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**Autosomal suppression and fitness costs of an old driving X chromosome in *Drosophila*
*testacea***

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Running title: Maintenance of an old driving X chromosome

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Abstract

Driving X chromosomes (X^D s) are meiotic drivers that bias their own transmission through males by killing Y-bearing gametes. These chromosomes can in theory spread rapidly in populations and cause extinction, but many are found as balanced polymorphisms or as “cryptic” X^D s shut down by drive suppressors. The relative likelihood of these outcomes, as well as the evolutionary pathways through which they come about, are not well-understood. An X^D was recently discovered in the mycophagous fly, *Drosophila testacea*, presenting the opportunity to compare this X^D with the well-studied X^D of its sister species, *Drosophila neotestacea*. Comparing features of independently evolved X^D s in young sister species is a promising avenue towards understanding how X^D s and their counteracting forces change over time. In contrast to the X^D of *D. neotestacea*, we find that the X^D of *D. testacea* is old, with its origin predating the radiation of three species: *D. testacea*, *D. neotestacea*, and their shared sister species, *Drosophila orientacea*. Motivated by the suggestion that older X^D s should be more deleterious to carriers, we assessed the effect of the X^D on both male and female fertility. Unlike what is known from *D. neotestacea*, we found a strong fitness cost in females homozygous for the X^D in *D. testacea*: a large proportion of homozygous females failed to produce offspring after being housed with males for several days. Our male fertility experiments show that while X^D male fertility is lower under sperm depleting conditions, X^D males have comparable fertility to males carrying a standard X chromosome under a free mating regime, which may better approximate conditions in wild populations of *D. testacea*. Lastly, we demonstrate the presence of autosomal suppression of X chromosome drive. Our results provide support for a model of X^D evolution where the dynamics of young X^D s are governed by fitness consequences in males, whereas in older X^D systems, both suppression and fitness consequences in females likely supersede male fitness costs.

Keywords: selfish genetic elements, genetic conflict, genomic conflict, gene drive, gamete-killing, X chromosome, sex ratio, segregation distortion, meiotic drive, *Drosophila*

Introduction

When an individual carries two alleles at a locus, Mendelian segregation ensures that both have a fair chance of being equally represented among the offspring. Although most genes follow the Mendelian expectation, there is a growing number of examples where genes do not, contriving instead to bias segregation in their favor (Lindholm et al., 2016). Employing diverse strategies, these genes – known as meiotic drivers – can spread rapidly in populations because of their transmission advantage, even if they carry a negative fitness cost. Moreover, meiotic drivers spread at the direct expense of their meiotic homologue, thus establishing conflict within the genome. Such intragenomic conflict can lead to evolutionary arms races over fundamental processes, such as meiosis and gametogenesis, making meiotic drivers a strong evolutionary force (Werren, 2011).

Driving X chromosomes (X^D s) are X-linked meiotic drivers that eliminate Y-bearing gametes in heterogametic males (XY). This ensures the near-exclusive transmission of the X^D , and also causes carrier males (X^D males) to produce almost all female offspring (Jaenike, 2001; Helleu et al., 2015). Unlike meiotic drivers on autosomes, whose biased transmission can easily go undetected, strong sex ratio distortion provides an obvious phenotype that has facilitated the discovery of numerous X^D s in a diversity of organisms, including plants, mammals, and insects (Jaenike, 2001).

Typical of meiotic drivers, X^D s are predicted to spread through populations once they arise. A consequence unique to sex ratio distorters, however, is that their spread pushes the population sex ratio increasingly towards one sex, threatening the species with extinction if the opposite sex becomes sufficiently rare (Gershenson, 1928; Hamilton, 1967). Nevertheless, extinction is not a necessary outcome. This is clearly demonstrated by the fact that several of the known X^D s in flies persist as balanced polymorphisms that can be stable in frequency across decades (Pinzone & Dyer, 2013; Price et al., 2014; Paczolt et al., 2017).

Polymorphism for an X^D can be explained by the presence of two non-mutually exclusive forces that counteract their spread (Jaenike, 2001). The first is natural selection against X^D carriers. Because X^D s eliminate Y-bearing sperm, X^D males produce fewer sperm relative to

males carrying the standard (*i.e.* non-driving) X chromosome (X^{ST} males) (Policansky & Ellison, 1970). This translates to a fitness cost when the male mating rate is high, such as when males are rare or if females frequently re-mate, because X^D males typically deplete their sperm faster than X^{ST} males (Jaenike, 1996; Atlan et al., 2004; Pinzone & Dyer, 2013; Unckless et al., 2015; but see Meade et al., 2019). If females do re-mate, the mating outcome of X^D males can be further hampered by post-copulatory mechanisms, such as cryptic female choice and sperm competition (Haig & Bergstrom, 1995; Angelard et al., 2008). Indeed, X^D males from several species are demonstrably poor sperm competitors against X^{ST} males, likely in part because they transfer fewer sperm (Wilkinson & Fry, 2001; Atlan et al., 2004; Angelard et al., 2008; Wilkinson et al., 2006; Price et al., 2008).

