X Chromosome Drive in *Drosophila testacea*

by

Graeme Keais
B.Sc., University of Victoria, 2015

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of

MASTER OF SCIENCE

in the Department of Biology

Supervisory Committee

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Abstract

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Selfish genes that bias their own transmission during gametogenesis can spread rapidly in populations, even if they contribute negatively to the fitness of their host. Driving X chromosomes provide a clear example of this type of selfish propagation. These chromosomes, which are found in a broad range of taxa including plants, mammals, and insects, can have important evolutionary and ecological consequences. In this thesis, I report a new case of X chromosome drive (X drive) in a widespread woodland fly, *Drosophila testacea*. I show that males carrying the driving X (SR males) sire 80-100% female offspring, and that the majority of sons produced by SR males are sterile and appear to lack a Y chromosome. This suggests that meiotic defects involving the Y chromosome may underlie X drive in this species. Abnormalities in sperm cysts of SR males reflect that some spermatids are failing to develop properly, confirming that drive is acting during gametogenesis. Further, I show that SR males possess a diagnostic X chromosome haplotype that is perfectly associated with the sex ratio distortion phenotype. Phylogenetic analysis of X-linked sequences from *D. testacea* and related species strongly suggests that the driving X arose prior to the split of *D. testacea* and its sister species, *D. neotestacea* and *D. orientacea*. Suppressed recombination between the X^{ST} and X^{SR} due to inversions on the X^{SR} likely explains their disparate evolutionary histories. By screening wild-caught flies using progeny sex ratios and a diagnostic X-linked marker, I demonstrate that the driving X is present in wild populations at a frequency of ~10% and that autosomal suppressors of drive are segregating in the same population. Both SR males and homozygous females for the driving X have reduced fertility, which helps to explain the persistence of the driving X over evolutionary timescales. The testacea species group appears to be a hotspot for X drive, and *D. testacea* is a promising model to compare driving X chromosomes in closely related species, some of which may even be younger than the chromosomes themselves.

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Acknowledgments

Thank you to Steve Perlman, a wonderful supervisor and mentor. Doing a MSc in Steve's lab has been an enjoyable and stimulating experience. I would also like to thank my committee members John Taylor and Ben Koop for their support. Both have been excellent teachers and mentors to me during my time at the University of Victoria. A number of other people have helped me during the course of my degree. In particular I would like to acknowledge Finn Hamilton, Mark Hanson, Christina Hodson, Ryan Gawryluk, Dr. Matthew J. Ballinger, Cuong Le, Caitlin Curtis, Chadi Saad-Roy, and Sarah Miller for their support, guidance, and knowledge. I am also grateful to NSERC and the University of Victoria for financial support.

Chapter 1 – X Chromosome Drive

Meiotic Drive

In diploid organisms, homologous chromosomes should be equally represented among gametes as a result of random meiotic segregation. Each homologue, and the genes they carry, should therefore obey Mendel's law of equal segregation and have the same probability of being transmitted to the next generation. While this is true for most genes, some genetic variants – termed meiotic drivers – manipulate gametogenesis in their favour, causing themselves to be transmitted far above the expected Mendelian ratio (*i.e.* they "drive")(Burt and Trivers, 2006). Two forms of meiotic driver exist: those that act in females, and those that act in males (Lindholm *et al*., 2016). In females, meiotic drive can occur when a chromosome preferentially migrates to the cell destined to become the egg, thus relegating its meiotic homologue to the polar body (an evolutionary dead-end). In males, meiotic drivers typically act by destroying or incapacitating sperm that carry their meiotic homologue (Burt and Trivers, 2006). Although in some ways fundamentally different, both forms of meiotic drive have the same result: the exclusive (or near-exclusive) transmission of gametes carrying the meiotic driver. This selfish form of propagation, which occurs at the direct expense of other genes in an individual's genome, allows meiotic drivers to increase in frequency without contributing to organismal fitness (Werren, 2011).

Sex Chromosome Drive

When a meiotic driver evolves on a sex chromosome, the transmission of one sex chromosome at the expense of the other causes members of the heterogametic sex to produce offspring that are heavily biased towards one sex. The biased transmission of sex chromosomes, called sex chromosome drive, is widespread, with well-characterized examples in plants, mammals, and insects (Jaenike, 2001). Sex chromosome drive is often strong: individuals carrying the "driving" sex chromosome produce offspring that

are nearly 100% of one sex. This dramatic sex ratio distortion has a number of ecological and evolutionary implications, especially considering the ability of meiotic drivers to spread in populations (Jaenike, 2001; Lindholm *et al*., 2016) – for example, if a driving sex chromosome were to reach fixation, the absence of one sex could cause extinction (Hamilton, 1967). As a practical matter, however, the sex ratio distortion is a clear phenotype that allows sex chromosome drive to be detected without the need for visible or molecular markers that are linked to the driver (as is the case for autosomal drivers)(Burt and Trivers, 2006). Even so, the discovery of sex chromosome drive requires large samples of progeny obtained from controlled crosses, and it is perhaps for this reason that our understanding of sex chromosome drive comes largely from flies (Diptera).

Over 20 driving sex chromosomes have been characterized in Diptera, including drive of both the X and Y chromosome (Jaenike, 2001). Although Y chromosome drive has been found in several species, X chromosome drive (X drive) is far more common: it has been found in numerous Drosophila and diopsid (stalk-eyed fly) species, and in a species of tsetse fly (Table 1-1)(Jaenike, 2001). Fifteen of the known driving X chromosomes are found in the genus *Drosophila*, where some species, such as *Drosophila simulans*, house multiple independently evolved X drive systems (Mercot *et al.*, 1995; Tao *et al.*, 2007b). The following will focus primarily on X drive in Diptera, where driving X chromosomes are traditionally called "*sex-ratio*" chromosomes (denoted X^{SR}) after the phenotype they produce (Gershenson, 1928). Males that carry an X^{SR} will be referred to as SR males, while males carrying a standard X chromosome (denoted X^{ST}) will be called ST males.

X Drive Mechanism

Cytological work in several dipteran species has revealed defects of male gametogenesis that underlie X drive. Policansky and Elison (1970) first showed using electron microscopy that sperm bundles in SR males contain half as many mature sperm compared to ST males in *Drosophila pseudoobscura*. Because SR males produce nearly 100% female offspring, this strongly suggested that the sperm failing to develop are those

carrying the Y chromosome. By fluorescently labelling sperm nuclei using probes specific to either the X or Y chromosomes, Montchamp-Moreau and Joly (1997) confirmed that sperm carrying the Y chromosome are failing to develop properly in SR males of *D. simulans* that carry the "Paris" X^{SR} (one of the three X drive systems in this species). Cazemajor *et al.* (2000) later showed that this stems from the Y chromatids failing to separate during meiosis II. The Y chromatids segregate to the same daughter cell, which subsequently fails to develop. In the stalk-eyed fly *Teleopsis whitei*, sperm nuclei are often found in abnormal positions in the sperm bundles of SR males, suggesting that X drive may work in a similar way in distantly related taxa (Wilkinson and Sanchez, 2001).

A generic model for the mechanism of X drive dictates that the X^{SR} produces a toxin that targets a site on the Y chromosome during gametogenesis, leading to the destruction of Y-bearing sperm (Jaenike, 2001). This model is borne out in the Paris X

Table 1-1. Known cases of X drive in flies.

Family	Species	Reference
Drosophilidae	*Drosophila obscura*	Gershensen (1928)
	D. subobscura	Jungen (1967)
	D. affinis	Voelker (1972)
	D. athabasca	Miller (1971)
	D. azteca	Sturtevant and Dobzhansky (1936)
	D. pseudoobscura	Sturtevant and Dobzhansky (1936)
	D. persimilis	Wu and Beckenbach (1983)
	D. simulans	Mercot *et al.* (1995)
	D. paramelanica	Stalker (1961)
	D. mediopunctata	De Carvalho *et al.* (1989)
	D. quinaria	Jaenike (1996)
	D. recens	Jaenike (1996)
	D. neotestacea	James and Jaenike (1990)
	D. testacea	Keais *et al.* (2017)
	D. orientacea	Pieper and Dyer (2016)
Diopsidae (stalk-eyed flies)	*Teleopsis dalmanni*	Presgraves *et al.* (1997)
	T. whitei	Presgraves *et al.* (1997)
	Diasemopsis sylvatica	Lande and Wilkinson (1999)
	Sphyracephala beccarrii	Lande and Wilkinson (1999)
Glossinidae (tsetse flies)	*Glossina morsitans*	Rawlings and Maudlin (1984)

drive of *D. simulans*, where the molecular basis of X drive is relatively well-characterized. The *sex ratio* phenotype has been mapped to two regions on the Paris X^{SR} that act together to express drive. Recently, Helleu *et al.* (2016) mapped one of these regions to a single gene and provide direct evidence that it contributes to drive. The drive gene is a mutant allele of a young, rapidly evolving heterochromatin protein called *HP1D2*. Transgenic SR males expressing a copy of the wild-type *HP1D2* produce offspring sex ratios that are nearly restored to 50:50. In addition, knock-down of the wild-type *HP1D2* with concurrent expression of the mutant driving *HP1D2* causes males to produce female-biased progeny. Helleu *et al.* (2016) show that the *HP1D2* protein localizes to the Y chromosome of male germ cells. As heterochromatin proteins are generally responsible for the proper packaging of DNA into heterochromatin, this suggests that the driving *HP1D2* allele produces a protein that improperly packages the Y chromosome heterochromatin for meiosis. This probably explains the failure of Y chromatids to separate properly during meiosis II in SR males. Recalling the generic model for X drive, the mutant *HP1D2* protein can be thought of as the "toxin", and the Y chromosome heterochromatin state as the "target". By preventing the development of Y-bearing sperm, the driving *HP1D2* allele subverts the fair process of meiosis, greatly biasing its own transmission.

The Evolutionary Dynamics of X Drive

The evolution of an X drive system is highly dynamic. Once X drive evolves, a number of outcomes are possible, including the fixation, loss, suppression, and stable persistence of the X^{SR} (Helleu *et al.*, 2014). Some of these fates are discussed below, as well as some of the ecological and evolutionary consequences that may be incurred during the evolution of an X drive system. (The maintenance of an X^{SR} as a stable polymorphism is discussed briefly in chapter 2, and in more detail in chapter 3).

Spread and Fixation

As with meiotic drivers generally, an X^{SR} is expected to spread rapidly in a population in the absence of counter-acting selection. In the case of X drive, the spread of

an X^{SR} will skew the population sex ratio increasingly towards females. Populations harbouring an X^{SR} may therefore have a higher per capita growth rate compared to competing species, which could serve as an advantage in interspecific competition (Unckless and Clark, 2014). However, if an X^{SR} reaches high frequencies, a lack of males could precipitate the extinction of local populations, or conceivably an entire species (Hamilton, 1967). Studying the involvement of X drive in the extinction of natural populations is difficult; and although there is one possible case of X drive causing the crash of a wild population of *Drosophila neotestacea*, the evidence is inferential (Pinzone and Dyer, 2013). The capacity of X drive to cause extinction has been demonstrated definitively in laboratory populations of *Drosophila pseudoobscura,* where the spread of an X^{SR} resulted in the extinction of multiple experimental populations due to an absence of males (Price *et al.*, 2010). Ultimately, whether X drive has caused species extinction is unknown; but the possibility of rapid population or species extinction could mean that X drive is more common than we are able to observe (Carvalho and Vaz, 1999).

Suppression

Any female bias in a population introduced by an X^{SR} will tend to select for autosomal genes that can suppress X drive. Following Fisher's principle, as a population becomes more female-biased, the fitness of males, being the rarer sex, will also increase (Fisher, 1930). Therefore, genes that increase the number of males in an individual's progeny will be favoured, including genes that suppress the X^{SR}. Autosomal suppressors that restore offspring sex ratios towards 50:50 in SR males are found in several of the known X drive systems in Diptera (Jaenike, 2001).

Suppressors on the Y chromosome can also evolve. Unlike autosomal suppressors, which are favoured only when the population sex ratio becomes female-biased, suppressing Y chromosomes are automatically selected for because by escaping the selfish action of the X^{SR} they have an advantage over susceptible Y chromosomes. Suppressing Y chromosomes are found in many, but not all, X drive systems, often alongside autosomal suppressors (Jaenike, 2001).

The common occurrence of either or both autosomal and Y-linked suppression illustrates the power of X drive in instigating antagonistic co-evolution between the X^{SR}

and other components of the genome (Helleu *et al.*, 2014). These "arms races" between drivers and suppressors can be a powerful evolutionary force shaping X^{SR} chromosomes and the species as a whole (Lindholm *et al.*, 2016). For example, in the event that the original X drive locus becomes suppressed, additional loci capable of neutralizing suppressors may arise on the X^{SR}. These new X^{SR} variants could then spread and replace the original. There is direct evidence that multiple loci are needed to fully express X drive in *D. simulans* and *D. subobscura* (Hauschteck-Jungen and Maurer, 1976; Montchamp-Moreau *et al.*, 2006). Furthermore, evidence for a recent selective sweep on wild Paris X^{SR} chromosomes supports the idea that new X^{SR} variants replace older ones (Derome *et al.*, 2004). Importantly, when multiple loci contribute to X drive, there will be selection for these loci to be inherited as a non-recombining unit (Prout *et al.*, 1973). This may be achieved through close physical linkage, as is the case in *D. simulans*: two interacting drive loci are close together on the X^{SR}, and recombination events that separate them are rarely observed (Cazemajor *et al*., 1997). However, the more common alternative appears to be the evolution of inversions coupling drive loci together. Inversions are found on most X^{SR} chromosomes examined in *Drosophila* (Jaenike, 2001). In some cases, multiple inversions distinguish the X^{SR} from the X^{ST}. The X^{SR} in *D. subobscura*, for instance, carries four inversions relative to the X^{ST} (Hauschteck-Jungen and Maurer, 1976). An arms race between a driver and suppressor can therefore have a large effect on the evolution of the X^{SR}.

Drive-suppressor co-evolution can lead to divergence within or between species if different populations/species experience independent drive-suppressor arms races. Cryptic X drive systems – wherein all individuals of a population or species carry a suppressed X^{SR} – can arise when a suppressed X^{SR} becomes fixed in a population. Cryptic X drive can then be revealed in crosses between suppressed individuals and individuals from other populations or species that do not have the suppressor (as they did not experience the same suppressor-driver arms race). Tao *et al.* (2001) uncovered cryptic X drive in crosses between *D. simulans* and *D. mauritiana*. They showed that the introgression of a portion of the *D. mauritiana* third chromosome into *D. simulans* replaced a dominant *D. simulans* suppressor allele with the non-suppressing allele from *D. mauritiana*, leading to the expression of X drive in *D. simulans* males. Intriguingly,

hybrid male sterility also mapped to the same chromosomal region responsible for suppressing X drive. This provides experimental support for the hypothesis that meiotic drive is an important factor in the evolution of hybrid male inviability and sterility (Haldane's rule) first proposed by Hurst and Pomiankowski (1991) and Frank (1991). This hypothesis is based on the notion that repeated cycles of suppression and drive could lead to the divergence of genes that are important for gametogenesis. Additional support for the role of X drive in hybrid male sterility was provided by Phadnis and Orr (2009), who showed that a single gene is responsible for X drive and hybrid male sterility in crosses between subspecies of *D. pseudoobscura*. X drive may therefore play an important role in speciation by contributing to reproductive isolation between populations/species via hybrid male sterility (McDermott and Noor, 2010).

