Transposable elements that cause dysgenesis also contribute to postzygotic isolation in the *Drosophila virilis* clade

Dean M. Castillo[*,1] and Leonie C. Moyle[*]

*.Department of Biology, Indiana University, Bloomington IN 47405

1. Current address:
School of Biological Sciences, University of Utah, Salt Lake City UT 84112

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Corresponding Author
Dean Castillo
School of Biological Sciences
257 South 1400 E, Room 201
Salt Lake City UT 84112

dean.castillo@utah.edu 801-581-7919

Abstract

Although hybrid incompatibilities are readily observed in interspecific crosses, the genetic basis of most incompatibilities is still unknown. One persistent hypothesis is that hybrid dysfunction is due to a mismatch between parental genomes in selfish elements and the genes that regulate the proliferation of these elements. In this study, we evaluated the potential role of transposable elements in hybrid incompatibilities by examining hybrids between *Drosophila virilis*, a species that is polymorphic for the transposable element *Penelope*, and a closely related species, *Drosophila lummei,* that lacks active *Penelope* elements. Given the established role of *Penelope* elements in hybrid dysgenesis among *D. virilis* strains, we predicted that interspecies crosses that involve *D. virilis* with *Penelope* elements will exhibit greater hybrid incompatibility than crosses involving *D. virilis* without *Penelope*. We observed both prezygotic and postzygotic isolation, the magnitude of which was dependent on the specific cross. Using F1 and backcross experiments to rule out alternative genetic incompatibilities, we demonstrated that amplified reproductive isolation in the interspecific cross involving *Penelope*-carrying *D. virilis* can be explained by the action of TEs that induce dysgenesis within *D. virilis*. These experiments demonstrate that TEs can contribute to the expression of hybrid incompatibilities via presence/absence polymorphisms. Further, our data indicate that the same TEs can cause distinct incompatible phenotypes in intraspecific crosses (male sterility) compared to interspecific hybrid crosses (inviability).

Introduction

Post-zygotic reproductive isolation in hybrid individuals is generally proposed to be a consequence of negative genetic interactions among two or more loci, as described by the Dobzhansky-Muller model of hybrid incompatibilities (Dobzhansky 1937; Muller 1942). Genic differences are obvious candidates to underlie these hybrid incompatibilities. However because the exact loci involved are known in only a few cases (Johnson 2010; Presgraves 2010; Castillo and Barbash 2017), the importance of other genetic mechanisms in generating hybrid incompatibilities under this model is still undetermined. One such alternative genetic mechanism involves the action of selfish genetic elements, including repetitive elements (Michalak 2009; Johnson 2010; Presgraves 2010; Werren 2011; Crespi and Nosil 2013). The proposal that transposable elements (TEs) contribute to reproductive isolation was met with early skepticism, largely due to a failure to detect their proposed mutational effects—including increases in the mutation rate—in crosses between different species with marked postzygotic isolation (Coyne 1986; Hey 1988; Coyne 1989). However, we now understand that phenotypes related to dysgenesis, a specific incompatibility phenotype caused by TEs, reflect de-repression of TE transcription (Martienssen 2010; Kharuna et al 2011), rather than mutational effects (Petrov *et al.* 1995; Blumenstiel and Hartl 2005). Additionally, the mechanisms that contribute to TE repression involve a complex machinery of proteins along with sequence-specific small RNAs (Brennecke *et al.* 2007; Slotkin and Martienssen 2007), providing a specific mechanism by which TEs could contribute to hybrid dysfunction: via parental mismatch between the number or identity of TEs and the machinery responsible for TE repression. This potential to cause negative genetic interactions via TE de-repression in hybrids, places TEs within the classical

Dobzhansky-Muller framework for the evolution of incompatibilities (Johnson 2010; Castillo and Moyle 2012; Crespi and Nosil 2013).

A role for selfish elements in postzygotic reproductive isolation has been strongly suggested by empirical studies that correlate differences in repetitive elements between species with increased repeat transcription and dysfunction in hybrids (Labrador *et al.* 1999; Martienssen 2010; Brown *et al.* 2012; Dion-Cote *et al.* 2014; Satyaki *et al*. 2014). Nonetheless there have been few attempts to directly assess the role of TEs in the expression of reproductive isolation. Clades with differences in TE content within and between species can be leveraged to infer the roles of TEs in reproductive isolation by conducting specific crosses that are predicted a priori to display elevated hybrid dysfunction based on the presence or absence of lineage specific TEs. Dysgenic systems in *Drosophila* are among the best characterized in terms of phenotypic effects of TEs that could be relevant to reproductive isolation, the specific TE families that are responsible for dysgenic phenotypes, and the known presence/absence of these TE families in different lines and species. Most notable among these, the *Penelope* dysgenic syndrome in *Drosophila virilis* and the *P-element* system in *D. melanogaster* are typically defined by gonad atrophy when a strain carrying transposable elements is crossed with a strain lacking these elements (Kidwell 1985; Lozovskaya *et al.* 1990). The dysgenic phenomenon depends on the direction of the cross and occurs when the female parent lacks the TE elements. These intraspecific dysgenic systems could be used to evaluate the contribution of TEs to reproductive isolation with other species, by contrasting the magnitude of interspecific hybrid dysfunction generated from lines that do and do not carry active dysgenic elements. This contrast requires two genotypes/lines within a species that differ in the presence of specific TEs known to cause dysgenesis in intraspecies crosses, and a second closely related species that lacks these same

TEs. If the TEs responsible for hybrid dysfunction within a species also influence reproductive isolation, interspecific crosses involving the TE-carrying line should exhibit greater hybrid incompatibility than crosses involving the lines in which TEs are absent.

In this study, we use this logic to evaluate whether TEs with known effects on intraspecific hybrid dysgenesis also affect interspecific reproductive isolation, using the *D. virilis* dysgenic system and the closely related species *D. lummei*. The dysgenic system in *D. virilis* was first described by Lozovskaya and colleagues (1990) when they observed gonadal atrophy and sterility in males and females, as well as male recombination (typically abnormal in *Drosophila*), in crosses between *D. virilis* strains that vary in transposable elements. Dysgenesis in *D. virilis* may be more complex than described in *D. melanogaster* because several elements might contribute to dysgenesis (Petrov *et al.* 1995; Blumenstiel 2014; Funikov et al. 2018), however one major element, *Penelope,* has been specifically implicated in the phenotype (Evgen'ev *et al.* 1997). Moreover, species in the *D. virilis* clade are closely related and easily crossed with one another and vary in whether they contain active *Penelope* elements, with *D. lummei* lacking full length active *Penelope* (Zelentsova *et al.* 1999; Evgen'ev *et al.* 2000).