Selection against X^D carriers can also occur independently of gamete-killing if deleterious alleles become linked to the driving locus (or loci). Most of the known X^D s are characterized by inversions that link together loci necessary for the expression of gamete-killing (James & Jaenike, 1990; Jaenike, 2001). Regions of low recombination such as inversions, however, can accumulate deleterious mutations (Gordo & Charlesworth, 2001), leading to selection against X^D carriers. In contrast to faster sperm depletion and poor sperm competitive ability, which are intrinsic consequences of gamete-killing in X^D males (Zanders & Unckless, 2019), selection against linked alleles can also affect females, especially females homozygous for the X^D ($X^D X^D$ females) (Wallace, 1948; Curtsinger & Feldman, 1980; Beckenbach, 1996). Moreover, this form of selection against X^D carriers should increase in severity over time as deleterious mutations accumulate (Jaenike, 1996; Dyer et al., 2007).

The second force counteracting the spread of an X^D is suppression. Suppressors on Y chromosomes or autosomes can evolve that prevent gamete-killing when paired with an X^D (Helleu et al., 2015). The majority of species that are polymorphic for X^D s are also polymorphic for suppressors, suggesting that the evolution of suppressors is a common route to balancing an X^D (Jaenike, 2001). Although suppression is common, polymorphism for an X^D can occur in their absence, as illustrated by two well-studied cases in *Drosophila* flies, *Drosophila pseudoobscura* and *Drosophila neotestacea*. Extensive searches in these species have revealed

no suppressors, yet their X^D s persist in natural populations as balanced polymorphisms (Pinzone & Dyer, 2013; Price et al., 2014).

Whether mediated by selection against X^D carriers or through suppression (or both), polymorphism for an X^D is likely transient over evolutionary timescales. For instance, the deleterious effects of an X^D may eventually become so severe that the X^D is lost (Dyer et al., 2007). Or, in cases where suppressors are present, an X^D could overcome suppression by evolving additional gamete-killing loci, and then spread to fixation to cause extinction. Still another possibility is that a suppressor spreads to fixation, masking the X^D entirely (Helleu et al., 2015). Here, if the X^D also fixes, the species will carry a “cryptic” X^D (Tao et al., 2001; Tao et al., 2007a,b). Despite the important evolutionary consequences of these various outcomes, their relative likelihoods are not well-understood, nor are the evolutionary pathways through which they come about (Lindholm et al., 2016). Understanding these dynamics has recently taken on added significance, as X^D s and other meiotic drivers offer naturally occurring analogues of synthetic drive systems currently being developed to control disease vectors and pests (Lindholm et al., 2016; Manser et al., 2019).

The testacea species group of the subgenus *Drosophila* provides an excellent system for exploring questions related to the ecological and evolutionary dynamics of X^D s. This group of mushroom-feeding flies has four known species: *D. putrida*, *D. neotestacea*, *D. orientacea*, and *D. testacea*. The latter three comprise a young, morphocryptic radiation with incomplete reproductive isolation (Grimaldi et al., 1992), and exceptionally, all three harbour X^D s (James & Jaenike, 1990; Pieper & Dyer, 2016; Keais et al., 2017). Current evidence suggests that at least two of these X^D s – those of *D. neotestacea* and *D. testacea* – are independently evolved (Keais et al., 2017); there is not yet published information about the X^D of *D. orientacea*. Species of the testacea group have similar ecologies and share a life history that is experimentally tractable under ecologically relevant conditions (James & Jaenike, 1990), permitting comparative studies into the ecological dynamics of independently evolved X^D s.

In this study, we take steps towards understanding the evolution and persistence of the X^D in *D. testacea*, which was only recently discovered (Keais et al., 2017), and we report the

presence of the X^D in a second European population. To investigate the evolutionary history of the X^D in *D. testacea*, we use phylogenetic analysis of X-linked gene sequences from X^D and X^{ST} *D. testacea* individuals, and individuals from the other known members of the testacea species group. As a start towards assessing how selection might operate against the X^D in this species, we perform fitness experiments in both male and female flies. Additionally, we characterize the genetic nature of the suppressors observed segregating in a population from Switzerland.

Methods

Flies

Three laboratory populations of *D. testacea* were used for the experiments in this study. The first population was established from several wild-caught flies collected in August 2012 near St. Sulpice, Vaud, Switzerland (St. Sulpice 2012 stock). A second population of *D. testacea* was founded using wild-caught flies collected in St. Sulpice in July 2016 (St. Sulpice 2016 stock). Lastly, we maintain a population of *D. testacea* that only contains the standard X chromosome (non-driving stock), established using flies from our St. Sulpice 2012 stock. In addition to our populations of *D. testacea*, we maintain an X^D chromosome according to the crossing scheme outlined in James & Jaenike (1990). 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*).

Phylogenetic analysis

To investigate the evolutionary history of the X^D in *D. testacea*, we used phylogenetic analysis to compare three X-linked genes (*pgd*, *Rpl36*, and *skpA*), an autosomal gene (*gl*), and a mitochondrial gene (*COI*) from the four known members of the testacea species group. Sequences for the gene *skpA* were previously generated from a sample of wild-caught *D. testacea* males collected in St. Sulpice, Switzerland, in July 2016 (Keais et al., 2017). We chose 11 XY males and three X^D Y males from this sample for further sequencing of *pgd*, *Rpl36*, *gl*, and

142 *COI*. In addition, we sequenced all five loci in an $X^D Y$ *D. testacea* male from our lab X^D line, and
143 an $X^{ST} Y$ male from a *D. testacea* line from Finland. To expand our sample of *D. testacea* X
144 chromosomes, we screened a sample of 27 *D. testacea* males collected in 2014 from Liblice,
145 Czech Republic for the presence of the X^D using *skpA* as a diagnostic marker (Keais *et al.*, 2017),
146 and subsequently sequenced the remaining loci in four of these males. For the other members
147 of the testacea species group, we sequenced all five loci in two *D. putrida* males, three *D.*
148 *neotestacea* males, and five *D. orientacea* males. The X-linked genes were successfully
149 sequenced in all individuals. However, not all individuals were successfully sequenced for *COI*
150 and *gl*; see **Table S1** for details.