Objectives

In this thesis, I characterize X chromosome drive in the fly, *Drosophila testacea.* My work is divided into two chapters, with experiments appearing roughly in the chronology that they were performed. In chapter 2, I investigate the nature of female-biased sex ratio distortion observed in *D. testacea*. I first show that sex ratio distortion in this species is due to a driving X chromosome. I examine the frequency of this X^{SR} in a wild population of *D. testacea* from Switzerland, and in doing so, I am able to screen for the presence of suppressors of X drive. This chapter has been published in the Journal of Evolutionary Biology (Keais *et al.*, 2017). In chapter 3, I reconstruct the evolutionary history of X drive in *D. testacea* using X-linked gene sequences from members of the testacea species group. I examine fitness costs associated with the *D. testacea* X^{SR} in both males and females and discuss how these may contribute to the maintenance of the X^{SR} as a polymorphism. The research presented in this thesis lays the foundation for future studies of X drive in *D. testacea*.

D. testacea is part of the testacea species group, which has four known members: *Drosophila putrida, Drosophila testacea, Drosophila neotestacea,* and *Drosophila orientacea* (Grimaldi *et al.*, 1992). The latter three represent a young radiation that were until recently thought to be a single species, as they are morphocryptic and have

incomplete reproductive isolation (Grimaldi *et al.*, 1992). Remarkably, all three (*D. testacea*, *D. orientacea*, and *D. neotestacea*) are now known to carry X drive polymorphisms. The close relations of these three species, and the fact that they all carry X^{SR} chromosomes makes this species group a unique system for the comparative study of X drive. Importantly, the ecology of testacea group species is relatively well-known. These flies convene at mushroom patches to find mates, females oviposit on mushrooms, and larvae subsequently feed on and develop in mushrooms until pupation. As most of their life-cycle is spent on mushrooms, research into X drive in this species group can therefore be expanded to natural contexts by using mushroom baits. The range of *D. testacea*, which spans from Europe to Japan (Grimaldi *et al.*, 1992), is also appealing, as it will permit the study of drive-suppressor co-evolution over a wide geographical space. Finally, the X^{SR} in *D. testacea* appears to be relatively stable in laboratory populations. This will facilitate a controlled dissection of the mechanisms that allow the X^{SR} to be maintained as a polymorphism. Ultimately, studying X drive in *D. testacea* and its sister species will broaden our understanding of the ecological and evolutionary impacts of selfish genetic elements.

Chapter 2 of this thesis is published in the Journal of Evolutionary Biology (Keais *et al.*, 2017). It has been modified for this thesis. The abstract of this thesis is also modified from Keais *et al.* (2017).

Citation:

Keais, G.L., Hanson, M.A., Gowen, B.E. & Perlman, S.J. 2017. X chromosome drive in a widespread Palearctic woodland fly, *Drosophila testacea*. *J. Evol. Biol.* **30**: 1185-1194.

Contributions to this work:

For the work presented in chapter 2, I was responsible for fly rearing and crosses, recording offspring sex-ratios, qPCR of *kl2*, and PCR and sequencing of *skpA*, *rpl36*, and *kl2*. I also interpreted and analyzed the data, prepared all figures and tables, and wrote the manuscript. Mark Hanson discovered the sex ratio bias (towards females) in the laboratory stock of *D. testacea*, and subsequently helped with genotyping flies for the pedigree. Brent Gowen produced the electron microcrographs. Steve Perlman isolated the X^{SR} from the laboratory stock of *D. testacea*, collected flies from Switzerland, and contributed to the writing and editing of the manuscript.

Chapter 2 – X Chromosome Drive in a Widespread Palearctic Woodland Fly, *Drosophila testacea*

Introduction

Mendelian segregation is effectively 'fair', meaning gene frequencies generally do not change during the process of gene transmission alone. However, some genes cheat, subverting the process of equal segregation (Crow, 1991). Such selfish genetic elements are able to increase their own transmission relative to the rest of the genome, ending up in more than the expected 50% of gametes. The power of non-Mendelian inheritance is striking: in the absence of countervailing selection, even a small transmission advantage above 50% will lead to the rapid spread and eventual fixation of a selfish 'driving' allele (Burt & Trivers, 2006).

X chromosome drive (X drive) provides a clear example of selfish genetic behaviour. This phenomenon was first described in *Drosophila* (where it is called the *sex-ratio* trait) (Gershenson, 1928), but has since been found in a wide range of taxa, including rodents, plants, and numerous flies (Diptera) (Jaenike, 2001). Broadly, X drive is characterized by the unequal transmission of the X chromosome, which can be achieved in a number of ways. In Diptera, X drive is achieved through the action of an X-linked gene product that destroys or incapacitates nearly all of an individual's Y-bearing sperm during gametogenesis. As a result, males carrying a driving X chromosome (X^{SR}) produce predominantly X-bearing gametes, and therefore sire almost exclusively daughters (Jaenike, 2001).

X drive engenders a diverse set of ecological and evolutionary consequences (Lindholm *et al.*, 2016). For example, it has been shown to significantly affect chromosome organization (Dyer *et al.*, 2007), the evolution of mating systems (Price *et al.*, 2008b; Pinzone & Dyer, 2013), and patterns of molecular evolution (Derome *et al.*, 2004; Phadnis & Orr, 2009; Kingan *et al.*, 2010). There is also increasing evidence that X drive plays a role in speciation through the evolution of hybrid incompatibilities (Frank, 1991; Hurst & Pomiankowski, 1991; Tao *et al.*, 2001; Phadnis & Orr, 2009; McDermott & Noor, 2010). Furthermore, an unhampered rise in the frequency of an X^{SR} chromosome

can lead to population sex ratios that are dramatically female biased. In theory, a severe lack of males caused by X drive could drive a species to extinction (Hamilton, 1967).

Despite the threat of extinction, some X drive systems are clearly very old (*e.g.* drive in *Drosophila pseudoobscura* [Babcock & Anderson, 1996]), raising the question of how X^{SR} chromosomes are maintained over evolutionary timescales. Several factors contributing to the long-term dynamics of X drive have been explored, both theoretically and experimentally. For example, because any deviation from a 1:1 sex ratio is not evolutionarily stable (Fisher, 1930), X drive instigates an extended genetics arms race over sex ratio, often leading to the evolution of resistant Y chromosomes and autosomal suppressors (reviewed in Jaenike, 2001). The presence of suppression is variable: some X drive systems are completely neutralized by suppression in the wild, and are only revealed by interspecific or inter-population crosses (Dermitzakis *et al.*, 2000; Tao *et al.*, 2001; Tao *et al.*, 2007a,b); other systems are polymorphic for suppression, and some lack it entirely (Jaenike, 2001).

In addition to suppression, selection also plays an important role in maintaining *sex-ratio* (SR) polymorphisms in populations. Inversions that couple driving loci together are frequently found on X^{SR} chromosomes. Due to a lack of recombination in these regions, there can be an accumulation of deleterious mutations linked to drive, which contribute negatively to the fitness of X^{SR} carriers (Jaenike, 2001). Therefore, selection against carriers, especially homozygous females ($X^{SR}X^{SR}$), can prevent the spread of an X^{SR} (Wallace, 1948; Edwards, 1961).

Selection against the X^{SR} may also act in a frequency-dependent manner. Males carrying the X^{SR} chromosome (hereafter, SR males) do poorly in sperm depletion assays when compared to standard (ST) males (*e.g.* Beckenbach, 1978; Jaenike, 1996; Atlan *et al.*, 2004; Wilkinson *et al.*, 2006; Unckless *et al.*, 2015), reflecting the fact that they are producing roughly half the amount of functional sperm. Therefore, if males become rare due to an increase in X^{SR} frequency, the male mating rate should increase, and ST males will be favoured, assuming a greater number of sperm translates to a greater number of offspring (Jaenike, 1996). Similarly, their reduced sperm production makes SR males poor sperm competitors compared to ST males (*e.g.* Wilkinson & Fry, 2001; Atlan *et al.*, 2004; Wilkinson *et al.*, 2006; Price *et al.*, 2008a); thus, the fitness of SR males is further

reduced if females re-mate (polyandry). Overall, the evolutionary maintenance of X drive systems is perplexing, in part because not all drive systems evolve suppression (Jaenike, 2001), nor do all species with drive exhibit polyandry (Verspoor *et al.*, 2016).

It has long been known that the reduced sperm production of SR males is due to the developmental failure of Y-bearing sperm (Policansky & Ellison, 1970), yet the underlying cause remains poorly understood in most species. The molecular basis of X drive has only been characterized in *Drosophila simulans*, which astonishingly houses at least three independent SR systems (Tao *et al.*, 2007a). Two of these systems, called Winters and Paris, have a known genetic basis. In the Paris SR system, X drive results from the missegregation of Y chromatids during meiosis II (Cazemajor *et al.*, 2000). Recently, Helleu *et al.* (2016) have shown that mutations in the X-linked heterochromatin protein, *HP1D2*, contribute to this abnormal Y chromosome behaviour, likely by improperly preparing the Y chromosome for meiosis. While this represents the only known molecular mechanism of X drive so far, abnormal behaviour of Y chromosomes during meiosis II has also been directly observed in *Drosophila pseudoobscura* (Novitski *et al.*, 1965; Cobbs *et al.*, 1991) and *Drosophila athabasca* (Novitski *et al.*, 1965), and is inferred in *Drosophila subobscura* (Hauschteck-Jungen *et al.*, 1972). This suggests that causing non-disjunction of the Y chromosome may be a common route for X^{SR} chromosomes to bias their own transmission (Helleu *et al.*, 2014). In contrast, the mode of action of the Winters SR trait in *D. simulans* occurs post-meiotically (Tao *et al.*, 2007a). In this case, the causative agent has been mapped to the X-linked gene *Dox*, which codes for a small RNA molecule with limited protein-coding potential (Tao *et al.*, 2007b). Although it is not well understood how the *Dox* gene product causes drive, these various studies of drive in *D. simulans* nevertheless tell us that different SR chromosomes act through different means and at different times during gametogenesis.

In this chapter I report the discovery of X drive in *Drosophila testacea*, a common and widespread Palearctic mushroom-feeding fly belonging to the testacea group of the subgenus *Drosophila*. I characterize several features of X drive in this species, with the aim of contributing to a broader understanding of the shared features of driving X chromosomes. I quantify sex ratio distortion in this species and confirm the inheritance pattern of the SR trait. I also examine the etiology of the X^{SR} by testing the fertility of

sons sired by SR males, as well as through transmission electron microscopy of developing sperm in both SR and ST males. Lastly, I examine the frequency of the driving X chromosome in the wild. Interestingly, the testacea group appears to be a hotspot of X drive, with three of its four members now known to harbour examples (James & Jaenike, 1990; Pieper & Dyer, 2016), including the well-studied *D. neotestacea*, whose driving X chromosome occurs at very high frequencies in nature, with no known suppressors (Dyer, 2012; Pinzone & Dyer, 2013). This presents an excellent opportunity to compare driving X chromosomes in closely related species, some of which may even be younger than the chromosomes themselves.

Materials and Methods

Fly stocks

Our lab stock of *D. testacea* was founded with multiple wild caught flies from St. Sulpice, Vaud, Switzerland in 2012. All flies are reared at 21°C with a 12-h light:dark cycle in vials containing instant *Drosophila* medium (Carolina Biological Supply, Burlington, NC), supplemented with commercial mushroom (*Agaricus bisporus*). I have also established a line lacking the X^{SR}.

Characterization of sex ratio distortion

We first detected a female bias in our stock of *D. testacea* during experiments requiring equal numbers of newly eclosed male and female flies. By quantifying the sex ratio of a single generation emerging from our stock, I found that ~77% were female. A series of initial crosses exploring the nature of this sex ratio distortion suggested that the trait was expressed only in males, a typical characteristic of X drive. Therefore, I took sons of a presumed heterozygous female ($X^{SR}X^{ST}$; X^{ST} refers to a standard or non-driving chromosome) and crossed them each individually to 2 virgin $X^{ST}X^{ST}$ females (all ST flies used in this study are from our non-driving line). After 4 days, males were removed, and mated females were transferred to fresh vials. Females were subsequently transferred to fresh vials every 4 days for an additional 12 days, and then discarded. The sex ratios of

emerging flies were scored. I tested for deviations from a 1:1 sex ratio using chi-squared tests implemented in R (version 3.3.0).

Inheritance of the SR trait

I performed a pedigree analysis to formally demonstrate that the SR trait in our lab population of *D. testacea* is due to an X-linked factor. This pedigree analysis eliminates the possibility that sex ratio distortion is caused by any of the following: a Y-linked gene, a cytoplasmic factor, or an autosomal gene (described in detail in James & Jaenike, 1990). Several daughters of a presumed $X^{SR}Y$ male were crossed with virgin $X^{ST}Y$ males. I assumed that these daughters were heterozygous for their X chromosome ($X^{SR}X^{ST}$), and I therefore expected them to produce both $X^{SR}Y$ and $X^{ST}Y$ sons. I crossed several of these sons to $X^{ST}X^{ST}$ females, and their progeny sex ratios were recorded. As expected, some sons produced offspring with normal (~1:1) sex ratios, while others produced female biased sex ratios. The latter were presumed to be $X^{SR}Y$, and their daughters were crossed with $X^{ST}Y$ males. Lastly, 20 sons resulting from this final cross were genotyped (see below) as either $X^{SR}Y$ or $X^{ST}Y$.

Fertility of sons sired by SR males

We assessed the fertility of the sons of SR males by mating 38 sons from seven different SR males to four virgin $X^{ST}X^{ST}$ females each. Each male was placed in a vial with two virgin $X^{ST}X^{ST}$ females and allowed to mate for five days. Males were then transferred by aspiration to an additional two virgin $X^{ST}X^{ST}$ females, and again left to mate for five days. All pairs of females were turned over to new vials every five days, to a total of three vials per pair of females.