Using these features and the established role of *Penelope* elements in hybrid dysgenesis among *D. virilis* strains, we contrast a cross where *Penelope* is absent from both *D. virilis* and *D. lummei* parental genotypes to a cross where *Penelope* is present in the *D. virilis* parent and absent in the *D. lummei* parent, to evaluate the connection between postzygotic reproductive isolation and differences in TE composition. The power of this experimental contrast also draws on the established body of work on intraspecific dysgenesis in this system. When female *D. virilis* parents in intraspecific crosses lack *Penelope* and male parents carry *Penelope,* strong dysgenesis is observed; therefore, in interspecific crosses we expect the strongest reproductive

isolation specifically between *D. lummei* females that lack *Penelope* and *D. virilis* males that carry *Penelope*—an asymmetrical pattern that is important to distinguish a model of TEs from alternative explanations. Additionally, the expression of dysgenesis within species is quantitative and determined by the copy number of *Penelope* elements (Lozovskaya *et al.* 1990; Vieira et al. 1998); this copy number effect can also further distinguish TE and non-TE causes of observed hybrid incompatibility in later generation interspecific crosses. With these expectations, using a series of directed crosses and backcrosses, here we infer that patterns of reproductive isolation observed in our study are consistent with a causal role for TEs in the expression of postzygotic species barriers.

Materials and Methods

Fly stocks

Two *D. virilis* stocks were used in the experiments. Strain 9 is a wild type strain that was collected in Georgia, USSR in 1970 and is consistently used as a parent in dysgenic crosses (Lozovskaya *et al.* 1990; Blumenstiel and Hartl 2005) as it differs from inducing strains by lacking several repetitive element families (Vieira *et al.* 1998; Blumenstiel 2014). For the purposes of this study we refer to this strain as TE-. The reference genome strain was used as our inducer strain (UCSD stock number 15010-1051.87) because it is known to induce dysgenesis (Blumenstiel 2014), and we refer to this strain as TE+. The genome strain was used instead of Strain 160, which is commonly used in dysgenic studies, because we experienced significant premating isolation between Strain 160 and *D. lummei* in single pair crosses, that precluded further genetic analysis and examination of postzygotic isolation. The reference genome strain is closely related to Strain 160 as both were created by combining several phenotypic markers to

make multiply marked lines (genome strain genotype *b; tb, gp-L2; cd; pe*) (Lozovskaya *et al.* 1990). The strain of *D. lummei* was acquired from the UCSD stock center (15010-1011.07).

Intra- and interspecies crosses to determine the nature of reproductive isolation

Virgin males and females from each stock were collected as they eclosed and aged for seven days prior to matings. All crosses involved single pairs of the focal female and male genotype combination (a minimum of 20, range 20-23, replicates were completed for a given female x male genotype combination). In total there were three intra-strain crosses that we completed to account for fecundity differences in subsequent analysis (TE- x TE-, TE+ x TE+, and *D. lummei* x *D. lummei*). As expected, in the three intra-strain crosses we did not see any evidence of male or female gonad atrophy (classical dysgenic phenotypes) or skewed sex ratios. There were differences in the total number of progeny produced, hatchability, and proportion of embryos that had reached the pre-blastoderm stage (hereafter 'proportion fertilized'). We used these values as baseline estimates of maternal effects which might influence these phenotypes in subsequent inter-strain analyses (Table 1; Table 2).

For inter-strain comparisons, we performed all reciprocal crosses between the three strains to analyze reproductive isolation on F1 progeny. These crosses fell into three categories. **I-Intraspecific Crosses Polymorphic for TEs:** Crosses between TE- and TE+ were used to confirm the expression of intraspecific male dysgenesis when the inducing (TE+) strain was used as a father. **II-Interspecies crosses with no TEs present:** Crosses between TE- and *D. lummei* were used to determine the nature of reproductive isolation between the species in the absence of dysgenic inducing TEs. **III-Interspecies crosses with TEs present:** Crosses between TE+ and *D. lummei* were used to determine the additional contribution, if any, of TEs to interspecific

post-mating reproductive isolation (Supplemental Fig 1). This contrast assumes that the two *D. virilis* strains, TE- and TE+, do not differ from each other in their genic Dobzhansky-Muller incompatibilities with *D. lummei*, an assumption that we could also evaluate with data from crosses performed using hybrid genotypes (described further below).

Backcross design to test models of hybrid incompatibility

We used a set of backcross experiments to compare the fit of several models of hybrid incompatibility to our data for intraspecies dysgenesis and interspecific reproductive isolation. F1 males from TE+ and *D. lummei* crosses were generated in both directions. These specific F1 males were created because this genotype was used previously to infer the genetic basis of dysgenesis (Lovoskaya et al. 1990; see below). These F1 males were then backcrossed individually to TE- and *D. lummei* females. We evaluated two main classes of model with fitness data from the resulting BC hybrids. Both classes broadly describe Dobzhansky-Muller incompatibilities, because they involve negative epistatic interactions between loci in the two parental strains, however they differ in whether they assume that the interactions occur between genes or between TEs and TE suppressors, and in several predictions that are specific to TEs that do not apply to genic models.

Genic models of hybrid incompatibilities

The first set of models against which we assessed our observations assume that alternative alleles at incompatible loci have diverged in each lineage and have a dominant negative interaction in F1 and backcross progeny that carry these alleles (Coyne and Orr 2004).

When two unlinked loci are involved we expect to see discrete classes of backcross (BC1) genotypes that exhibit hybrid incompatibilities, specifically half of the progeny would carry the incompatible genotype and half would not. If the hybrid incompatibility is not fully penetrant then, on average, any specific replicate BC1 cross would appear to have half the number of progeny exhibiting the hybrid incompatibility compared to F1s from the cross between the parental species.