151 The following thermal cycling conditions were used for PCR: 95 °C for 3 min, then
152 32 cycles of 94 °C for 1 min and 54 °C for 1 min, followed by a final 10 min at 72 °C. Primers
153 used for PCR can be found in **Table S2**. PCR amplicons were Sanger-sequenced (Sequetech,
154 USA), and sequence handling and analysis was performed using Geneious (version 5.1.7)
155 (<https://www.geneious.com>). For the autosomal gene *gl*, heterozygous sites were manually
156 identified on chromatograms, and converted to degenerate base symbols (IUPAC nucleotide
157 codes) prior to alignment.

158 Maximum likelihood phylogenetic analysis using PhyML 3.0 (Guindon *et al.*, 2010) was
159 performed on a concatenated gene alignment of the three X-linked gene sequences (1407
160 nucleotides), and on alignments of *gl* and *COI* sequences (538 and 658 nucleotides,
161 respectively). The best substitution model was determined for each nucleotide alignment using
162 Smart Model Select (Lefort *et al.*, 2017). SH-like approximate likelihood ratio test scores were
163 calculated for branch support.

164 **Suppressors**

166 The presence of X^D suppression has previously been reported in *D. testacea* (Keais *et al.*,
167 2017). Two of 37 wild-caught males collected in St. Sulpice, Switzerland in 2016 carried an X^D
168 alongside a suppressor, but whether suppression was autosomal or Y-linked was not reported.
169 We subsequently introgressed the Y chromosomes of both suppressed wild-caught males into
170 our non-driving laboratory genetic background for four generations, yielding two Y replacement

lines (Y^2 and Y^{63}). We then paired both Y chromosomes with an X^D by crossing $X^{ST}Y^2$ and $X^{ST}Y^{63}$ males to $X^D X^{ST}$ females. This cross generates males that carry a putatively suppressing Y chromosome (Y^2 or Y^{63}) and either the X^D or X^{ST} . These males were then mated to $X^{ST} X^{ST}$ females and their offspring sex ratios were scored. Strong female bias in the offspring of these males would preclude Y-linked suppression.

To test whether autosomal suppressors of drive are segregating in our St. Sulpice 2016 line we first set up four vials, each containing a single male from the St. Sulpice 2016 line and three $X^D X^{ST}$ females (derived from the St. Sulpice 2012 line, which has no suppressors). We then placed 31 of the emerging sons (aged 3-4 days old) individually in vials with two females (aged 4-7 day old) from our non-driving line ($X^{ST} X^{ST}$ with no suppressors). All flies were moved to new vials after four days. After a further four days, the males were removed for genotyping (see below), and the females were moved to a new vial. Females were placed in a fourth and final vial after four days, where they were kept for a final four days before being removed. The offspring emerging from all vials were sexed to screen for males that produce normal (50:50) sex ratios but also carry an X^D . The total number of offspring produced by each male was also recorded to estimate their fertility.

DNA extractions were performed on the males using PrepMan™ Ultra and their genotypes were determined using high-resolution melt-curve analysis (HRM). To develop HRM for genotyping, we targeted a 180bp region of the *skpA* gene that contains four nucleotide substitutions between the X^{ST} and X^D copies using the qPCR primers *skpA*-M-F and *skpA*-M-R (**Table S2**). Employing HRM using these primers yields significantly distinct melt temperature profiles for the two chromosome types, allowing for the accurate prediction of genotype based on melt temperature (**Figure S1**)(linear regression, $R^2 = 0.916$, $F_{1,39} = 425.5$, $P < 2.2e-16$). As a control, we 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 (**Table S2**). As expected, HRM using these primers yields melt temperature profiles that do not significantly differ between X chromosome types (**Figure S2**)(linear regression, $R^2 = 0.0640$, $F_{1,39} = 0.0487$, $P = 0.8265$). This protocol was developed using DNA from 22 X^D males and 19 X^{ST} males whose genotype had been pre-determined using either *skpA* gene sequences (12 X^D and 10 X^{ST}) or offspring sex ratios (10 X^D

and X^{ST}). Linear regressions were performed in R (version 3.4.1) using RStudio (<https://www.rstudio.com>) (R Core Team, 2017).

We used the following reaction mixture for HRM: 2 μ l of template DNA, 8 μ l of EvaGreen mastermix, 4.08 μ l of dH₂O, and 0.96 μ l of each 5 μ M forward and reverse primers (16 μ l reactions). The qPCR conditions were: 95 $^{\circ}$ C for 10m, followed by [95 $^{\circ}$ C for 15s, 60 $^{\circ}$ C for 45s] x 35, then 94 $^{\circ}$ C for 10m. This is immediately followed by HRM, which involves a temperature increase of 0.1 $^{\circ}$ C per cycle from 65 $^{\circ}$ C to 95 $^{\circ}$ C, at 5s per cycle.