To screen for a Y chromosome in these 38 sons, I extracted their DNA using Prepman™ Ultra (Applied Biosystems), and attempted to amplify the gene *kl-2* using quantitative PCR (qPCR). Previous work has shown this gene to be on the Y chromosome in testacea group flies (Dyer *et al.*, 2011). Gene sequences available from the National Center for Biotechnology Information (NCBI) were used to design a set of qPCR primers (kl2q-F and kl2q-R, Supplemental Table 2-2) within the *D. testacea kl-2* gene. I used DNA extracted from a virgin female as a negative DNA template control. The following qPCR thermal cycling conditions were used: 95 °C for 10 min, then

35 cycles of 95 °C for 15s followed by 60 °C for 45s, with the product confirmed using melt curve analysis and Sanger sequencing (Sequetech, USA). I confirmed that all DNA extractions were positive for DNA using a separate PCR reaction amplifying an X-linked gene, *rpl36* (primers in Supplemental Table 2-2). The presence of *rpl36* amplicons was confirmed on an agarose gel. An age-matched control for both the fertility assay and qPCR was performed using 24 sons of an ST male following the same procedures.

Genotyping

In an attempt to identify polymorphisms associated with sex ratio distortion, I extracted DNA from 17 males with known progeny sex ratios. Using PCR, I then amplified two X-linked genes (*rpl36* and *skpA*) from these males using primers previously developed in a study examining molecular evolution in the testacea species group (Dyer *et al.*, 2011). The following thermal cycling conditions were used: 95 °C for 3 min, then 32 cycles of 94 °C for 1 min and 54 °C for 1 min, followed by a final 10 min at 72 °C. PCR amplicons were then Sanger-sequenced (Macrogen, USA). Sequence handling and analysis was performed using Geneious v5.1.7 (Kearse *et al.*, 2012).

Electron microscopy of sperm cysts

To obtain images of sperm cysts using transmission electron microscopy (TEM), 7-day old ST (n = 3) and SR (n = 4) males were anesthetised with CO_2 and their testes were dissected out. DNA extractions were performed on the head and thorax of each fly and genotyped using the X-linked marker gene s*kpA* (see Genotyping, Methods). The isolated testes were processed using standard TEM methodology (Hayat, 1989): double-fixation and embedding into Epon. TEM sections were stained in uranyl acetate and lead citrate and viewed in a Jeol JEM 1400 TEM equipped with a Gatan SC-1000 digital camera.

Prevalence of X^{SR} in the wild

Wild *D. testacea* were caught near St. Sulpice, Switzerland, in August 2016 by aspirating flies off of mushroom baits. Forty-one wild caught males were mated to four laboratory $X^{ST}X^{ST}$ virgin females each and their progeny sex ratios were scored. I also generated *skpA* sequences (See Methods, Genotyping) for all males.

Given our results from these two methods, I suspected a suppressor may be acting in two of the wild caught males. I hypothesized that any males producing a normal offspring sex ratio, but carrying a driving X genotype, may also carry a suppressing element. To explore this possibility, I took daughters of putatively suppressing males (*i.e.* those producing a normal sex ratio but with a driving genotype) and mated them to $X^{ST}Y$ males from our lab stock (and thus containing no suppressors). Sons of these females were then mated to several virgin $X^{ST}X^{ST}$ females each and their progeny sex ratios were scored.

All unique DNA sequences for *skpA*, *rpl36*, and *kl-2* generated in this study were deposited in GenBank (accession numbers KY407222-KY407235 and KY774653-4).

Results

Characterization of sex ratio distortion

Female-biased sex ratios were common in our driving line. For example, of 23 *D. testacea* males mated to $X^{ST}X^{ST}$ females, 13 had a significant excess of female offspring relative to the expected 1:1 ratio (χ^2_1: $P < 0.005$)(Figure 2-1, Supplemental Table 2-1). These males produced offspring that were 81%-100% female. The remaining 10 males had normal offspring sex ratios (χ^2_1: $P > 0.05$), which ranged between 43% and 54% female (Figure 2-1, Supplemental Table 2-1).

Inheritance of the SR trait

Cross A (Figure 2-2) between a presumed SR male ($X^{SR}Y$) and an $X^{ST}X^{ST}$ female yielded 97% female offspring (F_1 females). These F_1 females were presumed heterozygous for their X ($X^{SR}X^{ST}$), and if mated to an $X^{ST}Y$ male (Figure 2-2, cross B), they should therefore produce both $X^{SR}Y$ sons and $X^{ST}Y$ sons (F_2 males). Indeed, some F_2 males produced offspring with a ratio of ~1:1 (Figure 2-2, cross C), and others produced predominantly female offspring (Figure 2-2, cross D). Mating daughters of cross D to $X^{ST}Y$ males generated F_4 males, 15 out of 20 of which were $X^{SR}Y$. This pedigree analysis eliminates the possibility that sex ratio distortion in *D. testacea* is

caused by a Y-linked, cytoplasmic, recessive autosomal, or dominant autosomal factor (see James & Jaenike, 1990).

Fertility of sons sired by SR males

Out of 38 sons sired by SR fathers, two produced viable offspring with normal sex ratios when mated to virgin laboratory females (Table 2-1, Supplemental Table 2-3). From these 38 sons of SR males, I was only able to amplify the Y-linked gene kl-2 from the two sons that produced offspring (Table 2-1, Supplemental Table 2-3). No kl-2 amplicon was detected from the 36 infertile sons (Supplemental Table 2-3). There is therefore a highly significant association between fertility and the presence of the gene kl-2 in sons of SR males (Fisher's exact test, P = 0.001422). In contrast, 23 of 24 sons sired by an ST male produced offspring (Supplemental Table 2-3). I successfully amplified kl-2 from all 24 control males (Supplemental Table 2-3).

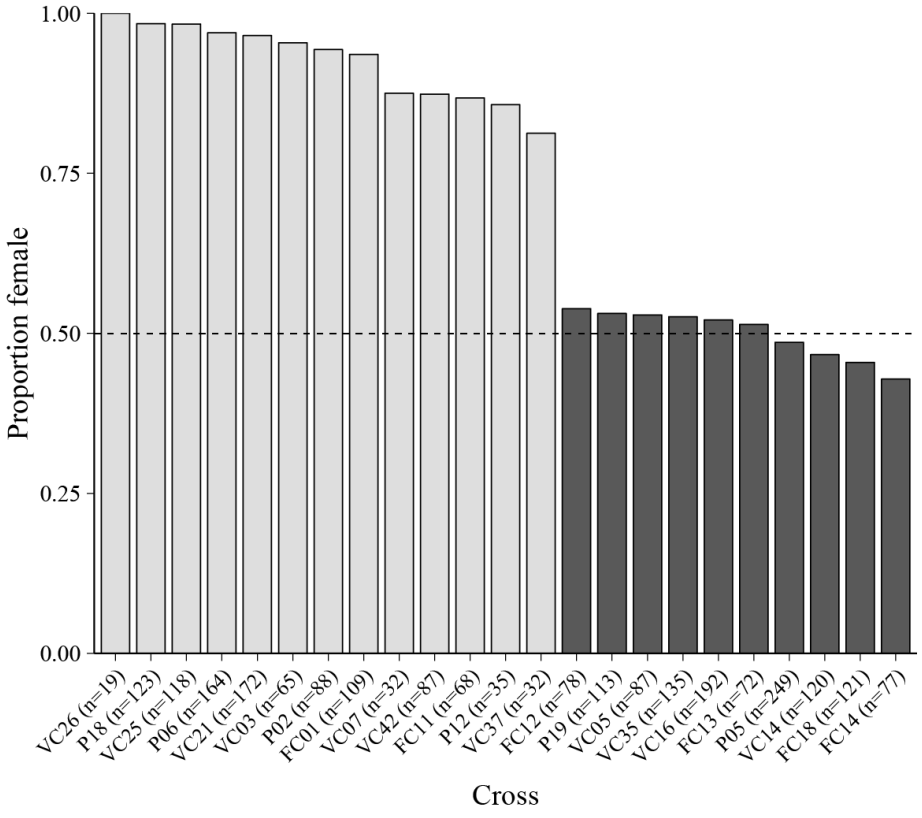


Figure 2-1. Progeny sex ratios of male *Drosophila testacea* (SR or ST). Each male was mated to two virgin females. The number of offspring sired by each male is shown in brackets. Only males that produced >15 offspring are included. Progeny sex ratios that significantly deviate from the expected 1:1 are shown as light bars (χ^2_1: $P < 0.05$). The dashed horizontal line denotes the expected proportion of female offspring (0.5).

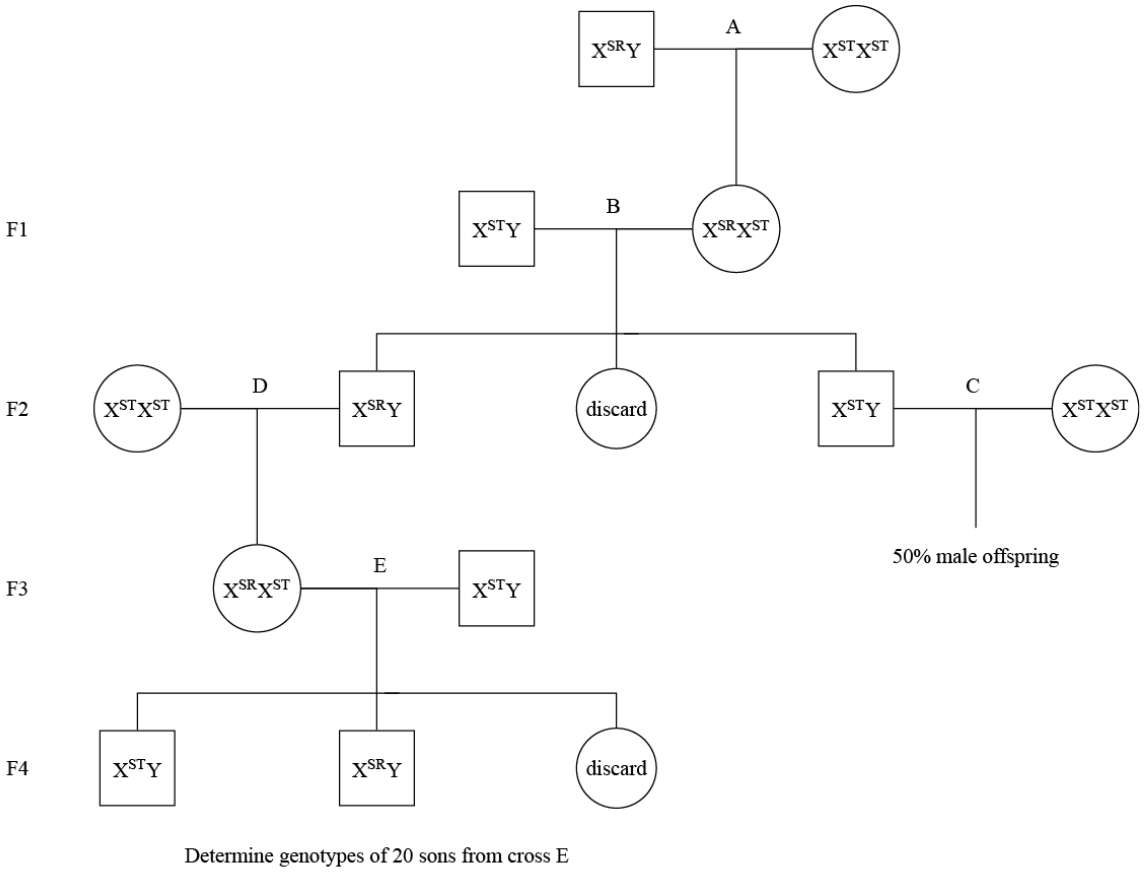


Figure 2-2. Crossing scheme used to determine the inheritance pattern of the *SR* trait in *Drosophila testacea.* Squares and circles denote males and females, respectively. Modified from James and Jaenike (1990).

Genotyping

Sequences generated for the *skpA* gene from males with known offspring sex ratios revealed the existence of two haplotypes, one perfectly associated with the SR trait (Fisher's exact test, P = 8.08e-05), which I call the X^{SR} haplotype, and the other perfectly associated with males producing normal offspring sex ratios, which I call the X^{ST} haplotype (Supplemental Table 2-1). The two haplotypes differ by six single nucleotide polymorphisms (SNPs) across 442 base pairs (bps), two of which are non-synonymous substitutions in the X^{SR} version. The X^{ST} haplotype is identical to previously obtained

skpA sequence from *D. testacea* from Germany (Dyer et al. 2011). Likewise, there were two *rpl36* haplotypes, associated with either the X^{ST} or X^{SR}, and differing at four synonymous sites across 323 bp.

Table 2-1. Fertility of 38 sons sired by seven different SR males. Sons were mated to four virgin laboratory females each to assess their fertility. DNA extractions were subsequently performed on these sons in order to screen for the Y-linked gene *kl-2* using qPCR.

SR male	Offspring sex ratio (proportion female)	Sons tested	Fertile	*kl-2* amplified
1	0.98	3	0	0
2	0.83	15	1	1
3	0.91	5	0	0
4	0.94	5	0	0
5	0.97	3	0	0
6	0.86	5	1	1
7	0.98	2	0	0

Spermatogenesis is abnormal in SR males

Cross sections of sperm cysts indicate that spermatogenesis proceeds normally in ST males: individualized spermatids are tightly arranged, and nearly all spermatids display an orderly axoneme-Nebenkern pair (Figure 2-3A). In contrast, sperm cysts of SR males are highly disorganized, and several spermatids have not developed normally or are absent (Figure 2-3B). For example, some spermatids appear to have fused axonemes, with the fused spermatids sharing a common cytoplasm (Figure 2-3B, asterisk). Another abnormality is the presence of underdeveloped mitochondrial derivatives (Figure 2-3B, arrow). Small vesicles, which may be multivesicular bodies (see Ramamurthy *et al.*, 1980), are also present in the sperm cysts of SR males, and not in ST males (Figure 2-3B, arrowhead).

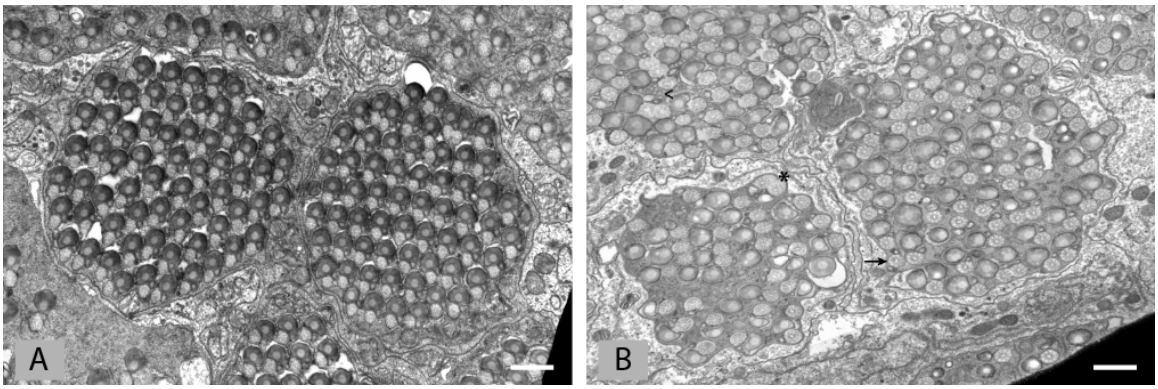


Figure 2-3. Electron micrographs of sperm cysts from 7-day old ST and SR males in cross section. (A) Cysts from ST males that have undergone individualization. Spermatids are tightly arranged, each with an orderly axoneme-Nebenkern pair. (B) Cysts from SR males are disorganized and show several abnormalities. Some spermatids have fused and share a common cytoplasm (*). Others have underdeveloped mitochondrial derivatives (arrow). Small vesicles, potentially multivesicular bodies, are present in the cytoplasm surrounding spermatids in SR males (arrowhead). Bars, 1μm.

