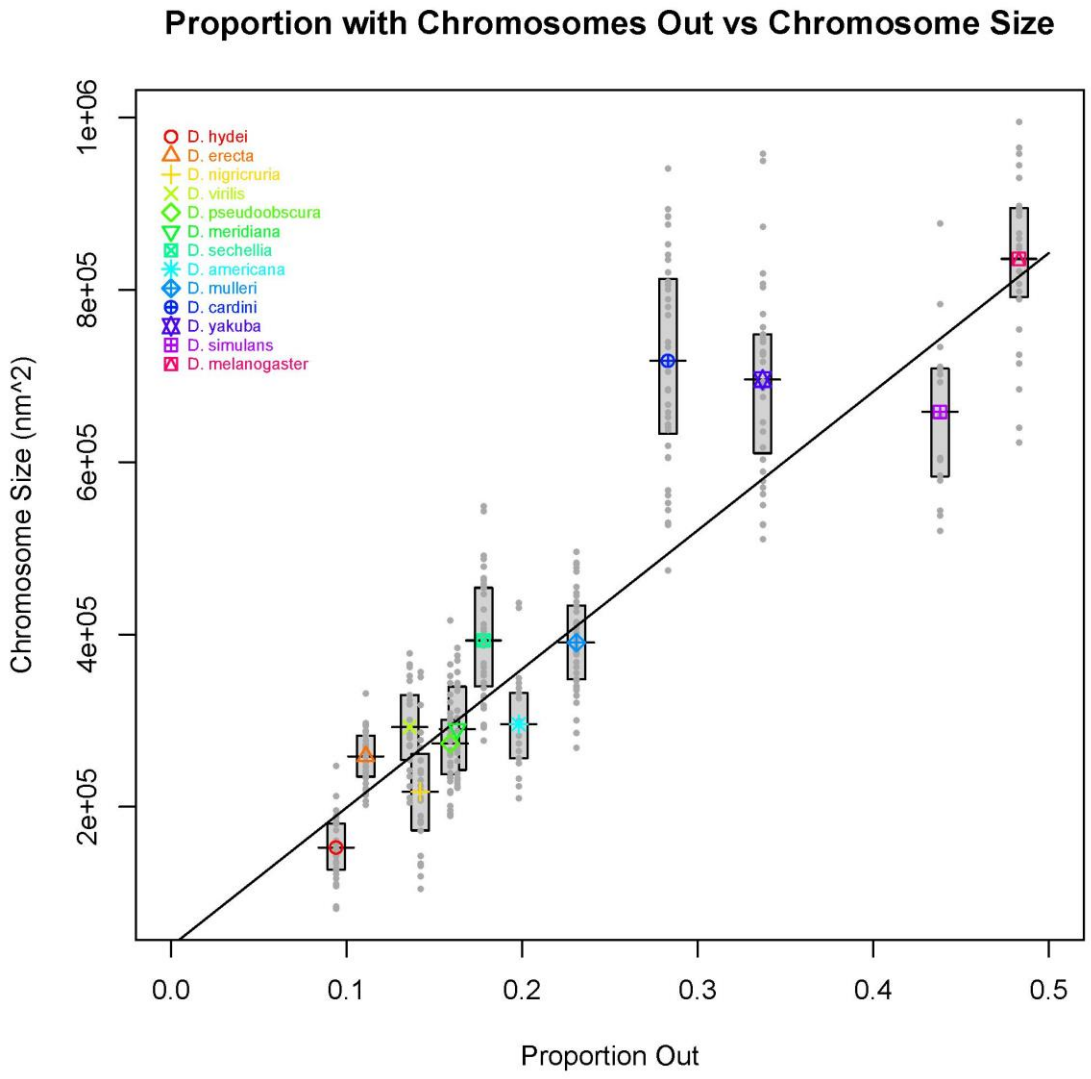


Figure 19: Graph showing the correlation between chromosomes out vs chromosome size excluding *D. similis*. The correlation coefficient is now 0.93.

DISCUSSION

In this project, we demonstrated that the dot chromosome separation distance first observed in *D. melanogaster* and *D. simulans* (Gilliland *et al.* 2015a) does not appear to be correlated with either the amount of heterochromatin or the abundance of inversions across *Drosophila* species. Because recent work identified the heterochromatic threads connecting nonexchange chromosomes as the leading candidate for how they achieve proper co-orientation (Hughes et. al 2009), it was suggested that the shortened *D. simulans* dot length and the longer *D. melanogaster* dot length might reflect a role of the thread in chromosome positioning (Gilliland *et al.* 2015b), leading us to our working hypothesis that increased amount of heterochromatin on the dot causes longer threads, which may cause the dot chromosomes to separate longer distances like in *D. melanogaster*. Our results failed to support the hypothesis that the shortened dot-dot distance was as a result of lower amount of heterochromatin, with no significant correlation between heterochromatin abundance and *dot-dot* distance among the 9 species assayed (p-value = 0.48). *D. virilis* has a higher heterochromatin content than *D. melanogaster*, but ended up with a shorter dot-dot length (Fig. 14).