48-hour sperm depletion assay

We performed a sperm depletion assay to test for fertility differences between X^DY and $X^{ST}Y$ males upon multiple matings. In order to generate a large sample of age-matched X^DY and $X^{ST}Y$ males, 30 X^DX^{ST} females were mated to 20 $X^{ST}Y$ males in a glass stock jar for 10 days. Eclosing males were collected over a two-day period, and 20 of these were then randomly chosen and housed individually in vials for seven days. Virgin females were collected prior-to and throughout the same period, housed in groups of five, and aged 8-12 days.

To commence the assay, 20 groups of five virgin females were transferred without anesthesia to vials with a standardized amount of *Drosophila* media and mushroom. A single male was then added to each vial without anesthesia. After 24 hours, each male was aspirated to a second vial containing another group of five virgin 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 PrepmanTM Ultra (Applied Biosystems) for genotyping using HRM. The females were transferred to new vials every two days, to a total of five vials per group of females. After two days in the fifth and final vial, the females were discarded. Flies eclosing from all vials were counted and sexed.

Homozygous female fertility

As a first step towards assessing female fertility effects associated with carrying an X^D , we generated both X^DX^D and X^DX^{ST} females (daughters of an X^DY male crossed with X^DX^{ST} females) and mated 80 of these females *en masse* to $X^{ST}Y$ males in pools of 10 females and 10

males. After seven days, 64 females (some females died in the mating vials) were transferred 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 inspected for the presence of eclosed offspring. At the end of the experiment, females were preserved in 95% ethanol for future genotyping (see below).

As a second step examining $X^D X^D$ female fertility, we again crossed $X^D X^{ST}$ females with an $X^D Y$ male to generate both $X^D X^D$ and $X^D X^{ST}$ females. Thirty-one of the emerging females ($X^D X^D$ or $X^D X^{ST}$) were aged 5-7 days and then housed two per vial with a single $X^{ST} Y$ male for seven days. Females were then placed in separate vials and transitioned to new vials every five days to a total of three vials per female. Females were housed in the final vial for 5 days and then removed for genotyping. All emerging offspring were sexed and counted.

To determine the genotype of each female from the above two experiments, their DNA was extracted using PrepmanTM Ultra (Applied Biosystems), and the *skpA* marker gene was PCR-amplified and Sanger-sequenced (Sequetech, USA).

Results

Frequency of the X^D in a second European population

Using a diagnostic marker, we found that two of 27 males collected in Liblice, Czech Republic in 2014 carried an X^D .

The X^D is an old polymorphism

Using phylogenetic analysis of X-linked gene sequences, we find that the two X chromosome types in *D. testacea* are highly divergent. In fact, our results suggest that the X^D of *D. testacea* diverged prior to the radiation of *D. orientacea*, *D. neotestacea*, and *D. testacea* (**Figure 1**). Our sample of X^D chromosomes, which includes chromosomes from the Czech Republic (**cz**, **Figure 1**) and Switzerland (**sw**, **Figure 1**), are identical across all three genes, and branch outside the radiation of *D. orientacea*, *D. neotestacea*, and *D. testacea* X^{ST} sequences. In

contrast to the absence of sequence variation on X^D chromosomes, X^{ST} chromosomes from *D. testacea* are variable, and show high similarity to *D. neotestacea*. Although the location of the drive locus (or loci) in *D. testacea* is unknown, the X^D versions of *pgd* and *skpA* are both perfectly associated with the sex-ratio distortion phenotype; thus, we infer that the region containing these genes also contains the drive locus (or loci). As expected, autosomal and mitochondrial sequences from X^D and X^{ST} males are indistinguishable, as depicted by their close grouping on the *gl* and *COI* phylogenies (**Figure S3 and Figure S4**).

X drive suppression is autosomal

Males carrying an X^D 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 (**Table S3**).

Crosses testing for the presence of X^D suppressors revealed that autosomal suppressors are segregating in our St. Sulpice 2016 stock (**Figure 2**). Our genotyping results showed that 10 of 31 males carried an X^{ST} chromosome. Nine of the 10 X^{ST} males produced offspring that ranged from 51-54% females (**Figure 2**, purple bars), while the tenth did not produce any offspring (not shown). The remaining 21 males were genotyped as X^D , and 14 of these males produced highly female-biased offspring, ranging from 85-100% females (**Figure 2**, yellow bars). However, five X^D males produced offspring sex ratios that were either indistinguishable from X^{ST} males (crosses 28 and 30), or slightly female-biased (crosses 12, 22, and 25) (**Figure 2**, grey bars), strongly suggesting they carry an X^D suppressor. The mean offspring sex ratio of these five X^D males is not significantly different from the mean sex ratio of the X^{ST} males (Welch's t-test, $t_{4.36} = 2.51$, $P = 0.0612$) but deviates significantly from the mean sex ratio of the non-suppressed X^D males (Welch's t-test, $t_{5.00} = 8.67$, $P = 0.0003$). Data from the final two males that were genotyped as X^D are not shown, as one produced only five offspring, while another was paired with a female that died prematurely without producing offspring.