Prevalence of X^{SR} in the wild

Most wild caught males produced normal offspring sex ratios when mated to laboratory females (Figure 2-4). However, 2 of 37 had significantly female biased offspring (86% and 95%), confirming the presence of the X^{SR} in natural populations of *D. testacea* (Figure 2-4, Supplemental Table 2-4). Both males with skewed offspring sex ratios carried the X^{SR} haplotype. Of the 37 males with normal sex ratios, 35 possessed *skpA* genotypes associated with X^{ST}. The remaining 2 males with normal sex ratios (61% and 52% female offspring) carried the X^{SR} haplotype. Thus, I suspected that these males may carry a suppressing element. Indeed, some F_2 sons from both putative suppressor males produced significantly skewed female offspring sex ratios when mated to females from our non-driving line (Supplemental Table 2-5), demonstrating that when put in our non-suppressing laboratory genetic background, both previously suppressed X's were able to drive. Finally, 4 males did not produce offspring; they all carried the X^{ST} *skpA* genotype. Thus, the frequency of X^{SR} in our wild sample is ~10% (4/41).

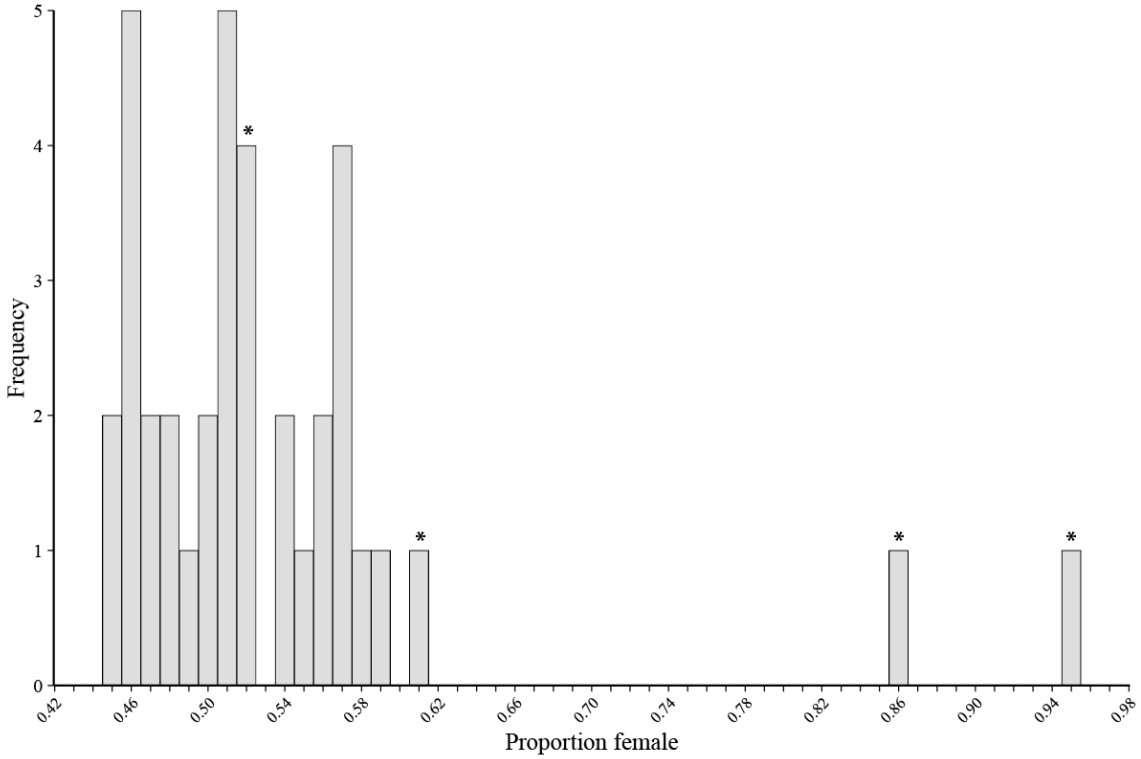


Figure 2-4. Offspring sex ratios of male *Drosophila testacea* from St. Sulpice, Switzerland, caught in August 2016. Each male was mated to four lab virgin females ($X^{ST}X^{ST}$). Asterisks indicate the four males with a driving X chromosome, two that produced heavily female-biased sex ratios, and two that were suppressed in the wild.

Discussion

This study is the first to identify X drive in the Palearctic woodland fly *Drosophila testacea*, a mycophagous species that ranges from western Europe to Japan. I show that males carrying the X^{SR} sire a significant excess of female offspring. I also show that the majority of male offspring sired by SR males are sterile and appear to lack a Y chromosome. The etiology of X drive in this species is characterized by the irregular development of sperm, as shown by the abnormal appearance of sperm cysts in all SR males. Finally, my results demonstrate that the X^{SR}, as well as suppressors of drive, are segregating in wild populations of *D. testacea* from Switzerland.

X drive in *D. testacea* is strong, with SR males producing 80-100% female offspring. However, drive is much stronger than it appears from progeny sex ratios

because nearly all sons produced by SR males are sterile (~95%) – of 38 sons sired by various SR males, only 2 produced offspring when mated to laboratory females. In addition, I was unable to detect the presence of a Y-linked fertility gene from all sterile sons. This strongly suggests that sterile sons of SR males do not possess a Y chromosome. If so, sterile sons are likely derived from nullo-XY sperm and are therefore XO.

The production of XO males as a result of X drive is a common pattern in *Drosophila*, having been found to occur in *Drosophila simulans* (Cazemajor *et al.*, 2000), *Drosophila pseudoobscura* (Sturtevant & Dobzhansky, 1936; Henahan & Cobbs, 1983; Cobbs, 1986), *Drosophila paramelanica* (Stalker, 1961), and *Drosophila athabasca* (Voelker & Kojima, 1971). X drive in *Drosophila neotestacea* is also presumed to result in the production of XO progeny, as inferred from the sterility of all sons produced by SR males in this species (James & Jaenike, 1990). While the production of XO males is clearly a common occurrence in X drive systems, there are subtle differences between them. For instance, in *D. pseudoobscura* all sons of SR males are XO and sterile (Henahan & Cobbs, 1983), whereas SR males in *D. simulans* produce both sterile XO and fertile XY males (Cazemajor *et al.*, 2000). Here I have shown that SR males in *D. testacea*, like *D. simulans*, can produce both sterile XO and fertile XY sons. However, fertile XY sons are far rarer in the progeny of *D. testacea* (~5%) when compared to *D. simulans*, where roughly two-thirds of male progeny of SR males are XY (Cazemajor *et al.*, 2000). The production of nullo-XY sperm by *D. simulans* SR males is explained by the missegregation of the Y chromosomes during meiosis II (Cazemajor *et al.*, 2000; Helleu *et al.*, 2016). As I observe XO males as a result of X drive in *D. testacea*, a similar abnormality could be occurring during meiosis in *D. testacea* SR males.

I found a haplotype that is unique to the X^{SR}, spanning several hundred base pairs within the genes *skpA* and *Rpl36*, demonstrating that there is reduced recombination between driving and non-driving X chromosomes. As both X-linked genes I sequenced showed so many sequence differences, it is likely that recombination is suppressed across a large portion of the *D. testacea* driving X chromosome. Suppressed recombination, often in the form of chromosomal inversions, is a common feature of driving X chromosomes, as it prevents the decoupling of interacting loci that contribute to drive

(Jaenike, 2001). A lack of recombination benefits the X^{SR} in the short term but will eventually lead to the accumulation of deleterious mutations (Dyer *et al.*, 2007). In accordance with this expectation, we find that even though *skpA* is a highly conserved gene, two of the six nucleotide changes found in X^{SR} *skpA* are nonsynonymous amino acid changes, one of which is not found in any sequenced Diptera.

The sequence differences existing between the X^{SR} and X^{ST} allow us to use *skpA* as a marker for SR, making it a powerful tool for studying X drive in *D. testacea*. In the present study, I took advantage of this marker to help screen for males carrying the X^{SR} in the wild, in the Swiss population where I first identified drive. I found that the X^{SR} exists at relatively low frequencies in males (~10%). When mated to laboratory females, 2 of 37 wild caught males expressed drive, both of which carried the X^{SR} chromosome. However, two males with normal sex ratios also carried the X^{SR}. By placing these X chromosomes in our laboratory genetic background, I show that both of these chromosomes exhibit drive, demonstrating that the initial wild caught males carried supressing elements. Attempting to characterize the genetic nature of suppression in *D. testacea* is beyond the scope of this paper. However, resistant Y chromosomes are predicted to evolve more readily than autosomal suppressors, as susceptible Y chromosomes have little to no fitness when paired with an X^{SR} (Helleu *et al.*, 2014). Future work will attempt to confirm whether suppression of drive is Y-linked (see chapter 3).

The testacea group is an especially promising system for studying the evolution and ecology of driving X chromosomes. Testacea group species are mycophagous, spending most of their life cycle on mushroom. Their ecology is therefore comparatively well understood, making them amenable to research under ecologically relevant contexts. Furthermore, three (*D. testacea*, *D. neotestacea,* and *D. orientacea*) of the four known members of the testacea species group now have been reported to harbour driving X chromosomes (James & Jaenike, 1990; Pieper & Dyer, 2016), indicating that this lineage may be a 'hotspot' for the evolution of X drive. These three species are very closely related, with incomplete reproductive isolation, and were only recently recognized to be different (Grimaldi *et al.*, 1992). Significantly, X drive in these closely related species differs in a number of important ways. The presence of suppression, its low frequency in the wild, and its divergence from the X^{ST} point to an ancient origin of the X^{SR} in *D.*

testacea, perhaps even pre-dating the origin of the species. In contrast, X drive in *D. neotestacea* appears to be comparatively young: it persists at high frequencies (>30%) in the wild and no suppressors of drive have been found in this species (Dyer, 2012). Also, recent dating work suggests that it evolved after *D. neotestacea* split from its relatives (Pieper & Dyer, 2016). X drive in *D. testacea* provides the unique opportunity to compare driving X chromosomes of different ages in closely related taxa, which should provide insight into the evolution of these selfish genetic elements, as well as their role in speciation.

Chapter 2 Supplemental Information

Supplemental Table 2-1. Offspring sex ratio and *skpA* haplotype data from 23 *Drosophila testacea* males (mated to two laboratory females each).

Cross	n	Female/male	Proportion female	p-value (chi-squared, null = 1:1)	*skpA* haplotype
VC26	19	19/0	1	1.307e-0.5	X^{SR}
P18	123	121/2	0.98374	2.2e-16	X^{SR}
VC25	118	116/2	0.983051	2.2e-16	X^{SR}
P06	164	159/5	0.969512	2.2e-16	X^{SR}
VC21	172	164/6	0.965116	2.2e-16	X^{SR}
VC03	65	62/3	0.953846	2.516e-13	X^{SR}
P02	88	83/5	0.943182	2.2e-16	X^{SR}
FC01	109	102/7	0.93578	2.2e-16	Not determined
VC07	32	28/4	0.875	2.209e-05	X^{SR}
VC42	87	76/11	0.873563	3.198e-12	X^{SR}
FC11	68	59/9	0.867647	1.333e-09	Not determined
P12	35	30/5	0.857143	2.381e-05	X^{SR}
VC37	32	26/6	0.8125	0.000407	X^{SR}
FC12	78	42/36	0.538462	0.4969	Not determined
P19	113	60/53	0.530973	0.5102	X^{ST}
VC05	87	46/41	0.528736	0.5919	X^{ST}
VC35	135	71/64	0.525926	0.5469	X^{ST}
VC16	192	100/92	0.520833	0.5637	X^{ST}
FC13	72	37/35	0.513889	0.8137	Not determined
P05	249	121/128	0.485944	0.6573	X^{ST}
VC14	120	56/64	0.466667	0.4652	X^{ST}
FC18	121	55/66	0.454545	0.3173	Not determined
FC14	77	33/44	0.428571	0.21	Not determined

Supplemental Table 2-2. Primers used in chapter 2.

Primer name	Sequence (5’ → 3’)	Source
SkpA-F	AAVATGCCBARYATYAARYTGCARTC	Dyer *et al.* (2011)
SkpA-R	CTTCTCCTCRCACCAYTCRTT	Dyer *et al.* (2011)
Kl2q-F	AAGAACGCCTACGAAAGCAA	This study
Kl2q-R	TGAGATGCCTCCACTTGTTG	This study
Rpl-F	CMRVGSCCACAAGACCWCSAARRTC	Dyer *et al.* (2011)
Rpl-R	CRTGRGTCTGRGCCTTCC	Dyer *et al.* (2011)

Supplemental Table 2-3. Fertility data for 38 sons sired by seven different SR fathers, and 24 sons sired by an ST father.

Father	Son	Produced offspring? (n = no, y = yes)	Offspring sex ratio (female/male)	*kl-2* amplified? (n = no, y = yes)
1 (SR male)	2-1	n	n/a	n
	2-2	n	n/a	n
2 (SR male)	4-1	n	n/a	n
	4-2	n	n/a	n
	4-3	n	n/a	n
	4-4	**y**	**20/17**	**y**
	4-5	n	n/a	n
	4-6	n	n/a	n
	4-7	n	n/a	n
	4-8	n	n/a	n
	4-9	n	n/a	n
	4-10	n	n/a	n
	4-11	n	n/a	n
	4-12	n	n/a	n
	4-13	n	n/a	n
	4-14	n	n/a	n
	4-16	n	n/a	n
3 (SR male)	8-1	n	n/a	n
	8-2	n	n/a	n
	8-3	n	n/a	n
	8-4	n	n/a	n
	8-5	n	n/a	n
4 (SR male)	P2-1	n	n/a	n
	P2-2	n	n/a	n
	P2-3	n	n/a	n
	P2-4	n	n/a	n
	P2-5	n	n/a	n
5 (SR male)	P6-1	n	n/a	n
	P6-2	n	n/a	n
	P6-3	n	n/a	n
6 (SR male)	P12-1	n	n/a	n
	P12-2	**y**	**41/36**	**y**
	P12-3	n	n/a	n
	P12-4	n	n/a	n
	P12-5	n	n/a	n
	P12-3	n	n/a	n
	P12-4	n	n/a	n
	P12-4	n	n/a	n
	P12-5	n	n/a	n
	P12-5	n	n/a	n
	P12-1	n	n/a	n
7 (SR male)	P18-1	n	n/a	n
	P18-2	n	n/a	n
1 (ST male)	6-1	y	Not counted	y

Supplemental Table 2-3. *Continued.*

Father	Son	Produced offspring? (n = no, y = yes)	Offspring sex ratio (female/male)	*kl-2* amplified? (n = no, y = yes)
	6-2	y	Not counted	y
	6-3	y	Not counted	y
	6-4	y	Not counted	y
	6-5	y	Not counted	y
	6-6	y	Not counted	y
	6-7	y	Not counted	y
	6-8	y	Not counted	y
	6-9	y	Not counted	y
	6-10	y	Not counted	y
	6-11	n	n/a	y
	6-12	y	Not counted	y
	6-13	y	Not counted	y
	6-14	y	Not counted	y
	6-15	y	Not counted	y
	6-16	y	Not counted	y
	6-17	y	Not counted	y
	6-18	y	Not counted	y
	6-19	y	Not counted	y
	6-20	y	Not counted	y
	6-21	y	Not counted	y
	6-22	y	Not counted	y
	6-23	y	29/22	y
	6-24	y	34/33	y

Supplemental Table 2-4. Offspring sex ratio and *skpA* haplotype data from wild caught male *Drosophila testacea* from St. Sulpice, Switzerland (mated to four laboratory females each).