We also expanded this general model to include interactions between specific chromosomes (X-autosome interactions), as well more than two interacting loci. For our purposes we considered an X-autosome model where there is a negative interaction between the X chromosome of the TE- or *D. lummei* parent and an autosome of the TE+ parent (Supplemental Fig. 3). We considered this specific X-autosome interaction because this generates dysgenesis/incompatibility in both sexes for the intraspecific dysgenic cross (TE- x TE+), but no male gonad dysgenesis/incompatibility in the reciprocal cross, consistent with previous reports (Lozovskaya *et al.* 1990). For models in which higher order (greater than 2 loci) interactions are responsible for the incompatibility observed in the F1s, we would expect to observe a smaller proportion of affected individuals in the backcross compared to the F1 cross (Supplemental Table 3); the specific expected degree of hybrid incompatibility in the backcross depends on the total number of loci required for hybrid incompatibility and the number of loci carried by each parental strain.

TE copy number models of hybrid incompatibility

To contrast with genic models of hybrid incompatibility we assessed the fit of our observations to a model is based on transposable elements. Under this model we expect

asymmetric reproductive isolation in crosses between parental species, but complete rescue of hybrid incompatibility in the backcrosses, a pattern that is not predicted from the genic hybrid incompatibility models. The prediction rescue of hybrid incompatibility in backcrosses comes from previous observations that in crosses between TE- females and males heterozygous for dysgenic factors (F1 males from TE+ x *D. lummei* crosses) the progeny are not dysgenic (Lozovskaya et al. 1990). We interpret this observation as indicating that the copy number of dysgenic factors (TEs) in the male parent contributes to the amount of dysgenesis, and is insufficiently high in F1 males of this cross to cause a dysgenic phenotypic in their offspring. This dosage/copy number effect was first suggested by (Vieira et al 1998) where they observed a positive relationship between *Penelope* copy number and the incidence of gonad atrophy. To connect this copy number pattern specifically to the backcross data from Lovoskaya et al (1990) we re-analyzed the data from Vieira et al (1998) to estimate the minimum number of *Penelope* elements required to produce a dysgenic phenotype (Supplemental Tables 1 and 2). We found a significant linear relationship between the number of *Penelope* elements and the proportion of dysgenic gonads (β=23.22; P<0.001, Confidence Interval=(13.9, 32.5)), with an intercept that was significantly different from zero (μ=23.25 P<0.001, Confidence Interval=(18.17,28.3)); the latter can be interpreted as the copy number at which dysgenesis is no longer expected. From the model we estimate that fewer than 28 copies of *Penelope* should not cause dysgenesis. F1 males from the cross between TE+ and *D. lummei* in the study by Lovoskaya et al (1990) would have ~23 copies of *Penelope* based on 46 active copies in TE+ Strain 160 and no active copies in *D. lummei* (Supplemental Table 1) and therefore are not predicted to cause dysgenesis when crossed to TE- females.

Phenotypes measured to infer dysgenesis and post-zygotic reproductive isolation

The phenotypes that we measured to infer levels of post-zygotic isolation were: egg to adult viability and the total number of progeny produced (both forms of inviability), the sex ratio of resulting progeny, and male gonad atrophy (a form of sterility common in dysgenic systems). These phenotypes were measured in both F1 and backcross experiments. After the initial pairing of individuals, the parents were kept for seven days. On day 4 the parental individuals were transferred to a new fresh vial. On day 7 the parents were transferred to a vial that had been previously dyed with blue food coloring to assist counting of eggs. All vials were cleared and parental individuals discarded at day 8. To ensure that we compared progeny of the same age among different crosses, we did not use any progeny from the first three days of the cross because both intra-strain and inter-strain crosses involving *D. lummei* females would often produce few or no progeny within this time. To be used in further analyses, an individual cross must have produced at least 10 progeny over the 4 day period (days 4-7).

To estimate the total number of progeny produced and sex ratio we counted the number of male and female progeny from days 4-7. We then analyzed the sex ratio for all progeny that eclosed (higher values indicating sex ratio skewed towards females). Lastly, we determined the viability of individuals for each cross by counting the number of eggs in the day 7 vial. We then allowed these eggs to hatch and develop and counted the number of pupae and adults. The number of progeny produced and egg to adult viability gave the same results; since the total number of progeny produced was collected from more replicates and could be summarized by normal distributions we only present total number of progeny produced. To estimate male gonad atrophy we collected all flies that eclosed from days 4-6 of the cross and saved males into new vials that we supplemented with extra yeast paste, with <10 males per vial. We aged these flies

on yeast for five days before scoring gonad atrophy. We scored gonad atrophy on a dissecting scope looking for the presence of atrophied testes (Blumenstiel and Hartl 2005). We also examined female ovary atrophy for a small sample from each interspecies cross but did not observe any atrophy; therefore we only report male gonad atrophy. In crosses between Strain 9 and Strain 160 it has been noted that both, one, or zero testes can be atrophied (Blumenstiel and Hartl 2005). Our estimate for analysis was the proportion of testes atrophied for the total number of testes scored.

To determine if differences in viability and total proportion of progeny produced for F1 crosses could be explained by differences in embryo lethality, we directly assayed hatchability and also assessed evidence for fertilization versus early embryo defects via embryo staining. First, to assay hatchability we set up population cages consisting of 25 males and 25 females using the same genotype combinations that produced F1s described above (3 replicate populations cages were set up per cross type). Males and females were left in the cages for one week and were given grape agar plates supplemented with yeast culture daily. After the acclimation period, a fresh agar plate was given to each population cage. After 24 hours the plates were replaced (Day 1). All the eggs on the plate were counted. Seventy-two hours later (Day 4) all eggs and larvae on the plate were counted. We calculated hatchability as the proportion of eggs that failed to hatch/total number of eggs. This was confirmed by counting larvae on the plates. The day after plates were collected for the hatchability assay (Day 2) another agar plate was collected (eggs had been oviposited for 24 hours) and embryos were collected, fixed, and stained with DAPI following basic protocols (Rothwell and Sullivan 2000; Rothwell and Sullivan 2007a). Briefly, embryos were collected in a nitex basket using 0.1% Triton X-100 Embryo Wash (7g NaCl, 3mL Triton X, 997 mL H20). Embryos were

dechorionated using 50% bleach for 3 minutes and rinsed well with water. Embryos were fixed with 37% formaldehyde and n-Heptane and then vitelline membrane was removed by serially shaking and washing in methanol. To stain embryos they were first rehydrated by rinsing with PBS and then blocked by incubating with PBTB, followed by rinsing with PBS before staining with PBTB+ 1:1000 DAPI for 5 minutes. Embryos were visualized using a fluorescent scope immediately after staining to determine if development had progressed. Embryos were considered to have begun development if we could see nuclei patterns typical of preblastoderm, blastoderm, and gastrula stages. Since we did not score fertilization by co-staining and looking for presence of sperm or whether the second phase of meiosis (formation of polar bodies) had been initiated, we may have missed any very early stage embryo lethality and considered these eggs unfertilized. Thus our estimates of embryo lethality are conservative (they potentially underestimate embryo lethality). All females were dissected after this last embryo collection to confirm they had mated by looking for the presence of sperm stored in the reproductive tract.