Ten of the 21 X^D males had the same father, and four of these (**Figure 2**, crosses 22, 25, 28, and 30) produced sex ratios that suggest the presence of a suppressor. This is consistent with their father being heterozygous for a dominant autosomal suppressor allele. Nine X^D males

had a different father, and only one of them (**Figure 2**, cross 12) produced an offspring sex ratio suggestive of a suppressor. The fact that not all male siblings showed evidence of suppression also rules out Y chromosome suppression.

Fertility differences between X^D and X^{ST} males

The crosses we performed to screen for suppressors in our St. Sulpice 2016 stock (see above) also allowed us to compare the fertility of X^D and X^{ST} males under a free mating condition. There was a wide range of total offspring produced. While X^D males produced slightly more offspring, there was no significant difference in offspring production between genotypes (**Figure 3A**; Welch's t-tests: X^DY vs. $X^{ST}Y$, $t_{19.86} = 0.95$, $P = 0.3764$; X^DY vs. X^DY suppressed, $t_{7.85} = -0.68$, $P = 0.5184$; $X^{ST}Y$ vs. X^DY suppressed, $t_{8.96} = -1.37$, $P = 0.2044$). In contrast, results from the 48-hour sperm depletion assay show that X^D males have severely reduced fertility compared to X^{ST} males under sperm depleting conditions (**Figure 3B**). On average, X^D males ($N = 7$) produced significantly fewer offspring than X^{ST} males ($N = 8$) in our sperm depletion assay (Welch's t-test, $t_{7.37} = 3.20$, $P = 0.0141$). Four males (three X^D males and one X^{ST} male) produced no offspring and were excluded from the analysis.

Homozygous female fertility

Across both experiments assessing X^DX^D female fertility, a significantly higher proportion of X^DX^D females did not produce offspring relative to X^DX^{ST} females (**Figure 4A**; Z-test, $\chi^2_1 = 10.99$, $P = 0.0005$). Only ten of 42 X^DX^D females from the first experiment (~23.8%), and five of 18 from the second experiment (~27.8), produced offspring. In contrast, 11 of 15 (~73.3%) and 6 of 11 (~54.5) X^DX^{ST} females produced offspring in the first and second experiments, respectively. Despite the majority of X^DX^D females failing to produce offspring, the subset of X^DX^D females that did produce offspring produced a similar amount compared to the subset of X^DX^{ST} females that produced offspring (**Figure 4B**; Welch's t-test, $t_{8.93} = 1.55$, $P = 0.1568$). We were unable to successfully genotype nine females (seven and two females from the first and second experiment, respectively).

Discussion

Driving X chromosomes (X^D s) that bias their own transmission in males by incapacitating or destroying Y-bearing sperm are found in numerous species. X^D s can occur as stable polymorphisms, can become “cryptic” due to the fixation of genes that suppress their gamete-killing phenotype, or can spread to fixation and cause extinction due to a lack of males. The conditions required to reach these separate outcomes, however, are not well understood. Here, we further develop the testacea species group as a model for studying the ecological and evolutionary dynamics of X^D s by examining the evolutionary history of the X^D of *D. testacea* using phylogenetics, and by identifying factors that have allowed for the persistence of the X^D in this species.

Phylogenetic analysis of X-linked sequences has revealed a striking evolutionary history of the X^D in *D. testacea*. This X^D appears to have diverged before the split of *D. testacea*, *D. neotestacea*, and *D. orientacea*. Interestingly, both *D. neotestacea* and *D. orientacea* also have X^D chromosomes (James & Jaenike, 1990; Pieper & Dyer, 2016), raising the question of whether these X^D s share a common origin or mechanism (Pieper et al., 2018). Work by Pieper & Dyer (2016) shows that the X^D of *D. neotestacea* evolved from a *D. neotestacea* X^{ST} chromosome, which, in conjunction with our present results, demonstrates that the X^D s of *D. neotestacea* and *D. testacea* are distinct. The relationship between the X^D s of *D. orientacea* and *D. testacea*, on the other hand, remains to be determined (there is no published *D. orientacea* X^D sequence yet). Regardless, the evolution of X^D s is clearly common in this young radiation. Pieper & Dyer (2016) estimate that the X^D of *D. neotestacea* arose 330,000-500,000 years ago. Using the same dating technique (a substitution rate of 1.7% per million years; Caccone et al., 1988), we tentatively date the divergence of the X^D in *D. testacea* at ~920,000 years ago. Although we use these dates with caution, intriguingly the interval between the origin of these two X^D s is close to the estimated frequency of new putative autosomal meiotic drivers in *Arabidopsis* (arising every ~200,000-300,000 years) (Corbett-Detig et al., 2019), and at least two X^D s have evolved in *Drosophila simulans* in the last ~800,000 years (Kingan et al., 2010; Fouvry et al., 2011).