It was also speculated that the dot-dot distances of each species may correlate with whether the species has common polymorphic inversions or not (Gilliland *et al.* 2015b). Since inversions block crossing over, increasing the abundance of inversions is expected to make meiosis with nonexchange chromosomes more common. Because nonexchange chromosomes are positioned between the dots near the spindle poles and exchange chromosomes at the metaphase plate, it was suggested that having the dots

further out may provide more space for additional nonexchange chromosome to also fully move out on the spindle. If this additional space is important then we hypothesized that species with common inversions may have longer dot-dot distances. However, we found no clear association between inversion and dot-dot distances among *Drosophila* groups. Our results showed no significant association between dot-dot distances and the amount of inversions (P-value > 0.079). If we examine closely related sister species like *D. hydei* and *D. mulleri* we can observe this effect clearly. While *D. hydei* has many common polymorphic inversions, *D. mulleri* is monomorphic with no common inversions (Sperlich and Pfriem 1986). However, we found that *D. hydei* has a shorter dot-dot distance (4.74 μm) compared to *D. mulleri* (5.37 μm). Therefore, neither of our initial hypotheses appear to be supported by our data (Fig. 13).

However, while doing this work our fixed DAPI images revealed considerable variation in the dot chromosome sizes among our sampled *Drosophila* species (Fig. 15). This led us to quantify the pixel sizes of these dot chromosomes for all species. We found that *D. similis* has the largest dot chromosome while *D. hydei* had the smallest. The size of *D. similis* chromosome was so huge that it is possible that it has fused with another chromosome, similar to what happened in *D. willistoni*. We also found considerable variation in the proportion of oocytes with chromosomes out on the spindle during prometaphase. *D. hydei* has its dot chromosomes out on the spindle about 9% of the time, compared to *D. melanogaster* which has its dots out on the spindle about 48 % of the time (Fig. 16). This quantification showed that there is a strong correlation between the dot chromosome size and the proportion of chromosomes out on the spindle across all

species. (Correlation coefficient = 0.798). If we exclude *D. similis* from our results, the correlation increases to 0.93 across the remaining species.

Why does proportion of chromosomes out on the spindle matter?

It is important to note that this project made use of fixed images for the prometaphase dot chromosome measurements. Each fixed oocyte is essentially frozen at one point in time during the process of oogenesis. This means that the more time cells spend in any given configuration, the more often we expect to get figures fixed in that state. Therefore, we interpret these observations to mean that *Drosophila* species with more chromosomes out on the spindle must take longer to complete the chromosome movements during prometaphase I. Overall, our observations suggests that the time that chromosomes spend out on the spindle is strongly correlated with the size of the dot chromosome.

CONCLUSION

Even though our initial hypotheses that heterochromatin and inversions might have an effect on the dot-dot chromosome distance were rejected, this study is the first to show that *Drosophila* species have varying dot chromosome sizes which correlates with their proportion out on the spindle during female meiotic prometaphase. Previous studies (Leung *et al*. 2015) have also examined the dot chromosome of 4 different *Drosophila* species using polytene chromosomes from their salivary glands to study the evolution of the Muller F element and its genes. They did find that the dot chromosome has maintained characteristics distinct from other autosomes in the *Drosophila* lineage like higher repeat density, larger genes due to larger introns and lower codon bias. This is a different result compared to what we found, since heterochromatic regions are not amplified in polytene chromosomes.

Future work is needed with respect to the size of *D. similis* dot chromosome. One modification of the primitive karyotype of *Drosophila* is the absence of dot chromosomes and the origin of this modification is diverse. The fate of this dot chromosome can be inferred simply from cytogenetic analysis but in some cases like in *D. willistoni* a genetic or a combined molecular and cytogenetic analysis is needed. It is important to know if *D. similis* has undergone autosome dot fusion or its dot chromosome are just really that large. Likewise, if the dot is facilitating prometaphase chromosome movements, it predicts that the *D. willistoni* chromosome 3 (which is a fusion of the E and F elements, which

corresponds to a 3R/4 fusion in *D. melanogaster)* may also move out on the spindle. We plan to examine prometaphase I oocytes from *D. willistoni* females to see if the fusion chromosome also moves out on the spindle like the dot chromosomes in the species we sampled here.

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