Wild male	n	Female/male	Proportion female	*skpA* haplotype
S2*	300	184/116	0.6133333	X^{SR}
S3	279	140/139	0.5017921	X^{ST}
S4	227	118/109	0.5198238	X^{ST}
S5	0	n/a	n/a	X^{ST}
S36	220	122/98	0.5545455	X^{ST}
S37	160	82/78	0.5125	X^{ST}
S38	151	76/75	0.5033113	X^{ST}
S39	238	204/34	0.8571429	X^{SR}
S40	207	118/89	0.5700483	X^{ST}
S41	128	57/71	0.4453125	X^{ST}
S42	306	174/132	0.5686275	X^{ST}
S43	109	61/48	0.559633	X^{ST}
S44	193	99/94	0.5129534	X^{ST}
S45	315	155/160	0.4920635	X^{ST}
S47	0	n/a	n/a	X^{ST}
S48	189	102/87	0.5396825	X^{ST}
S49	122	56/66	0.4590164	X^{ST}
S50	159	91/68	0.572327	X^{ST}
S51	0	n/a	n/a	X^{ST}
S52	140	82/58	0.5857143	X^{ST}
S53	130	60/70	0.4615385	X^{ST}
S54	136	69/67	0.5073529	X^{ST}
S55	101	46/55	0.4554455	X^{ST}
S56	216	100/116	0.462963	X^{ST}
S57	226	108/118	0.4778761	X^{ST}
S58	110	51/59	0.4636364	X^{ST}
S59	254	115/139	0.4527559	X^{ST}
S60	129	62/67	0.4806202	X^{ST}
S61	219	102/117	0.4657534	X^{ST}
S62	0	n/a	n/a	X^{ST}
S63*	190	98/92	0.5157895	X^{SR}
S64	108	55/53	0.5092593	X^{ST}
S65	179	103/76	0.575419	X^{ST}
S66	88	50/38	0.5681818	X^{ST}
S67	242	131/111	0.5413223	X^{ST}
S68	155	80/75	0.516129	X^{ST}
S69	157	82/75	0.522293	X^{ST}
S70	113	63/50	0.5575221	X^{ST}
S71	133	127/6	0.9548872	X^{SR}
S72	124	58/66	0.4677419	X^{ST}
S73	259	133/126	0.5135135	X^{ST}

*putatively suppressed males, see supplemental table 2-5

Supplemental Table 2-5. Offspring sex ratio and *skpA* haplotype data from F_2 sons of wild-caught males carrying a suppressing element. F_2 males are siblings produced by crossing $X^{SR}X^{ST}$ - females (F_1 daughters of the wild-caught males S2 and S63) to laboratory $X^{ST}Y$ males.

Wild-caught male (see table S4)	F_2 son	n	Female/male	Proportion female	*skpA* haplotype
S2	2-2	64	34/30	0.53125	X^{ST}
	2-38	25	24/1	0.96	X^{SR}
S63	63-2	75	38/37	0.50667	X^{ST}
	63-6	41	41/0	1.0	X^{SR}

Chapter 3 – Maintenance of an ancient X drive polymorphism in *Drosophila testacea*

Introduction

X chromosome drive, or X drive, occurs when an X-linked gene causes the biased transmission of the X chromosome in members of the heterogametic sex. This phenomenon has been described in mammals, plants, and insects, and is particularly well-characterized in flies (Diptera), where there are at least 20 known X drive systems (Jaenike, 2001). In Diptera, X drive causes Y-bearing sperm to develop improperly, so that males carrying the driving X chromosome (called SR males) transmit predominantly X-bearing sperm (Policansky and Elison, 1970; Cazemajor *et al*., 2000). SR males sire heavily female-biased offspring as a result. The transmission advantage enjoyed by the driving X chromosome (X^{SR}) leads to the prediction that it should spread in a population until it replaces the standard X chromosomes (X^{ST}), unless opposed by counter-acting natural selection (Gershenson, 1928; Hamilton, 1967). In this scenario, a paucity of males as a consequence of high X^{SR} frequencies could eventually precipitate the extinction of local populations, and in theory this could also cause the extinction of entire species (Hamilton, 1967).

Despite the expectation that they should spread rapidly to fixation, many of the well-studied X^{SR} chromosomes persist as balanced polymorphisms (Jaenike, 2001). For example, longitudinal data shows that X^{SR} frequencies can be stable on a timescale of decades: the X^{SR} of *Drosophila pseudoobscura* has persisted at a frequency of ~20-30% in some populations for at least 70 years (Price *et al*., 2014); sampling efforts made 23 years apart in a Malaysian population of the stalk-eyed fly *Teleopsis whitei* shows that the X^{SR} frequency in this species has remained at ~14% (Paczolt *et al*., 2017); and X^{SR} frequency in *Drosophila neotestacea* has been at 20-25% over a ~25 year period (James and Jaenike, 1990; Pieper and Dyer, 2016). Molecular dating of X^{SR} chromosomes has revealed that this level of stability has likely been the case for hundreds of thousands of years. Dating of the X^{SR} in *D. pseudoobscura*, for example, suggests that this X drive system may be over 1 million years old (Babcock and Anderson, 1996; Kovacevic and

Schaeffer, 2000). Another example is the X^{SR} of *D. neotestacea*, which Pieper and Dyer (2016) have estimated to be 330,000 – 580,000 years. Clearly, then, some X^{SR} chromosomes can persist in species over evolutionary timescales without causing extinction. The disparity between what is expected (rapid spread to fixation), and what is observed (stable persistence), has instigated several theoretical and experimental investigations into the mechanisms that may explain the evolutionary maintenance of these extreme natural polymorphisms.

Natural selection acting against the X^{SR} is likely to play an important role in maintaining stable X drive polymorphisms in populations. Because the Y-bearing sperm of SR males fail to develop, SR males produce half as many sperm as ST males. The spread of an X^{SR} could be stemmed if this reduction in sperm production causes a reduction in the fertility of SR males. Beckenbach (1978) showed that SR male *D. pseudoobscura* do not suffer a reduction in fertility compared to ST males when males only mate once. Crucially, however, when males are made to mate more than once, SR males show a pronounced reduction in fertility compared to ST males (Beckenbach, 1978). This pattern is true not only in *D. pseudoobscura*, but also in several other species carrying X drive polymorphisms (*e.g.* Jaenike, 1996; Atlan *et al.*, 2004; Wilkinson *et al.*, 2006; Unckless *et al.*, 2015). Jaenike (1996) has suggested that this pattern may allow selection to act against the X^{SR} in a frequency-dependent manner. He proposes that if the male mating rate is low, SR and ST males may have the same fertility, resulting in the spread of the X^{SR}. The population will become more female-biased as the X^{SR} spreads, leading to an increase in the male mating rate, and a concurrent decrease in the relative fertility of SR males. Thus, the X^{SR} is favoured when at low frequency, but punished by selection when at high frequency due to the depletion of sperm in SR males after multiple mating (Jaenike, 1996). Note that the male mating rate increases simply as a result of a higher female-to-male ratio in this model and does not take into account the propensity of females to re-mate (polyandry). The male mating rate will be higher when females are polyandrous, regardless of the female-to-male ratio; therefore, polyandry is likely an important additional component of the frequency-dependent process outlined above (Price *et al.*, 2010).

Selection against the X^{SR} will also occur when deleterious alleles are linked to the driver. X^{SR} chromosomes are often characterized by inversions that differentiate the X^{SR} from the X^{ST} (Jaenike, 2001). These inversions suppress recombination between the X^{SR} and X^{ST} and are thought to couple interacting X drive loci as a non-recombining unit (Prout *et al.*, 1973). The lack of recombination in these regions can lead to an accumulation of deleterious mutations due to Hills-Robertson interference (Gordo & Charlesworth, 2001). There will then be selection against X^{SR} carriers, in particular against $X^{SR}X^{SR}$ females. Selection against $X^{SR}X^{SR}$ females is an important component in the maintenance of an X drive polymorphism (Curtsinger and Feldman, 1980). In many X drive systems, homozygous females are sterile and/or have greatly reduced viability compared to heterozygous or standard females (Wallace, 1948; Curtsinger and Feldman, 1980; Beckenbach, 1996; Dyer *et al.* 2007).

An additional mechanism that may explain the persistence of an X drive polymorphism is the evolution of drive-suppressing elements. Because Y chromosomes are the direct target of an X^{SR}, there is strong selection for Y chromosomes that can suppress or resist the selfish action of the X^{SR}. Suppressing Y chromosomes are found in many X drive systems in Diptera (Jaenike, 2001). Carvalho *et al*. (1997) proposed a model that explains the persistence of an X^{SR} alongside a suppressing Y chromosome. In this model, there are two Y chromosomes (suppressing and susceptible) and two X chromosomes (X^{SR} and X^{ST}). Any rise in the frequency of an X^{SR} increases the fitness of the suppressing Y chromosome relative to the susceptible Y chromosome. The suppressing Y chromosome will then spread, leading to a decline in X^{SR} frequency. Yet as the X^{SR} declines in frequency, so too does the suppressing Y chromosome. In other words, the frequencies of the X^{SR} and the suppressing Y chromosome cycle in a manner that depends on the frequency of the other, allowing both to persist (Carvalho *et al.*, 1997).

Autosomal suppressors are also found in many SR systems, and must be taken into account when trying to understand the evolutionary dynamics of X drive (Jaenike, 2001). The evolution of autosomal suppressors is explained by Fisher's principle, which states that the rarer sex is the more fit sex (Fisher, 1930). Males will be the rarer sex whenever an X^{SR} is at appreciable frequencies, as the population sex ratio will be skewed

towards females. Autosomal genes that suppress the X^{SR} increase the number of males in an individual's progeny and are thus selected for when the population is female-biased. Although autosomal and Y-linked suppressors are found in many X drive systems, they are by no means essential for maintaining a drive polymorphism. Two of the best studied X drive systems, *D. pseudoobscura* and *D. neotestacea*, do not harbour suppressors, despite extensive searches (Jaenike, 2001).

Drosophila testacea is a widespread mycophagous fly found in woodlands ranging from Europe to Japan (Grimaldi *et al.*, 1992). I have recently shown that this species harbours an X drive polymorphism. Here, I reconstruct the evolutionary history of the X^{SR} using phylogenetics. I also explore the mechanisms that may allow this X^{SR} to be maintained as a polymorphism in both laboratory and natural populations. It remains inconclusive whether common mechanisms exist for maintaining X drive polymorphisms in nature. Examining the factors that facilitate the persistence of the X^{SR} in *D. testacea* will contribute to a more general understanding of the evolutionary maintenance of X drive polymorphisms. X drive in *D. testacea* appears to be relatively stable in lab populations; thus, the factors underlying its apparent stability in the lab can be extrapolated to natural populations to explain the maintenance of this polymorphism on evolutionary timescales.

Materials and Methods

Fly stocks

Two laboratory populations of *D. testacea* were used in the work performed for this chapter. The first population was established from several wild-caught flies collected in August 2012 near St. Sulpice, Vaud, Switzerland. The X^{SR} chromosome was isolated in the summer of 2014 from this stock and has since been maintained according to the crossing scheme outlined in Keais *et al.* (2017) and chapter 2 of this thesis. The second laboratory population was also founded with flies caught near St. Sulpice – but these were caught in July 2016. The populations are denoted in the text by their year of collection. All stocks are reared at 21°C with a 12-h light:dark cycle in vials containing

instant *Drosophila* medium (Carolina Biological Supply, Burlington, NC), supplemented with commercial mushroom (*Agaricus bisporus*).

Phylogenetic analysis of the X^{SR}

To investigate the evolutionary history of the X^{SR} in *D. testacea*, I compared X-linked gene sequences from the four known members of the Drosophila testacea species group (testacea group) using phylogenetic analysis. I chose to analyse two X-linked genes (*skpA* and *pgd*) and an autosomal gene (*gl*), thus allowing me to compare the history of the X chromosomes against that of the autosomes. These genes were chosen because all three have sequences available from GenBank for all four species found in the testacea group. Sequences for *D. testacea* were generated for this analysis from a sample of males caught in the wild in the summer of 2016. For both *pgd* and *gl*, PCR amplicons were generated and sequenced (Sequetech, USA) from a subsample (N=14) of these wild-caught males using primers designed in Pieper and Dyer (2016). PCR reaction mixtures, conditions, and primers can be found in the supplemental material. Sequences for the gene *skpA* had been obtained previously from the same *D. testacea* individuals (see chapter 2). Of the 14 wild-caught males chosen for sequencing, four were known to carry an X^{SR}. To compare these 2016 X^{SR} chromosomes with the X^{SR} maintained in the lab since 2012, I sequenced *skpA*, *pgd* and *gl* from a single male from our lab X^{SR} line. Sequences for *D. neotestacea*, *D. orientacea*, and *D. putrida* were obtained from GenBank.

To generate a data matrix for phylogenetic analysis, I concatenated *skpA* and *pgd* sequences for all individuals and aligned them using Geneious. Maximum likelihood phylogenetic analysis was then performed using PhyML with 100 bootstraps for node support (Guindon *et al.*, 2010). The same process was performed to produce a phylogeny of the autosomal gene *gl*. Sequence information can be found in supplemental tables 3-3 and 3-4.