We sought to better understand how the X^D in *D. testacea* has persisted over evolutionary timescales by examining potential fitness consequences in X^D carriers. We have

found that X^D males in *D. testacea* suffer a large decrease in fertility relative to X^{ST} males when males are made to mate multiple times, mirroring results from several other species (Jaenike, 1996; Atlan et al., 2004; Pinzone & Dyer, 2013; Unckless et al., 2015). However, we also find that this fertility difference is not apparent when males are allowed to freely mate with females. Because the X^D is at very low frequency in the wild (actively expressed in only 5% of males), its presence likely does not have an appreciable impact on the population sex ratio. In turn, males in the wild may not mate frequently enough to deplete their sperm stores. Therefore, although we recognize the possibility of subtler fitness consequences in X^D males (Angelard et al., 2008), we suspect that male fertility plays only a minor role in explaining the contemporary frequency of the X^D in *D. testacea*.

In addition to male fertility, we examined the fertility of females that are hetero- and homozygous for the X^D ($X^D X^{ST}$ and $X^D X^D$, respectively). When given ample time to mate with males, we found that a far greater proportion of $X^D X^D$ females did not produce offspring compared to $X^D X^{ST}$ females. There are at least two explanations for why a greater number of $X^D X^D$ failed to produce offspring. First, it is possible that a higher fraction of $X^D X^D$ females simply did not mate, which would imply they have an impaired ability to obtain successful matings. This effect has been shown for X^D males of *D. subobscura* (Verspoor et al., 2016). However, it is also possible that alleles have accrued on the X^D that negatively impact fertility in females, and whose effects are more likely to be expressed when homozygous. Regardless of the cause, our results point to a relatively strong fitness cost for $X^D X^D$ females in *D. testacea*. We are aware of four other species of *Drosophila* where $X^D X^D$ fitness has been examined. Curtsinger & Feldman (1980) found viability and fertility selection occurring against $X^D X^D$ females in *D. pseudoobscura*, and Dyer et al. (2007) found factors on the X^D in *D. recens* that cause complete sterility in females when homozygous. No fitness costs were found for $X^D X^D$ females in *D. quinaria* (Jaenike, 1996), or, notably, *D. neotestacea* (James, 1992). Although we do not observe complete sterility of $X^D X^D$ females, which would limit the frequency obtainable for an X^D to 25% (Dyer et al., 2007), we infer that $X^D X^D$ fertility is nevertheless a large contributor to the observed frequency of the X^D in *D. testacea*, as well as its stability as a polymorphism due to negative frequency-dependent selection against $X^D X^D$ females (Curtsinger & Feldman, 1980).

In addition to fitness costs in X^D carriers, suppression is an important counteracting force in X^D systems. We have shown here that X^D suppression previously reported in *D. testacea* is autosomal rather than Y-linked. Vaz & Carvalho (2004) showed that for a neutral autosomal suppressor to exist as a polymorphism alongside an X^D polymorphism, X^D males must have a fitness that is greater than 0.3 but less than 1 relative to X^{ST} males. This condition is likely satisfied in the *D. testacea* system. The Vaz & Carvalho (2004) model also requires that $X^D X^{ST}$ females have a greater fitness than $X^{ST} X^{ST}$ females, a comparison that we did not investigate in this paper, but one that should be considered in future work. Because the X^D was found in the Swiss population in samples taken four years apart (Keais et al., 2017), and has now been found at a similar, low frequency in the Czech Republic, the X^D may be at a non-local equilibrium frequency, and this may be contingent on a polymorphism for the autosomal suppressor (Vaz & Carvalho, 2004).

Overall, the results presented here imply a starkly different regime of counteracting forces in the *D. testacea* X^D system compared to that of *D. neotestacea*. In contrast to the low level of drive expression in *D. testacea*, the X^D in *D. neotestacea* can reach very high frequencies – in some populations, over 40% of X chromosomes are X^D (Pinzone & Dyer, 2013) – and all X^D s are expressed, as there are no known suppressors in *D. neotestacea*. Such high X^D frequency has an impact on *D. neotestacea* population sex ratios (James & Jaenike, 1990; Pinzone & Dyer, 2013). Fertility selection resulting from sperm depletion of X^D males – both because of the population sex ratio, and because of female remating – is therefore thought to be a key factor in limiting the spread of the X^D in *D. neotestacea* (Pinzone & Dyer, 2013). Unlike in *D. testacea*, $X^D X^D$ females of *D. neotestacea* do not appear to have reduced fitness (James, 1992). This reflects a difference in X^D chromosomal organization between the two species, itself a function of X^D age: Recombination events between the X^D and X^{ST} chromosomes have occurred recently in *D. neotestacea*, limiting the accumulation of deleterious alleles on the X^D (Pieper & Dyer, 2016), whereas our phylogeny of X-linked genes implies complete suppression of recombination between the X^D and X^{ST} in *D. testacea*.

The above observations comparing X^D s in *D. testacea* and *D. neotestacea* are broadly consistent with the model proposed by Jaenike (1996) regarding the stages in the evolution of

an X^D , where younger X^D s – if stabilized at all – are limited by the intrinsic costs of gamete-killing, whereas older X^D s are limited by deleterious costs to females as a result of an increasing and irreversible genetic load on the X^D , and by co-evolution with suppressors. By comparing independently evolved X^D s at different stages in their evolution, and found in young species with similar ecologies, we begin to provide a clearer, finer-scale picture of how X^D s and their counteracting forces change over time.

Data Accessibility

The data associated with this manuscript are available on Dryad (<https://doi.org/10.5061/dryad.7h44j0zqw>). Nucleotide sequences generated for this study have been deposited on GenBank under accession numbers KY-KY.