Statistical analysis

F1 reproductive isolation

All analyses were performed in R v3.3.2. For traits measured in the F1 crosses we performed three separate analyses, based on the identity of the female used in the cross.

To determine if there were differences in trait values based on the male parent in the cross we used linear and generalized linear models. All models had the form

$$y = \mu + \beta_i x_i + \beta_j x_j$$

μ represents the trait value for the intra-strain cross. β_i and β_j represent the effect sizes for males that were from different strains/species than the female in the specific analysis. For example,

when we analyzed data where TE- was the female parent, β_i could represent the deviation caused by having the TE+ strain as the father and $\beta_j$could represent the deviation caused by having *D. lummei* as the father. For a given phenotype there was significant reproductive isolation when $\beta_i$or β_j was significantly less than zero, indicating lower trait value than the intra-strain cross. In the case of sex-ratio any deviation of the correlation coefficient from zero could be interpreted as sex specific effects. The dysgenesis and total progeny produced variables were analyzed with simple linear regressions. Since the sex ratio data are more accurately represented by binomial sampling we analyzed these data using binomial regression (a generalized linear model).

To examine whether there was asymmetry in reproductive isolation for the *D. lummei* and TE+ strain cross, it was necessary to convert these phenotypes into relative values, based on the intra-strain cross. For example, TE+ female x *D. lummei* male measurements can be converted to relative values by finding the difference from the TE+ intra-strain cross average value. These relative measurements were compared in pairwise t-tests.

Backcross reproductive isolation

The results for the backcross experiment were analyzed in the same way as the F1 crosses. We compared phenotypes for crosses based on the female parent. Specifically, we compared the phenotype values for the control TE- (*D. virilis*) intra-strain cross with the crosses between TE- females and TE+ or F1 male genotypes. We then compared the phenotype values for the control *D. lummei* intra-strain cross with the crosses involving *D. lummei* females mated to TE+ or F1 male genotypes.

For a two locus genic model of incompatibility with a negative interaction in F1 progeny between two unlinked autosomal loci, we predict there would be no sex specific effects (Supplemental Fig. 2). When F1 males are used as parents only ½ of the resulting progeny, regardless of sex, should exhibit the hybrid incompatibility. If the epistatic interaction does not have complete penetrance then the effect size in this BC should be ½ compared to the parental cross. For example if 30% of F1 progeny are dysgenic then only 15% of the backcross progeny are expected to be dysgenic. Under a genic model with an X-autosome interaction between the X chromosome of the TE- parent and an autosome of the TE+ parent, sex specific effects will only be observed in the F1 progeny and not in the backcross (Supplemental Fig. 3). Lastly, for the TE model of incompatibility there are no expected sex-specific effects and the level of incompatibility in the backcross will be the same as the intra-strain control, and significantly less than what is predicted from the genic models of incompatibility.

Comparison of hatchability and embryo development measurements

To determine if lower hatchability was a product of unfertilized eggs or embryo lethality, we compared these independent measures using contingency tables. We used the proportion of eggs that had begun development past the pre-blastoderm stage as our measure of fertilization rate. Since there were few eggs for a given embryo collection replicate, we pooled across all three replicates. We then compared the proportion of eggs hatching to the proportion of eggs fertilized using a Chi-square test of independence. We could also compare differences in the proportion of eggs fertilized between crosses that shared a common female genotype, to infer premating isolation.

Data availability

All data and R code used is available through Figshare. doi to be determined

Results

Dysgenesis confirmed in intraspecies crosses polymorphic for TEs

In the classic dysgenic cross (TE- female X TE+ male) we observed 38% testes atrophy (Fig 1). This frequency of gonad atrophy was significantly different from the TE- intra-strain cross (β=0.3807; P<0.0001). In the reciprocal non-dysgenic cross (TE+ female X TE- male) we observed rare gonad atrophy events at a frequency not significantly different from zero. We did not observe skew in offspring sex ratio in either F1 cross, but the non-dysgenic cross produced significantly more offspring than the intra-strain comparison (β=26.732; P=0.0136); this increased productivity may reflect some outbreeding vigor in these long-established lab lines. There was no deviation in the proportion of eggs hatching compared to the proportion fertilized in either cross, indicating there was no embryo inviability (Table 2).

Prezygotic isolation occurs in interspecies crosses when TEs are absent

The TE- x *D. lummei* cross produced, on average, 22 fewer progeny than the TE- intra-strain control cross (μ=123.318; P<0.0001; β=-22.509; P=0.0341). This reduction in progeny production likely represents reduced fertilization (prezygotic isolation) rather than reduced F1 embryo viability in this cross, based on data from two comparisons. First we compared the estimates for the proportion of fertilized embryos to the proportion of embryos hatching within the cross, and found these did not differ, indicating a lack of embryo lethality. Next we compared the proportion of fertilized embryos between this cross and the intra-strain control, and found

that the proportion of fertilized embryos was significantly less for the TE- x *D. lummei* cross compared to the TE- intra-strain control cross (Table 2).

In comparison, the reciprocal cross (*D. lummei* female x TE-) had no evidence of prezygotic isolation or F1 embryo lethality. We actually observed a higher proportion of hatched embryos compared to our estimate for proportion fertilized; this likely reflects an underestimate of fertilization rate because this measure relies on handling embryos with multiple washing and fixing steps which can lead to loss of embryos in the final sample (Rothwell and Sullivan 2007b). In contrast to prezygotic isolation, gonad atrophy was not observed for males or females in either reciprocal cross between TE- and *D. lummei*.