48-hour sperm depletion assay

To test for fertility differences between SR and ST male *D. testacea*, I performed a sperm depletion assay, in which males were allowed to mate with an excess of females for a prescribed period of time. In order to generate a large sample of aged-matched SR

and ST males, 30 $X^{SR}X^{ST}$ females were mated to 20 $X^{ST}Y$ males in a glass mason jar (Bernardin, Canada) for 10 days. Eclosing males were collected over a two-day period, and 19 of these were then randomly chosen and housed individually in vials for seven days. Virgin females were collected during the same period, housed in groups of five, and aged 8-12 days.

To commence the assay, 20 groups of five virgin females (denoted "first females") were transferred without anesthesia to standardized vials (~1.45g instant Drosophila media rehydrated with 3mL of water and supplemented with a piece of *Agaricus bisporus* mushroom weighing 0.6-0.8g). Single males were then added to these vials without anesthesia. After 24 hours, each male was aspirated to a vial containing a second group of five virgin females (denoted "second females") and left to mate for 24 hours. Males were removed at the end of the second mating period and their DNA was extracted using Prepman™ Ultra (Applied Biosystems) for genotyping. The females were left to oviposit for two days, after which they were transferred to new vials every two days, to a total of five vials per group of females. Females were left to oviposit for two days in the fifth (and final) vial and were then discarded. Flies eclosing from all vials were counted and sexed. The fertility of each male, therefore, was assessed by the number of offspring emerging from a total of 10 vials (five from the first females and five from the second females). Each male's genotype was determined using a high-resolution melt-curve analysis (HRM) protocol (supplemental methods), and by their offspring sex ratios.

Fecundity of $X^{SR}X^{SR}$ females

In order to test for female-specific fitness defects caused by the X^{SR}, I generated both $X^{SR}X^{SR}$ and $X^{SR}X^{ST}$ females and examined their fecundity by mating them to $X^{ST}Y$ males. As these females were the offspring of an $X^{SR}Y$ male and $X^{SR}X^{ST}$ females, the $X^{SR}X^{SR}$ and $X^{SR}X^{ST}$ genotypes were expected in equal proportion. I collected 80 females and mated them *en masse* to $X^{ST}Y$ males in pools of 10 females and 10 males. After seven days, I transferred a total of 64 females (several females died in the mating vials) to individual vials for oviposition. Females were transferred to new vials every four days for

16 days, for a total of four vials each. The vials were visually inspected for the presence of larvae and pupae, and all eclosing adults were counted.

To determine the genotype of the 64 females, their DNA was extracted using Prepman™ Ultra (Applied Biosystems), and the *skpA* marker gene (see chapter 2) was PCR-amplified and Sanger-sequenced (Sequetech, USA). To avoid contamination from any sperm stored post-mating, DNA extractions were performed using only the head and thorax. For PCR reaction conditions, see the supplemental material.

Testing for Y-linked suppression

The presence of X^{SR} suppression in a natural population of *D. testacea* from Switzerland is reported in chapter 2 of this thesis and in Keais *et al.* (2017). To reiterate, two of 37 wild-caught males carried an X^{SR} chromosome but produced normal offspring sex ratios (1:1). These two X^{SR} chromosomes were shown to drive in a non-suppressing genetic background. A drive-suppressing element that is able to restore progeny sex-ratios to normal in SR males is therefore segregating in wild populations of *D. testacea*. I hypothesized that the suppressing element might be Y-linked; therefore, I introgressed the Y chromosomes from both suppressed males into a laboratory genetic background for four generations, yielding two Y replacement lines, Y^2 and Y^{63}. Note that this strategy does not attempt to isolate autosomal suppressors.

To test for drive suppression by Y^2 and Y^{63}, I paired each with an X^{SR} by crossing XY^2 and XY^{63} males separately to $X^{SR}X$ females. This cross generates males that carry a putatively suppressing Y chromosome (Y^2 or Y^{63}) and either the X^{SR} or X^{ST}. These males were then mated to $X^{ST}X^{ST}$ females and their progeny sex ratios were screened for female bias. Strong female bias in the offspring of these males would preclude Y-linked suppression.

Screening for the X^{SR} in the 2016 stock

To screen for the X^{SR} in our 2016 stock of *D. testacea*, I sampled 20 males emerging from a single stock vial and performed DNA extractions on them using Prepman™ Ultra (Applied Biosystems). I then genotyped them using HRM (supplemental methods).

Results

The X^{SR} is an ancient polymorphism

Genes on the X chromosome are highly divergent between the two X chromosome types in *D. testacea*. The X^{SR} sequences branch outside of a monophyletic clade containing *D. orientacea*, *D. neotestacea*, and the *D. testacea* X^{ST} sequences (Figure 3-1), suggesting that the X^{SR} diverged prior to the radiation of these three species. As the X^{SR} versions of *pgd* and *skpA* are both perfectly associated with the sex-ratio distortion phenotype, this region must also contain the X drive locus (or loci). As expected, autosomal sequences from SR male and ST are indistinguishable, as depicted by their close grouping on the *gl* phylogeny (Figure 3-2).

SR males have severely reduced fertility

I assessed the fertility of SR (N = 7) and ST (N = 8) males by the number of offspring they produced with the first females (Figure 3-3, first females), the number of offspring they produced with the second females (Figure 3-3, second females), and the total number of offspring they produced (Figure 3-3, total). ST males did not produce significantly more offspring than SR males with the first females (although this approached significance) (Figure 3-3, first females) (t_{obs} = 2.1767, P = 0.0647). However, ST males did produce significantly more offspring than SR males with the second females (Figure 3-3, second females) (t_{obs} = 2.6685, P = 0.03104), indicating that the sperm stores of SR males deplete faster than those of ST males. The same trend is reflected by the fact that the mean number of offspring produced by ST males did not significantly decrease between the first and second females (t_{obs} = 1.1947, P = 0.2594), whereas it did for SR males (t_{obs} = 3.0658, P = 0.01366). In total, SR males produced on average significantly fewer offspring than ST males (Figure 3-3, total)(t_{obs} = 3.1999, P = 0.01407), with an average of 17% the fertility of ST males. Four individuals (three SR males and one ST male) produced no offspring with either group of females – these were excluded from the analysis.

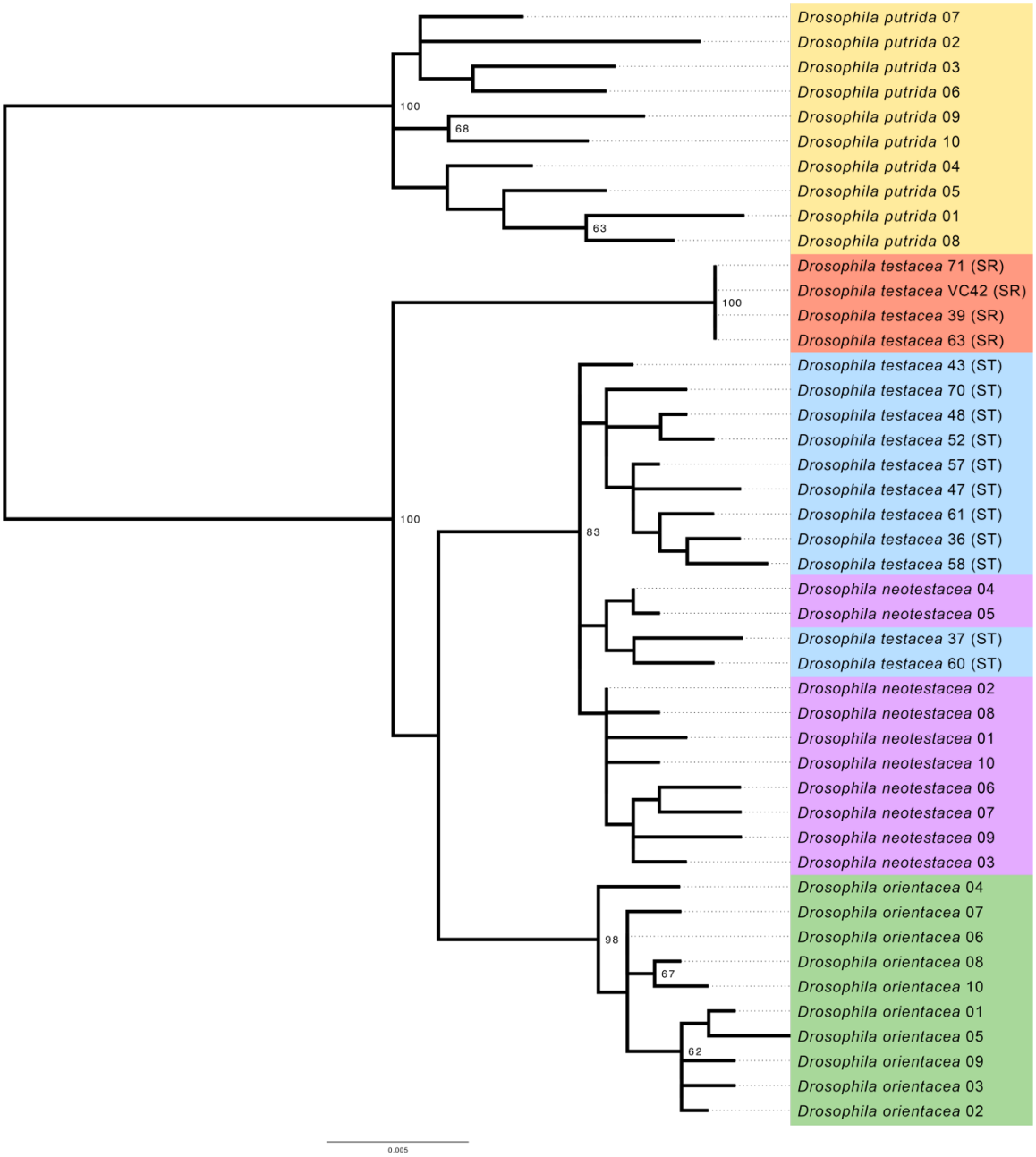


Figure 3-1. Maximum-likelihood phylogeny of the testacea species group generated by PhyML using sequences from the X-linked genes, *pgd* and *skpA* (1087 nucleotides), with bootstrapped node support. Only bootstraps values >60 are shown. The sample of *D. testacea* males with an X^{SR} (SR) are highlighted in red, whereas males with an X^{ST} (ST) are shown in blue. *D. putrida*, *D. neotestacea*, and *D. orientacea* are highlighted in yellow, blue, and green, respectively.

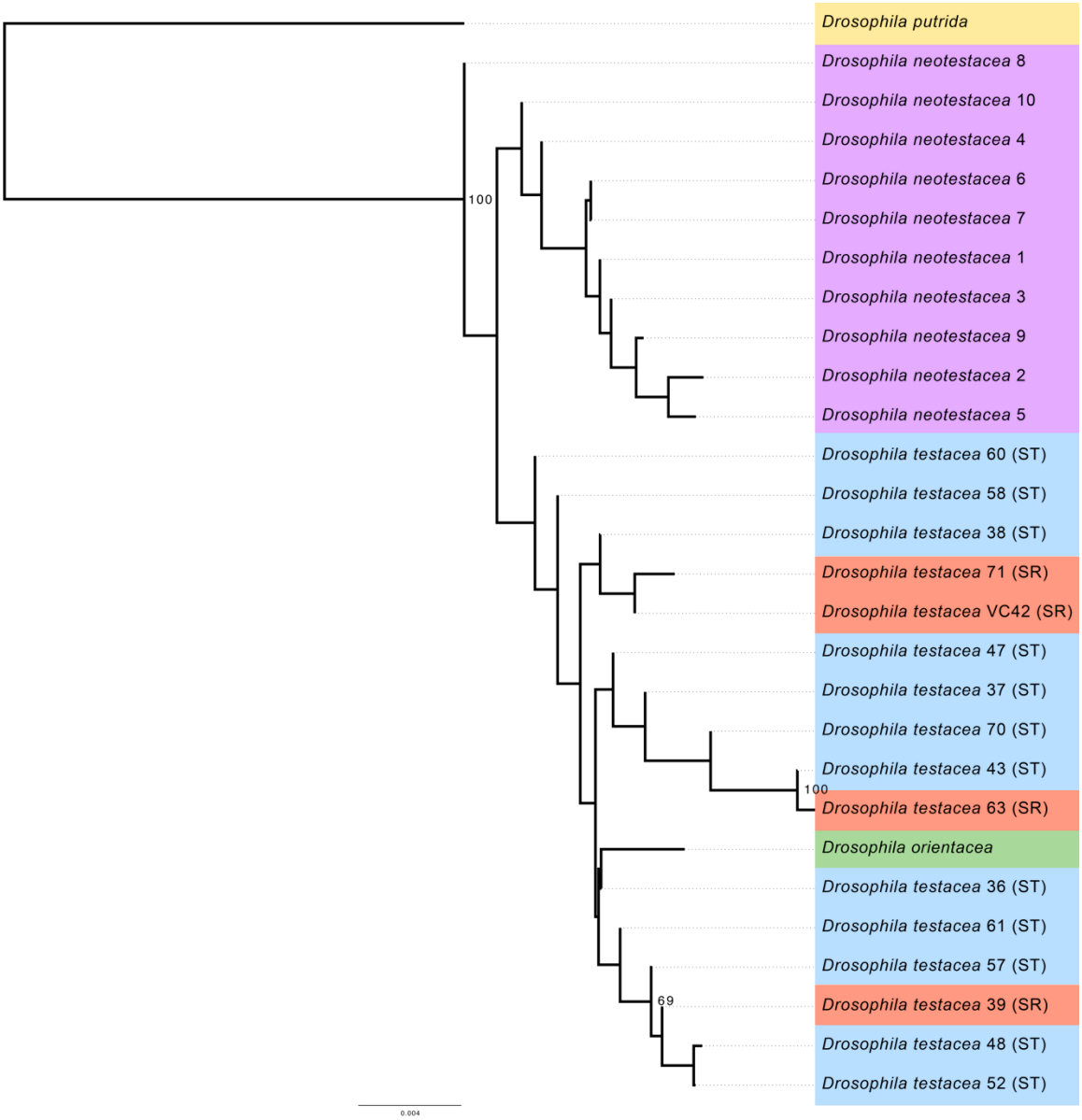


Figure 3-2. Maximum-likelihood phylogeny of the testacea species group generated by PhyML using sequences from the autosomal gene *gl* (402 nucleotides), with bootstrapped node support. Only bootstraps values >60 are shown. The sample of *D. testacea* males with an X^{SR} (SR) are highlighted in red, whereas males with an X^{ST} (ST) are shown in blue. *D. putrida*, *D. neotestacea*, and *D. orientacea* are highlighted in yellow, blue, and green, respectively.

$X^{SR}X^{SR}$ females have reduced fecundity

Of 64 females, 57 were successfully genotyped as either $X^{SR}X^{SR}$ or $X^{SR}X^{ST}$ using the X-linked marker, *skpA*. Chromatograms generated for the remaining 7 females were too noisy to confidently determine their genotype. Unexpectedly, 42 of the 57 genotyped females were $X^{SR}X^{SR}$, whereas only 15 were $X^{SR}X^{ST}$. Of the 42 $X^{SR}X^{SR}$ females, only 10 (~24%) produced offspring (Table 3-1). In contrast, the majority (11 out of 15, or ~73%) of $X^{SR}X^{ST}$ females produced offspring. Furthermore, when $X^{SR}X^{SR}$ females did produce offspring, they produced significantly fewer per vial than $X^{SR}X^{ST}$ females (as not all females received the same number of vials, offspring production was measured per vial).