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Figure 1. The X^D of *Drosophila testacea* is an ancient polymorphism. Shown is a maximum-likelihood phylogram of nucleotide sequences from three X-linked genes (*pgd*, *Rpl36*, and *skpA*; 1407 nucleotides) from the four known members of the testacea species group. The X^D sequences (shown in yellow), which include chromosomes from Switzerland (sw) and the Czech Republic (cz), branch outside the clade containing sequences from *Drosophila orientacea*, *Drosophila neotestacea*, and *D. testacea* X^{ST} chromosomes (X^{ST} sequences shown in purple; fi = Finland). Branches are labeled with SH-like approximate likelihood ratio test scores greater than 0.75.

Figure 2. Offspring sex ratios of male *Drosophila testacea* showing autosomal suppression of the X^D . Most males that carry an X^D have strongly female-biased offspring sex ratios (yellow). However, five X^DY males have offspring sex ratios that are close to 50:50, or that are marginally female-biased (grey). Males carrying the X^{ST} chromosome have normal offspring sex ratios (purple). The horizontal dashed line represents the expected 50:50 offspring sex ratio.

Figure 3. The relative fertility of X^{ST} and X^D male *Drosophila testacea* depends on mating condition. **(A)** Free mating condition. The mean number of offspring produced by X^{ST} males (purple), X^D males (yellow), and X^D males carrying a suppressor (grey) does not significantly differ under a free mating condition (males had access to two virgin females each for eight days) (t-tests: $X^{ST}Y$ vs. X^DY , $P = 0.3764$; X^DY vs. X^DY suppressed, $P = 0.5184$; $X^{ST}Y$ vs. X^DY suppressed, $P = 0.2044$). **(B)** Sperm depletion condition. X^D males (yellow) produce significantly fewer offspring compared to $X^{ST}Y$ males (purple) when sperm-depleted (males were mated to 10 females each over a 48-hour period)(t-test, $*P = 0.0141$).

Figure 4. **(A)** A significantly larger proportion of X^DX^D females failed to produce offspring compared to X^DX^{ST} females after being housed with males for several days (Z-test, $***P < 0.001$). **(B)** Of the females that produced offspring, there was no significant difference in the mean number of offspring produced by X^DX^D or X^DX^{ST} females (ns, not significant; t-test, $P = 0.1568$).

Produced no offspring Produced offspring

Table S1. Individuals from the testacea species group used for phylogenetic analysis. The sources of *skpA*, *RpI36*, *pgd*, *gl*, and *COI* gene sequences are indicated (nt, nucleotides).

Species	Individual	Collection location	<i>skpA</i> (442 nt)	<i>RpI36</i> (323 nt)	<i>pgd</i> (642 nt)	<i>gl</i> (538 nt)	<i>COI</i> (658 nt)
<i>D. putrida</i>	S-	Pittsford, NY, USA	this study	this study	this study	this study	no sequence
<i>D. putrida</i>	CT	Hartford, CT, USA	this study	this study	this study	no sequence	no sequence
<i>D. putrida</i>	HQ982078	n/a	n/a	n/a	n/a	n/a	GenBank (HQ982078)
<i>D. orientacea</i>	R2	Nanae, Hokkaido, Japan	this study	this study	this study	this study	no sequence
<i>D. orientacea</i>	R3	Nanae, Hokkaido, Japan	this study	this study	this study	this study	this study
<i>D. orientacea</i>	R4	Nanae, Hokkaido, Japan	this study	this study	this study	no sequence	no sequence
<i>D. orientacea</i>	R5	Nanae, Hokkaido, Japan	this study	this study	this study	this study	this study
<i>D. orientacea</i>	lab	Japan	this study	this study	this study	no sequence	no sequence
<i>D. neotestacea</i>	S-east	Hartford, CT, USA	this study	this study	this study	no sequence	this study
<i>D. neotestacea</i>	Fernie	Fernie, BC, Canada	this study	this study	this study	this study	this study
<i>D. neotestacea</i>	NV15	Victoria, BC, Canada	this study	this study	this study	no sequence	this study
<i>D. testacea</i>	f101	Lahti, Finland	this study	this study	this study	this study	this study
<i>D. testacea</i>	VC42	St. Sulpice, Switzerland	Keais <i>et al.</i> (2017)	Keais <i>et al.</i> (2017)	this study	no sequence	this study
<i>D. testacea</i>	sw36	St. Sulpice, Switzerland	Keais <i>et al.</i> (2017)	this study	this study	this study	this study
<i>D. testacea</i>	sw37	St. Sulpice, Switzerland	Keais <i>et al.</i> (2017)	this study	this study	this study	this study
<i>D. testacea</i>	sw39	St. Sulpice, Switzerland	Keais <i>et al.</i> (2017)	this study	this study	this study	this study
<i>D. testacea</i>	sw43	St. Sulpice, Switzerland	Keais <i>et al.</i> (2017)	this study	this study	this study	this study
<i>D. testacea</i>	sw47	St. Sulpice, Switzerland	Keais <i>et al.</i> (2017)	this study	this study	this study	this study
<i>D. testacea</i>	sw48	St. Sulpice, Switzerland	Keais <i>et al.</i> (2017)	this study	this study	this study	this study
<i>D. testacea</i>	sw52	St. Sulpice, Switzerland	Keais <i>et al.</i> (2017)	this study	this study	this study	this study
<i>D. testacea</i>	sw57	St. Sulpice, Switzerland	Keais <i>et al.</i> (2017)	this study	this study	this study	this study
<i>D. testacea</i>	sw58	St. Sulpice, Switzerland	Keais <i>et al.</i> (2017)	this study	this study	this study	this study
<i>D. testacea</i>	sw60	St. Sulpice, Switzerland	Keais <i>et al.</i> (2017)	this study	this study	this study	this study
<i>D. testacea</i>	sw61	St. Sulpice, Switzerland	Keais <i>et al.</i> (2017)	this study	this study	this study	this study
<i>D. testacea</i>	sw63	St. Sulpice, Switzerland	Keais <i>et al.</i> (2017)	this study	this study	this study	this study
<i>D. testacea</i>	sw70	St. Sulpice, Switzerland	Keais <i>et al.</i> (2017)	this study	this study	this study	this study
<i>D. testacea</i>	sw71	St. Sulpice, Switzerland	Keais <i>et al.</i> (2017)	this study	this study	this study	this study
<i>D. testacea</i>	cz15	Liblice, Czech Republic	this study	this study	this study	no sequence	this study
<i>D. testacea</i>	cz17	Liblice, Czech Republic	this study	this study	this study	no sequence	this study
<i>D. testacea</i>	cz18	Liblice, Czech Republic	this study	this study	this study	no sequence	this study
<i>D. testacea</i>	cz21	Liblice, Czech Republic	this study	this study	this study	no sequence	this study