Strong F1 postzygotic isolation occurs in interspecies crosses when TEs are present

The cross between TE+ females and *D. lummei* males produced rare male gonad atrophy, similar to the intraspecies non-dysgenic cross. We also saw no atrophy in the reciprocal *D. lummei* x TE+ cross where gonad atrophy might be expected (Fig 1). Instead, reduced viability was observed in both the reciprocal crosses, resulting in significantly fewer progeny produced compared to control crosses (Fig 3). The TE+ female x *D. lummei* male cross produced 19 fewer progeny compared to the TE+ intra-strain control cross (mu=50.52; P<0.0001; β=-31.37; P<0.0001). In the reciprocal cross, *D. lummei* female x TE+ male, 17 fewer progeny were produced compared to the *D. lummei* control cross (mu=34.50; P<0.0001; β=-16.95; P=0.0027). In both crosses the reduced number of progeny was due to early embryo lethality, as the proportion of embryos hatched was significantly lower than the proportion of embryos fertilized (Table 2). In the TE+ female x *D. lummei* male cross, inviability was also specifically increased for males, resulting in a significant excess of females and a sex ratio that was 63% female

(β=0.1171; P<0.0001; Fig 3). This deficit of males is not consistent with an X-autosome incompatibility between the *D. lummei* X and the TE+ autosome, because the males that are inviable carry the TE+ X chromosome.

We also compared the relative reduction in viability in these crosses, taking into account the differences in intra-strain and maternal effects. For the TE+ female x *D. lummei* male cross, progeny production was reduced by 62%; for the reciprocal cross (*D. lummei* x TE+) progeny production was reduced by 50%. These reductions were not significantly different from one another (t=1.547; df=41;P=0.1527).

Backcross experiments support a role for TEs in postzygotic isolation

Using data on gonad atrophy and hybrid inviability in our backcrosses, we aimed to determine whether the observed patterns of hybrid incompatibility were more consistent with a TE copy number model or alternative two-locus genic incompatibility models (Supplemental Fig. 2 and 3). First we quantified the relationship between TE copy number and gonad atrophy with the specific strains used in this experiment by testing whether F1 males from crosses between *D. lummei* and TE+, which would have half the copy number of the TE+ parent, produced dysgenic sons when crossed to TE- females (similar to Lovoskaya et al. 1990). In the classic intraspecific dysgenic cross (TE- x TE+ cross) we observed 38% gonad atrophy which served as our baseline expectation. For the TE incompatibility model we would expect to observe rescue of gonad atrophy in the backcrosses when using F1 males because these males will have fewer TEs than required to cause dysgenesis. For the genic incompatibility models we would expect ~19% gonad atrophy compared to the TE- x TE+ cross because only 1/2 of the progeny would carry both incompatible alleles. We found that both backcrosses had significantly

less than 19% gonad atrophy, results that are inconsistent with the two locus genic models. Specifically, gonad atrophy was dramatically reduced when F1 males were used as parents in crosses with TE- females. When F1 males carried the X^{lum} chromosome the level of dysgenesis was not significantly different than zero (β=0.0264; P=0.1366). When F1 males carried the X^{TE+} chromosome the level of dysgenesis was greater than zero, but significantly less than the TE+ parent (β=0.0638; P=0.0011; CI= 0.0261, 0.1016), and less than 19% atrophy. Therefore data for gonad atrophy in our backcrosses are consistent with the prediction from the TE incompatibility model.

Given our results for gonad atrophy we proceeded to examine support for the different incompatibility models in the context of hybrid inviability in the interspecies hybrid crosses. Specifically, we examined progeny production and sex ratio in backcrosses between *D. lummei* females and F1 males. For the TE copy number model we expect complete rescue of progeny viability when either F1 male is used as the male parent, and no sex specific lethality. For the autosome-autosome model and X-autosome model we expect to observe ~26 progeny produced, which is ½ of the reduction in progeny observed when comparing the *D. lummei* female x TE+ male control cross (where ~17.5 progeny were produced) to the intrastrain control cross (where ~34.5 progeny were produced). In both backcrosses the number of progeny produced was rescued to a level consistent with the TE copy number model but not either two-locus incompatibility model. Specifically, progeny production was significantly greater than the cross between *D. lummei* females and TE+ males and was not significantly different than the intrastrain *D. lummei* control cross (Table 3; Fig 3). In both cases the backcross produced significantly more than 26 progeny, based on a one sample *t*-test; the F1 male carrying the *D. lummei* X chromosome produced 39.76 on average and the F1 male carrying the X chromosome

from the TE+ produced 38.41 progeny on average when crossed with the *D. lummei* female (Table 3).

Two-locus genic incompatibility models might be an oversimplification when considering hybrid inviability, so we also considered models with higher order (greater than two loci) interactions. In general, if more than two loci are responsible for the incompatibility observed in the F1s, we expect to observe smaller effects in the backcross (Supplemental Table 3). For a three-locus autosomal interaction the greatest expected rescue would be 1/4 of the original effect (therefore ~30.26 progeny produced), while for a four-locus autosomal interaction the largest rescue would be 1/8 the original effect (and 32.38 progeny produced) (Supplemental Table 3). We pooled all backcrosses for this analyses since they produced the same number of progeny on average (see above). Using a one sample t-test we rejected the hypothesis that our data can be explained by a three-locus incompatibility (H0: μ>30.26; t=2.36, df=28, P=0.0126) or four-locus incompatibility (H0: μ >32.38; t=1.802, df=28, P=0.041). We did not explore interactions beyond four loci because *D. virilis* only has four major autosomes and, since we used F1 males that lack recombination, each chromosome is transmitted as a single locus in our crosses. Regardless, unlike all genic models, the TE copy number model predicts complete rescue of progeny production when either F1 male is used as the male parent; our observation that the backcross produced the same number of progeny as the *D. lummei* intra-strain control cross (Table 3) is therefore consistent with this model.