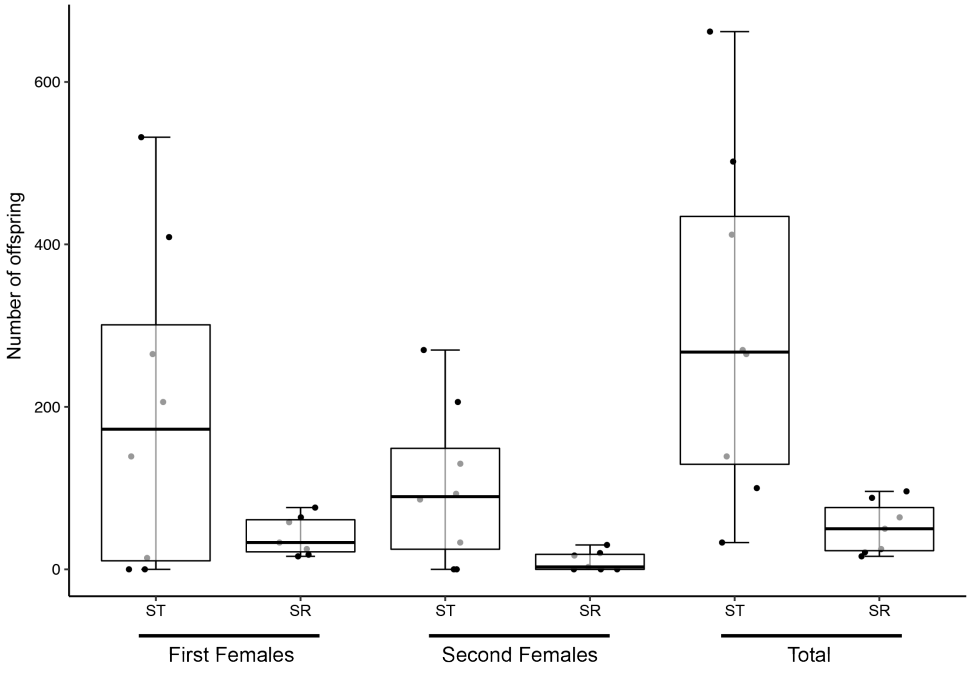


Figure 3-3. Fertility relations of ST and SR males determined from a 48-hour sperm depletion assay. The number of offspring produced by each male with the first and second groups of females are denoted by First Females and Second Females, respectively. Total number of offspring produced by each male is denoted by Total.

Suppression of X^{SR} in the wild is not Y-linked

Males carrying an X^{SR} and either of two putatively suppressing Y chromosomes (Y^2 or Y^{63}) were shown to produce significantly female-biased progeny, indicating that neither Y-chromosome is able to suppress drive (supplemental table 3-1).

Presence of the X^{SR} in the 2016 stock

Two of 20 males screened from the 2016 stock carried an X^{SR}, revealing that the X^{SR} has persisted in the laboratory from July 2016 until January 2018. This represents ~38 generations, assuming a generation time of 2 weeks.

Table 3-1. Fecundity of $X^{SR}X^{ST}$ and $X^{SR}X^{SR}$ females.

Female genotype	N	N fertile	Mean offspring per vial
$X^{SR}X^{SR}$	42	10	3.85
$X^{SR}X^{ST}$	15	11	13.81

Discussion

Driving X chromosomes that bias their own transmission in males by destroying Y-bearing sperm are found in numerous species of flies. Despite the expectation that these chromosomes should spread rapidly in populations, in many cases they are found as stable polymorphisms. Here, I show that X drive in *D. testacea* is ancient and likely arose before the split of *D. testacea* and its sister species, *D. neotestacea* and *D. orientacea*. I also present results showing that the X^{SR} carries a fitness cost for both SR males and homozygous females for the X^{SR}. These fitness experiments represent an initial investigation into the factors contributing to the evolutionary maintenance of X drive in this species.

Phylogenetic analysis of X-linked sequences has revealed a striking evolutionary history of the X^{SR} in *D. testacea*. The branching pattern shown in Figure 3-1 strongly suggests that the X^{SR} in *D. testacea* evolved in an ancestor of *D. testacea*, *D. neotestacea*,

and *D. orientacea*. Two sites among the 1,087 examined are unique to *D. putrida* and the X^{SR}, and an additional four are shared between the X^{SR}, *D. putrida* and *D. orientacea*. These sites – especially those shared with *D. putrida* – represent the ancestral character state of the testacea species group (Pieper and Dyer, 2016). The persistence of this ancestral haplotype is a clear indication that the X^{SR} and X^{ST} in *D. testacea* do not recombine along a portion of their length. Inversions that suppress recombination between X^{SR} and X^{ST} chromosomes are common in X drive systems. It is therefore likely that an inversion evolved on the X^{SR} in *D. testacea* prior to the split of *D. testacea*, *D. neotestacea*, and *D. orientacea*, capturing with it a snapshot of ancestral variation.

A similar scenario has occurred during the evolution of X drive in *D. pseudoobscura*. Babcock and Anderson (1996) examined the evolutionary history of the X^{SR} chromosome found in *D. pseudoobscura*, which houses multiple overlapping inversions unique to the X^{SR} on the right arm of the X chromosome. By dating loci linked to X drive in *D. pseudoobscura*, they estimate that the X^{SR} in this species is 800,000 – 1 million years old. Furthermore, they show that the evolution of the X^{SR} predates the split of *D. pseudoobscura* and its sister species, *D. persimilis*. Using a substitution rate of 1.7% per million years (Caccone *et al.*, 1988), and a divergence of 1.56% between the X^{SR} and X^{ST} in *D. testacea*, I estimate that the X^{SR} in *D. testacea* is roughly 920,000 years old.

How has X drive been maintained in *D. testacea* over this extended period of time? I have shown here that SR males have on average 17% the fertility of ST males when mated to multiple females. Additionally, the fertility of SR males declines more than the fertility of ST males after multiple mating, suggesting that the sperm stores of SR males are more rapidly depleted. I therefore infer that the impaired fertility of SR results directly from the destructive nature of the X^{SR}: SR males have fewer functional sperm because their Y-bearing sperm are failing to develop. According to Jaenike (1996), this effect could limit the spread of the X^{SR} in *D. testacea* if the male mating rate is sufficiently high. The fertility experiment performed here does not address the fertility differences between SR and ST males after a single mating, which is a key parameter in the frequency-dependent selection model proposed by Jaenike (1996). Further work

should address this to provide a more complete understanding of the fertility differences of SR and ST males in *D. testacea*.

The X^{SR} also incurs a pronounced fitness cost in homozygous females. After being housed with males for an extended period of time, a high proportion (~76%) of $X^{SR}X^{SR}$ females did not produce offspring when allowed to oviposit. The same was not true for $X^{SR}X^{ST}$ females: in the same experiment, only ~27% of $X^{SR}X^{ST}$ did not produce offspring. The higher proportion of $X^{SR}X^{SR}$ not producing offspring could be explained by two non-mutually exclusive reasons. First, it is possible that a higher fraction of $X^{SR}X^{SR}$ females did not mate. As females were given a sufficient period for mating to have occurred, this would indicate that $X^{SR}X^{SR}$ females have an impaired ability to obtain mates compared to $X^{SR}X^{ST}$ females. Second, the high proportion of $X^{SR}X^{SR}$ females not producing offspring could be due to recessive deleterious loci on the X^{SR} that cause the loss of female fecundity – that is, many of the $X^{SR}X^{SR}$ females may have mated, but did not produce offspring because they are sterile. The lack of recombination on X^{SR} chromosomes due to inversions can lead to the accumulation of deleterious mutations due to Hill-Robertson interference (Gordo and Charlesworth, 2001). Females homozygous for the X^{SR} in *D. recens* are sterile due to a recessive sterility factor that is fixed on the X^{SR} (Dyer *et al.*, 2007). This is strong evidence that the X^{SR} in *D. recens* has accumulated deleterious loci linked to the driver (Dyer *et al.*, 2007). Interestingly, the *D. testacea* $X^{SR}X^{SR}$ females that did produce offspring produced far fewer on average than $X^{ST}X^{ST}$ females. Clearly, then, the X^{SR} has accumulated deleterious mutations that reduce fecundity in homozygous females – whether these can also cause the full sterility of females as suggested above requires further investigation.

Suppressors can play an important role in the evolutionary dynamics of an X^{SR}. I have demonstrated here that suppression first identified in two wild-caught males from Switzerland (chapter 2) is not Y-linked. Because the X^{SR} chromosomes carried by these males was not suppressed by their Y chromosomes, suppression must be autosomal. If suppression not only restores offspring sex ratios to normal, but also restores the fertility of SR males, the co-existence of the X^{SR} with suppressors could facilitate higher frequencies of the X^{SR} (Capillon & Atlan, 1999). Thus, examining the fertility of SR

males carrying suppressors is warranted, as well as determining suppressor frequency in wild-populations.

By sampling and genotyping 20 males from the 2016 stock of *D. testacea*, I found that two males carried an X^{SR}. Therefore, an X^{SR} has persisted in this stock for close to 40 generations since the time it was founded in July of 2016. In light of this – and considering the bottlenecks in population size that regularly occur during stock maintenance – I conjecture that the frequency of the X^{SR} must be relatively stable under laboratory conditions, as any major fluctuations in its frequency would have resulted in its loss from our stock population by chance. Uncovering the parameters that have facilitated the maintenance of the X^{SR} under laboratory conditions should provide the foundation for understanding how the X^{SR} has persisted in the wild for nearly 1 million years.

Chapter 3 Supplemental Information

Supplemental Methods

Genotyping using High Resolution Melt Curve Analysis

I developed a high-resolution melt curve analysis protocol (high resolution melt, or HRM) for determining the X chromosome type of male *Drosphila testacea*. During HRM, DNA is first amplified with qPCR. The resulting PCR product is denatured by increasing the temperature sequentially by a set temperature increment, yielding a melt temperature profile that depends on the size and composition of the amplified sequence. *D. testacea* has two major X chromosome types (X^{ST} and X^{SR}) that differ by several nucleotides within the gene *skpA*. I targeted a 180bp region of *skpA* that contains four nucleotide differences between the X^{ST} and X^{SR} with the qPCR primers SkpA-M-F and SkpA-M-R (supplemental table 3-2). Employing HRM using these primers yields significantly distinct melt temperature profiles for the two chromosome types (supplemental figure 3-1)(linear model: $p < 2.2e\text{-}16$), thus allowing me to determine the X chromosome type of male *D. testacea*. Note that this genotyping method is only used with males, as it has not been optimized to genotype females. As a control, I targeted a 189 bp region in *skpA* that is identical between the chromosome types using the qPCR primers SkpA-N-F and SkpA-N-R (supplemental table 3-2). HRM using these primers yields melt temperature profiles that do not significantly differ between X chromosome types (supplemental figure 3-2)(linear model: $p = 0.8265$). This protocol was developed using DNA from males with known X chromosome type determined by their *skpA* gene sequence (see chapter 2 methods).

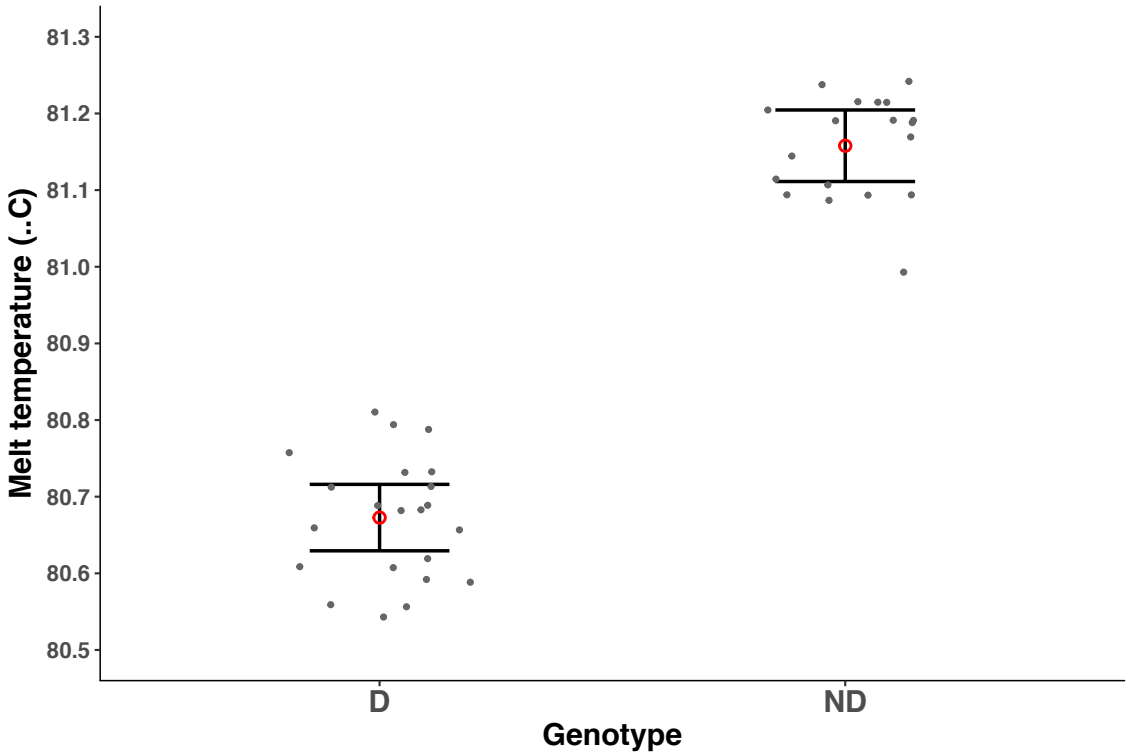
I used the following reaction mixture for HRM: 2 µl of template DNA, 8 µl of EvaGreen mastermix, 4.08ul of dH_20, and 0.96 µl of each 5 µm forward and reverse primers (16 µl reactions). The qPCR conditions were: 95 ºC for 10m, followed by [95 ºC for 15s, 60 ºC for 45s] x 35, then 94 ºC for 10m. This is immediately followed by HRM, which involves a temperature increase of 0.1 ºC per cycle from 65 ºC to 95 ºC, at 5s per cycle.