Table S2. Primers used in this study.

Primer name	Sequence (5' → 3')	Source
SkpA-F	AACATGCCGACCATTAAGTTG	Dyer <i>et al.</i> (2011)
SkpA-R	CTTCTCCTCACACCGTTCGTT	Dyer <i>et al.</i> (2011)
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	CGGGAGTGAAGTCCTTTTGT	This study
neo-pgd-F	CGCCTGGTGACGTCATTATT	This study
neo-pgd-R	CTCACGCATCAGCATGAATC	This study
pgd-F	ATYGATGGYGGCAACTC	Pieper and Dyer (2016)
pgd-R	CNCGCATWAGCATRAAKCCYTG	Pieper and Dyer (2016)
gl-F	TTTCGATTGCGGCGGNTGYTTYGA	Pieper and Dyer (2016)
gl-R	GCCGTGGTGCATGGTCATRTTCAT	Pieper and Dyer (2016)

Table S3. Offspring sex ratio data from two representative $X^D Y^2$ and $X^D Y^{63}$ males showing the absence of Y-linked suppression.

Male	Genotype	Female/male offspring	Proportion female
Y2-2	$X^D Y^2$	72/1	0.986301
Y63-15	$X^D Y^{63}$	50/3	0.943396

Figure S1. Melt temperatures (°C) of a *skpA* gene region (180 bp) amplified from $X^D Y$ ($n = 22$) and $X^{ST} Y$ ($n = 19$) *Drosophila testacea* males using qPCR. The amplified region contains four nucleotide substitutions between the X^D and X^{ST} copies of the gene. Each male's X chromosome status (genotype; either X^D or X^{ST}) was pre-determined from Sanger sequencing of the *skpA* gene (12 X^D and 10 X^{ST}) or through offspring sex ratios (10 X^D and 9 X^{ST}). The mean melt temperatures of the two genotypes are significantly different (linear regression, $R^2 = 0.916$, $F_{1,39} = 425.5$, $P < 2.2e-16$). Bars represent 99% confidence intervals from the linear model. Open red circles indicate the mean.

Figure S2. Melt temperatures (°C) of a *skpA* gene region (189 bp) amplified from $X^D Y$ ($n = 22$) and $X^{ST} Y$ ($n = 19$) *Drosophila testacea* males using qPCR. The amplified region is identical between the X^D and X^{ST} chromosomes. Each male's X chromosome status (genotype; either X^D or X^{ST}) was pre-determined from Sanger sequencing of the *skpA* gene (12 X^D and 10 X^{ST}) or through offspring sex ratios (10 X^D and 9 X^{ST}). The mean melt temperatures of the two genotypes are not significantly different (linear regression, $R^2 = 0.0640$, $F_{1,39} = 0.0487$, $P = 0.8265$). Bars represent 99% confidence intervals from the linear model. Open red circles indicate the mean.

Figure S3. Shown is a maximum-likelihood phylogram of nucleotide sequences from the autosomal gene, *gl* (538 nucleotides), from the four known members of the testacea species group. Male *D. testacea* carrying an X^D and X^{ST} are shown in yellow and purple, respectively. Branches are labeled with SH-like approximate likelihood ratio test scores greater than 0.75. (sw = *D. testacea* from Switzerland; fi = *D. testacea* from Finland).

Figure S4. Shown is a maximum-likelihood phylogram of nucleotide sequences from the mitochondrial gene, *COI* (658 nucleotides), from the four known members of the testacea species group. Male *D. testacea* carrying an X^D and XST are shown in yellow and purple, respectively. Branches are labeled with SH-like approximate likelihood ratio test scores greater than 0.70. (cz = *D. testacea* from Czech Republic; sw = *D. testacea* from Switzerland; fi = *D. testacea* from Finland).