Apart from progeny production, we also had data on sex ratio in our backcrosses. Interestingly, in the cross involving *D. lummei* females and F1 males that carried the X^{TE+} chromosome there was a significant deviation from 50% female sex ratio (β =0.25; p=0.037). Neither the TE incompatibility model or the specific X chromosome incompatibility model we

considered (see methods) predicted sex specific effects past the F1 generation. Since we had already observed a pattern of sex specific inviability from the cross between TE+ females and *D. lummei* males, we also used the backcross data to determine the genetic factors responsible for this additional male specific hybrid inviability. The only two other crosses where we observed a skewed sex ratio were also female-biased and involved F1 males that carried the X^{TE+} and *D. lummei* Y crossed with *D. lummei* or TE- females (Fig. 2). In each of these three crosses, males had different X chromosome genotypes but consistently had the *D. lummei* Y chromosome. From this, we can conclude that this additional incompatibility is caused by an autosome-Y interaction.

Discussion

In this study, we used a powerful set of crosses to test whether TEs with known effects on intraspecies hybrid dysgenesis also influence reproductive isolation. Using the *D. virilis* dysgenic system we predicted that the *D. virilis* strain carrying TEs should show increased reproductive isolation when crossed with *D. lummei* lacking these TEs, compared to the cross involving the *D. virilis* strain that lacks dysgenic-inducing TEs. Taken together, our data support the inference that TEs contribute to elevated reproductive isolation observed in the interspecific cross that involves dysgenic TEs, while rejecting alternative hybrid incompatibility models to explain this elevated isolation.

A role for accumulated selfish genetic elements in the expression of postzygotic reproductive isolation is consistent with the Dobzhansky-Muller model of hybrid incompatibility (Johnson 2010; Castillo and Moyle 2012; Crespi and Nosil 2013), but little emphasis has been placed on the difficulty of disentangling the effects of these selfish elements from the effects of

other hybrid incompatibilities. For example, one way TEs are thought to contribute to reproductive isolation is through transcriptional misregulation that causes sterility or inviability (Michalak 2009; Dion-Cote et al. 2014; Martienssen 2010). However, this is not a unique feature of TE-based hybrid incompatibility; divergence in trans and cis-regulatory elements are common and can also cause misregulation and hybrid incompatibilities (reviewed in Mack and Nachman 2017). To disentangle TE-specific from other effects it would therefore also be necessary to differentiate whether TE misregulation is symptomatic of a common misregulation phenomena in hybrids or if TE divergence drives global misregulation. Instead of focusing on analyzing patterns of misregulation, here we took the approach of isolating phenotypic effects due to TEs by using strains with pre-defined differences in TE abundance, making explicit predictions about which hybrid classes should exhibit increased reproductive isolation, and evaluating these using classical genetic crosses.

The strength of this approach relies on our ability to make and test predictions that are exclusive to a TE incompatibility model, compared to alternative genic models, and thereby differentiate effects due to TE- and non-TE causes. For example, we observed that postzygotic incompatibility occurred only in crosses involving the TE-carrying *D. virilis* strain, and not in the cross between TE- *D. virilis* and *D. lummei*. However, this pattern of increased reproductive isolation alone does not exclude the possibility that other incompatibility loci are contributing to inviability, including TE and non-TE causes. Indeed, from a model of incompatibilities based on TEs alone we expected to see asymmetry in reproductive isolation, such that more isolation should occur specifically when the TE+ strain was the male parent (Supplemental Fig. 1); the observation of equally strong postzygotic isolation in both directions of the cross suggests the additional contribution of other non-TE factors between our *D. virilis* TE+ line and *D. lummei*.

Our ability to disentangle the effects of TEs vs non-TE incompatibilities in backross offspring rested on the observation that F1 males failed to produce dysgenic sons when crossed to TE- females (Lovoskaya 1990). The mechanism we propose based on previous studies (Vieira et al 1998), is that F1s have sufficiently low copy number of TEs (half that of their TE+ fathers) such that dysgenesis does not occur when F1 males are themselves used as fathers. This pattern allowed us to contrast the TE incompatibility model with genic incompatibility models because they make contrasting predictions of the level of incompatibility expected. We found that both the rescue of non-atrophied testes in crosses between TE- females and F1 males, and the rescue of viability in crosses between *D. lummei* females and F1 males, is consistent with the TE incompatibility model because it is significantly greater than the expectation for the alternative genic incompatibility models. Using these backcrosses we were also able to determine that additional non-TE loci contributed to the incompatibility observed in the TE+ x *D. lummei* cross. This inviability was male specific, suggested by a female biased sex ratio, and resulted from an autosome-Y incompatibility. Together these observations support the operation of two distinct mechanisms of hybrid inviability in our crosses, one of which directly implicates TEs in the expression of this postzygotic isolating barrier.

A second inference from our results is that TEs can cause different hybrid incompatibility phenotypes—that is, sterility or inviability—depending on context. TEs misregulation can cause substantial cellular or DNA damage which has the potential to produce incompatibility phenotypes (Phadnis et al 2015; Tasnim and Kelleher 2018). While the mechanistic link between damage and hybrid incompatibility needs further exploration, these observations suggest that the occurrence of hybrid sterility vs hybrid inviability could result from misregulation of the same TEs at different developmental stages. While transposable elements have typically been thought

to restrict their damage to the germline, this expectation is based on elements that are generally germline specific such as the *P*-element and *I*-element in *D. melanogaster*. In contrast, *Penelope* transcripts can be found in both somatic and germline tissue (Blumenstiel and Hartl 2005) and when *Penelope* is introduced to a naïve genome, transposition initially occurs in somatic tissue (Rozhkov et al 2012), where such activity could cause inviability phenotypes. Direct evidence that TEs that are normally active in the germline can cause inviability phenotypes when they become active in somatic tissue comes from a *P-element* mutant that was engineered to have expression in somatic cells (Engles et al 1987); in this case, inviability that is dependent on *P*-element copy number is observed in crosses involving this somatically-expressed *P-element* mutant line. Overall, given the potential for somatic mobilization of *Penelope* in *D. virilis* it is mechanistically plausible that this element is responsible for both of our observed incompatible phenotypes.