PCR conditions for pgd, gl, and skpA

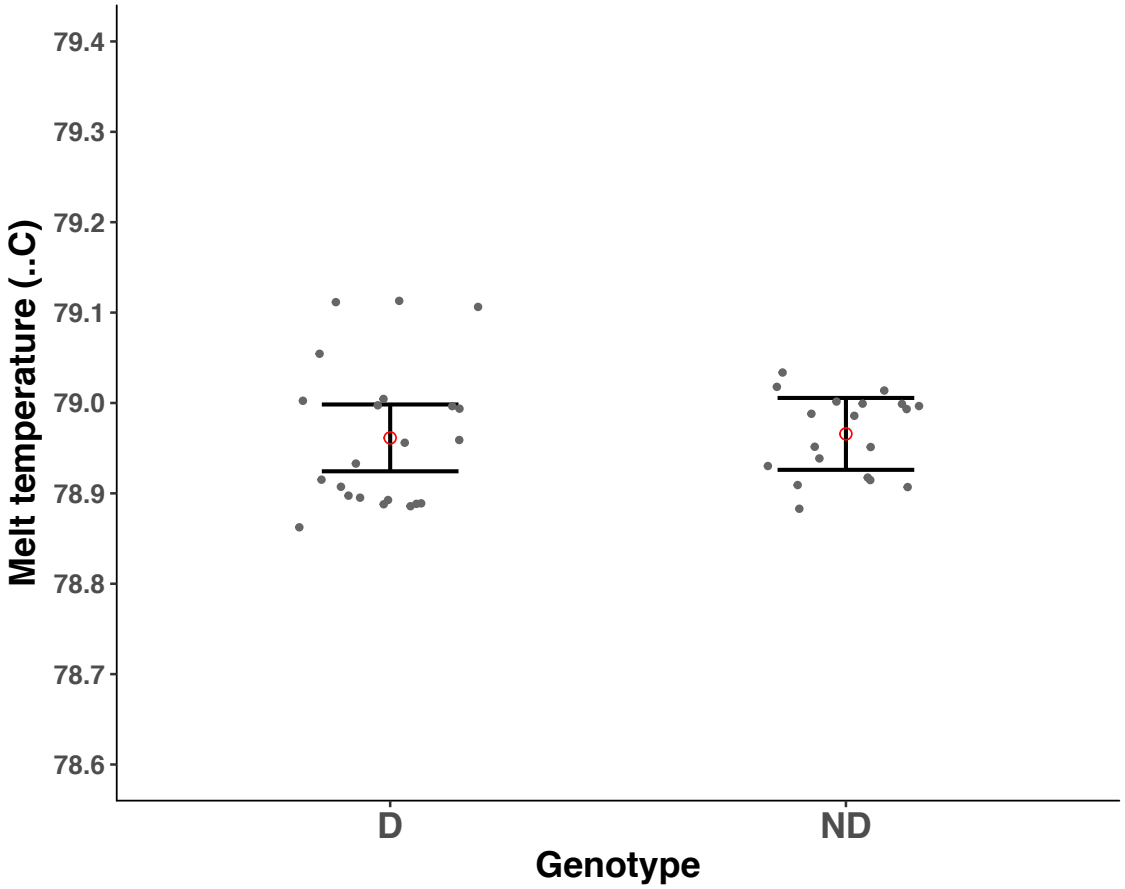
The thermal cycling conditions for the amplification of the genes *pgd*, *gl*, and *skpA* are as follows: 95 °C for 3 min, then 32 cycles of 94 °C for 1 min and 54 °C for 1 min, followed by a final 10 min at 72 °C. PCR amplicons were then Sanger-sequenced (Sequetech, USA). Sequence handling and analysis was performed using Geneious v5.1.7 (Kearse *et al.*, 2012). Primers used can be found in supplemental table 3-2.

Supplemental Table 3-1. Offspring sex ratios (proportion female) of two representative males from the two Y replacement lines, Y^2 and Y^{63}.

Individual	Y chromosome	Proportion female
Y2-14	Y^2	0.9 (n=20)
Y63-15	Y^{63}	0.94 (n=106)



Supplemental Figure 3-1. Melt temperatures of a *skpA* gene region (189 bp) amplified from *Drosophila testacea* males using qPCR. Males carried either a driving X chromosome (D) or a standard X chromosome (ND), which differ by four nucleotides in the *skpA* gene region targeted with qPCR. Bars represent 99% confidence intervals from the linear model. Open red circles indicate the mean.



Supplemental Figure 3-2. Melt temperatures of a *skpA* amplicon (189 bp) amplified from a region that is identical between the X^{SR} and X^{ST} chromosomes. Amplified from *Drosophila testacea* males carrying either a driving X chromosome (D) or a standard X chromosome (ND) males using qPCR. Bars represent 99% confidence intervals from the linear model. Open red circles indicate the mean.

Supplemental Table 3-2. Primers used in chapter 3.

Primer name	Sequence (5' → 3')	Source
SkpA-F_exact	AACATGCCGACCATTAAGTTG	This study
SkpA-R_exact	CTTCTCCTCACACCGTTCGTT	This study
SkpA-M-F	CAAGTGCTCTGGCACAATTC	This study
SkpA-M-R	GTGCGTTTCTCCTTGCTCTC	This study
SkpA-N-F	TCACATCATGGGATGCTGAT	This study
SkpA-N-R	CGGGAGTGAAGTCCTTTTTG	This study
gl-F	TTTCGATTGCGGCGGNTGYTTYGA	Pieper and Dyer (2016)
gl-R	GCCGTGGTGCATGGTCATRTTCAT	Pieper and Dyer (2016)
pgd-F	ATYGATGGYGGCAACTC	Pieper and Dyer (2016)
pgd-R	CNCGCATWAGCATRAAKCCYTG	Pieper and Dyer (2016)

Supplemental Table 3-3. Individuals from the *Drosophila* testacea species group used for phylogenetic analysis of the autosomal gene *gl*.

Individual in phylogeny	Gene	Accession	Reference
Drosophila putrida	*gl*	KX637921.1	Pieper and Dyer (2016)
Drosophila orientacea	*gl*	KX637920.1	Pieper and Dyer (2016)
Drosophila neotestacea 1	*gl*	KX637880.1	Pieper and Dyer (2016)
Drosophila neotestacea 2	*gl*	KX637881.1	Pieper and Dyer (2016)
Drosophila neotestacea 3	*gl*	KX637882.1	Pieper and Dyer (2016)
Drosophila neotestacea 4	*gl*	KX637883.1	Pieper and Dyer (2016)
Drosophila neotestacea 5	*gl*	KX637884.1	Pieper and Dyer (2016)
Drosophila neotestacea 6	*gl*	KX637885.1	Pieper and Dyer (2016)
Drosophila neotestacea 7	*gl*	KX637886.1	Pieper and Dyer (2016)
Drosophila neotestacea 8	*gl*	KX637887.1	Pieper and Dyer (2016)
Drosophila neotestacea 9	*gl*	KX637888.1	Pieper and Dyer (2016)
Drosophila neotestacea 10	*gl*	KX637889.1	Pieper and Dyer (2016)
***Drosophila testacea* VC42 (X^{SR})**	*gl*	-	This study
Drosophila testacea 36 (X^{ST})	*gl*	-	This study
Drosophila testacea 37 (X^{ST})	*gl*	-	This study
Drosophila testacea 38 (X^{ST})	*gl*	-	This study
***Drosophila testacea* 39 (X^{SR})**	*gl*	-	This study
Drosophila testacea 43 (X^{ST})	*gl*	-	This study
Drosophila testacea 47 (X^{ST})	*gl*	-	This study
Drosophila testacea 48 (X^{ST})	*gl*	-	This study
Drosophila testacea 52 (X^{ST})	*gl*	-	This study
Drosophila testacea 57 (X^{ST})	*gl*	-	This study
Drosophila testacea 58 (X^{ST})	*gl*	-	This study
Drosophila testacea 60 (X^{ST})	*gl*	-	This study
Drosophila testacea 61 (X^{ST})	*gl*	-	This study
***Drosophila testacea* 63 (X^{SR})**	*gl*	-	This study
Drosophila testacea 70 (X^{ST})	*gl*	-	This study
***Drosophila testacea* 71 (X^{SR})**	*gl*	-	This study

Supplemental Table 3-4. Individuals from the *Drosophila* testacea species group used for phylogenetic analysis of the X-linked genes *skpA* and *pgd*.

Individual in phylogeny	Gene	Accession	Reference
Drosophila putrida 01	*skpA*	HQ685127.1	Dyer *et al.* (2011)
	pgd	HQ685537.1	Dyer *et al.* (2011)
Drosophila putrida 02	*skpA*	HQ685128.1	Dyer *et al.* (2011)
	pgd	HQ685538.1	Dyer *et al.* (2011)
Drosophila putrida 03	*skpA*	HQ685129.1	Dyer *et al.* (2011)
	pgd	HQ685539.1	Dyer *et al.* (2011)
Drosophila putrida 04	*skpA*	HQ685130.1	Dyer *et al.* (2011)
	pgd	HQ685540.1	Dyer *et al.* (2011)
Drosophila putrida 05	*skpA*	HQ685131.1	Dyer *et al.* (2011)
	pgd	HQ685541.1	Dyer *et al.* (2011)
Drosophila putrida 06	*skpA*	HQ685132.1	Dyer *et al.* (2011)
	pgd	HQ685542.1	Dyer *et al.* (2011)
Drosophila putrida 07	*skpA*	HQ685133.1	Dyer *et al.* (2011)
	pgd	HQ685543.1	Dyer *et al.* (2011)
Drosophila putrida 08	*skpA*	HQ685134.1	Dyer *et al.* (2011)
	pgd	HQ685544.1	Dyer *et al.* (2011)
Drosophila putrida 09	*skpA*	HQ685135.1	Dyer *et al.* (2011)
	pgd	HQ685545.1	Dyer *et al.* (2011)
Drosophila putrida 10	*skpA*	HQ685136.1	Dyer *et al.* (2011)
	pgd	HQ685546.1	Dyer *et al.* (2011)
Drosophila orientacea 01	*skpA*	HQ685137.1	Dyer *et al.* (2011)
	pgd	HQ685547.1	Dyer *et al.* (2011)
Drosophila orientacea 02	*skpA*	HQ685138.1	Dyer *et al.* (2011)
	pgd	HQ685548.1	Dyer *et al.* (2011)
Drosophila orientacea 03	*skpA*	HQ685139.1	Dyer *et al.* (2011)
	pgd	HQ685549.1	Dyer *et al.* (2011)
Drosophila orientacea 04	*skpA*	HQ685140.1	Dyer *et al.* (2011)
	pgd	HQ685550.1	Dyer *et al.* (2011)
Drosophila orientacea 05	*skpA*	HQ685141.1	Dyer *et al.* (2011)
	pgd	HQ685551.1	Dyer *et al.* (2011)
Drosophila orientacea 06	*skpA*	HQ685143.1	Dyer *et al.* (2011)
	pgd	HQ685552.1	Dyer *et al.* (2011)
Drosophila orientacea 07	*skpA*	HQ685144.1	Dyer *et al.* (2011)
	pgd	HQ685553.1	Dyer *et al.* (2011)
Drosophila orientacea 08	*skpA*	HQ685145.1	Dyer *et al.* (2011)

Table 3-3. *continued.*

Individual in phylogeny	Gene	Accession	Reference
Drosophila orientacea 08	*pgd*	HQ685554.1	Dyer *et al.* (2011)
Drosophila orientacea 09	*skpA*	HQ685146.1	Dyer *et al.* (2011)
	pgd	HQ685555.1	Dyer *et al.* (2011)
Drosophila orientacea 10	*skpA*	HQ685142.1	Dyer *et al.* (2011)
	pgd	HQ685556.1	Dyer *et al.* (2011)
Drosophila neotestacea 01	*skpA*	HQ685156.1	Dyer *et al.* (2011)
	pgd	HQ685557.1	Dyer *et al.* (2011)
Drosophila neotestacea 02	*skpA*	HQ685157.1	Dyer *et al.* (2011)
	pgd	HQ685558.1	Dyer *et al.* (2011)
Drosophila neotestacea 03	*skpA*	HQ685158.1	Dyer *et al.* (2011)
	pgd	HQ685559.1	Dyer *et al.* (2011)
Drosophila neotestacea 04	*skpA*	HQ685159.1	Dyer *et al.* (2011)
	pgd	HQ685560.1	Dyer *et al.* (2011)
Drosophila neotestacea 05	*skpA*	HQ685160.1	Dyer *et al.* (2011)
	pgd	HQ685561.1	Dyer *et al.* (2011)
Drosophila neotestacea 06	*skpA*	HQ685161.1	Dyer *et al.* (2011)
	pgd	HQ685562.1	Dyer *et al.* (2011)
Drosophila neotestacea 07	*skpA*	HQ685162.1	Dyer *et al.* (2011)
	pgd	HQ685563.1	Dyer *et al.* (2011)
Drosophila neotestacea 08	*skpA*	HQ685163.1	Dyer *et al.* (2011)
	pgd	HQ685564.1	Dyer *et al.* (2011)
Drosophila neotestacea 09	*skpA*	HQ685164.1	Dyer *et al.* (2011)
	pgd	HQ685565.1	Dyer *et al.* (2011)
Drosophila neotestacea 10	*skpA*	HQ685165.1	Dyer *et al.* (2011)
	pgd	HQ685566.1	Dyer *et al.* (2011)
***Drosophila testacea* VC42 (X^{SR})**	*skpA*	-	This study
	pgd	-	This study
Drosophila testacea 36 (X^{ST})	*skpA*	-	This study
	pgd	-	This study
Drosophila testacea 37 (X^{ST})	*skpA*	-	This study
	pgd	-	This study
Drosophila testacea 38 (X^{ST})	*skpA*	-	This study
	pgd	-	This study
***Drosophila testacea* 39 (X^{SR})**	*skpA*	-	This study
	pgd	-	This study
Drosophila testacea 43 (X^{ST})	*skpA*	-	This study

Table 3-3. *continued.*

Individual in phylogeny	Gene	Accession	Reference
Drosophila testacea 43 (X^{ST})	*pgd*	-	This study
Drosophila testacea 47 (X^{ST})	*skpA*	-	This study
	pgd	-	This study
Drosophila testacea 48 (X^{ST})	*skpA*	-	This study
	pgd	-	This study
Drosophila testacea 52 (X^{ST})	*skpA*	-	This study
	pgd	-	This study
Drosophila testacea 57 (X^{ST})	*skpA*	-	This study
	pgd	-	This study
Drosophila testacea 58 (X^{ST})	*skpA*	-	This study
	pgd	-	This study
Drosophila testacea 60 (X^{ST})	*skpA*	-	This study
	pgd	-	This study
Drosophila testacea 61 (X^{ST})	*skpA*	-	This study
	pgd	-	This study
***Drosophila testacea* 63 (X^{SR})**	*skpA*	-	This study
	pgd	-	This study
Drosophila testacea 70 (X^{ST})	*skpA*	-	This study
	pgd	-	This study
***Drosophila testacea* 71 (X^{SR})**	*skpA*	-	This study
	pgd	-	This study

Chapter 4 – References

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