Finally, our observations also identify a mechanism that could contribute to intraspecific variation in the strength of isolation between species. Evidence for polymorphic incompatibilities is typically inferred from analyzing variation in the strength of post-zygotic isolation between species, because the identity of the alleles underlying this variation is usually unknown (Reed and Markow 2004; Kozlowska *et al.* 2012). These polymorphic incompatibility loci are typically assumed to be presence/absence polymorphisms of particular alleles that underlie genic Dobzhansky-Muller incompatibilities (Cutter 2012). In this study, we show that the presence/absence of TEs segregating in populations and species can also contribute to this pattern. Presence/absence polymorphism for specific TEs may be transient since TEs can invade populations rapidly (reviewed in Blumenstiel 2011). Nevertheless, this pace indicates that such differences could rapidly accumulate between species, and in the *D. virilis* clade at least one

other species, *D. kanekoi,* also lacks active *Penelope* elements (Zelentsova *et al.* 1999), suggesting that this mechanism could extend to other species pairs that experience dynamic turnover in the identity of their active TEs.

Overall, here we have provided evidence for a role of TEs in increased reproductive isolation between lineages that differ in the presence of specific active TE families, using a framework that allowed us to differentiate TE vs non-TE effects by leveraging knowledge of TE biology. Our results indicate that the generality of when and how TEs contribute to reproductive isolation could be further addressed by focusing on *a priori* cases where species are known to vary in the number or identity of TEs, rather than relying on interpreting patterns of TE misregulation that arise in hybrid crosses. Our findings suggest that the rapid evolution of TEs in different lineages could potentially explain the presence of polymorphic incompatibilities in many systems. The addition of studies that examine lineages at variable stages of TE differentiation will also provide more insight into the contribution of TEs to the accumulation of hybrid incompatibilities, both hybrid sterility and inviability, over evolutionary time.

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Table 1. Average trait means for control intrastrain crosses. These values are used as baseline in statistical models testing for increased reproductive isolation in interspecis crosses. For male dysgensis (n) refers to the number of male progeny scored. se=standard error.

Female	Male	Dysgenesis (n)	Sex Ratio (se)	Viability (se)
TE-	TE-	0.0 (1398)	0.488 (0.016)	0.844 (0.055)
TE+	TE+	0.0 (575)	0.516 (0.014)	0.571 (0.057)
D. lummei	*D. lummei*	0.0 (335)	0.521 (0.015)	0.411 (0.067)

Table 2. F1 postzygotic reproductive isolation, as observed in crosses between TE+ and *D. lummei,* is a product of embryo inviability. Inviability is inferred when fewer eggs hatch than expected based on the proprtion of eggs that initiated development (proportion fertilized). The samples sizes (n) are the pooled number of embryos examined across replicate crosses. * represents significant differences in the proportion fertilized from proportion hatched. † represents a significant difference in the proportion fertilized for an interspecific cross compared to the control cross of the maternal strain.

Female	Male	Proportion Fertilized (n)	Proportion Hatched (n)	χ_1^2
TE-	TE-	0.85 (179)	0.79 (344)	2.76
TE+	TE+	0.58 (155)	0.55 (224)	0.2
D. lummei	*D. lummei*	0.61 (218)	0.67 (260)	1.75
TE-	TE+	0.85 (180)	0.80 (283)	1.00
TE+	TE-	0.57 (153)	0.57 (206)	0.00
TE-	*D. lummei*	0.33† (389)	0.39 (79)	0.59
D. lummei	TE-	0.60 (161)	0.75 (337)	12.18*
TE+	*D. lummei*	0.55 (126)	0.42 (235)	5.41*
D. lummei	TE+	0.43† (212)	0.31 (208)	6.06*

Table 3. Progeny production is rescued when F1 males are crossed to *D. lummei* females. For the backcrosses we tested whether progeny rescue was greater than the expectation for two locus genic incompatibility models. F1-1 are males from the *D. lummei* x TE+ cross and F1-2 males are from the reciprocal cross. *indicates a significant difference in progeny production from the intrastrain control.

Female	Male	Mean Progeny Produced	*t* statistic H0: mu=26 (df)	*P*-value
D. lummei	*D. lummei*	34.50	NA	NA
D. lummei	TE+	17.54*	NA	NA
D. lummei	F1-1	39.76	2.61 (16)	0.009
D. lummei	F1-2	38.41	2.22 (11)	0.023

Figure 1. Gonad atrophy characteristic of the classic dysgenic phenotype. A) Intrastrain control crosses, intraspecific crosses, and interspecific crosses demonstrating the lack of dysgenesis except in the intraspecific cross. B) Dysgenesis in backcross progeny compared to TE- x TE+ cross showing that the F1 males do not induce dysgenesis to the same extent as the pure TE+ males. F1-1 are males from the *D. lummei* x TE+ cross and F1-2 males are from the reciprocal cross. * indicates significantly different from zero. ** indicates significant difference between GS and F1 males. The dashed line represents the expected effect size based on incompatibility models.

Figure 2. The sex ratio of each cross used to examine deviations from a 50:50 sex ratio caused by sex specific lethality. A) Intrastrain control crosses, intraspecific crosses, and interspecific crosses. B). The sex ratio of backcross progeny showing sex biased progeny ratios occur when *D. lummei* Y is in combination with some complement of TE+ autosomes. F1-1 are males from the *D. lummei* x TE+ cross and F1-2 males are from the reciprocal cross. * indicates significant deviation from 50:50 sex ratio.

Figure 3. The number of progeny produced for each cross demonstrating significantly fewer progeny produced in crosses between *D. lummei* and TE+ in both directions. A) Intrastrain control crosses, intraspecific crosses, and interspecific crosses. B). Backcross progeny compared to the *D. lummei* intrastrain cross showing that the F1 males do not induce progeny lethality, and progeny production is recused. F1-1 are males from the *D. lummei* x TE+ cross and F1-2 males are from the reciprocal cross * indicates significantly different from the intrastrain cross of the maternal line.

Figure 1

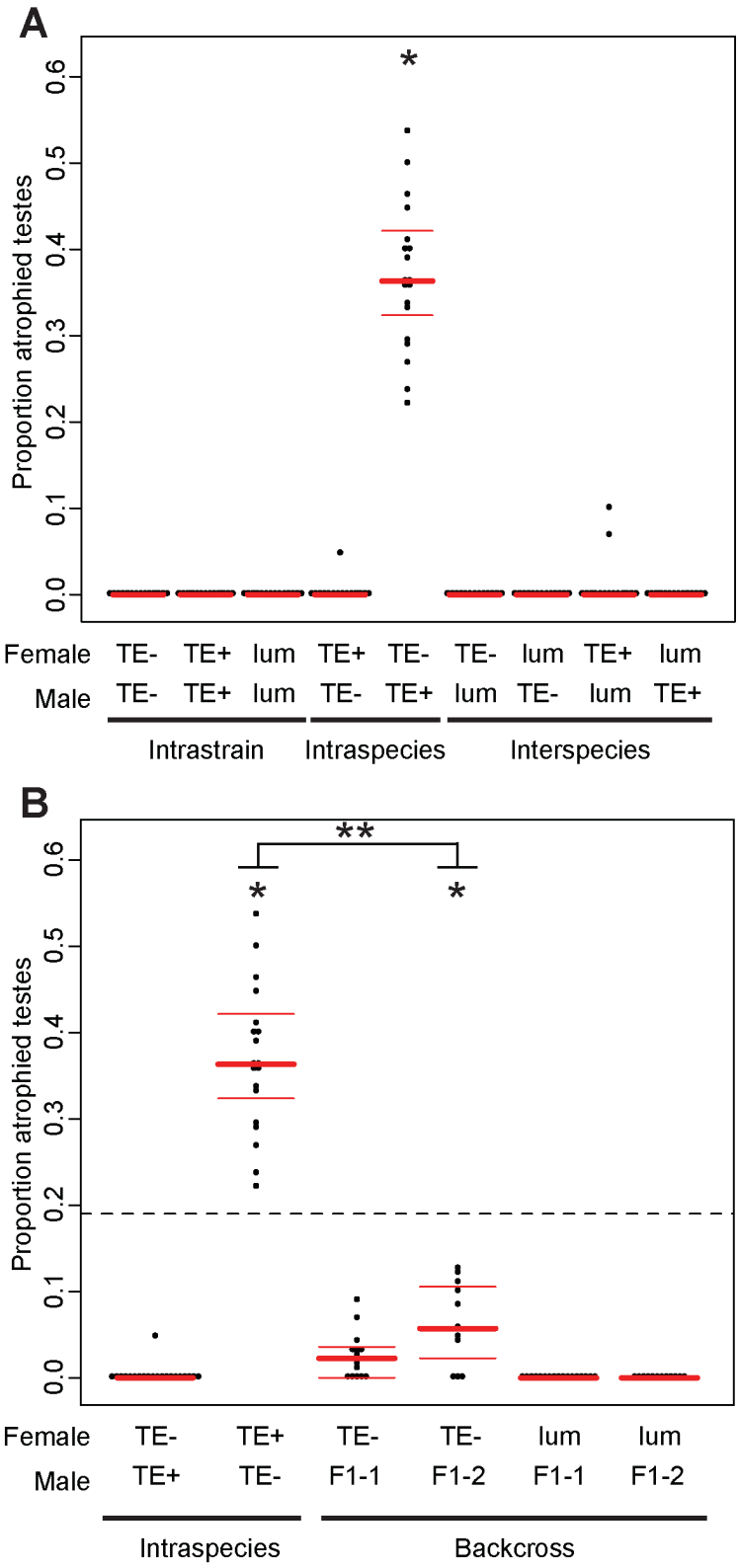

Figure 2

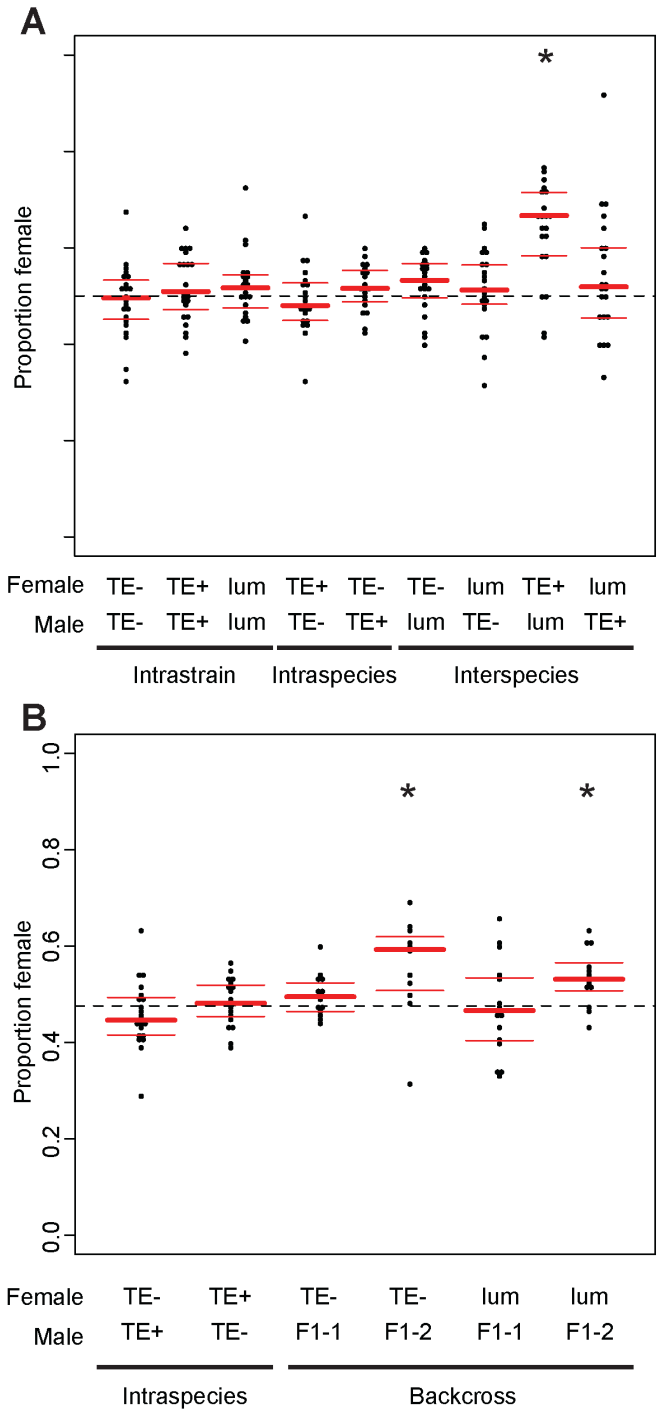

Figure 3