Rapid divergence of the male reproductive proteins in the *Drosophila dunni* group and implications for postmating incompatibilities between species

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Abstract

Proteins involved in post-copulatory interactions between males and females are among the fastest evolving genes in many species, usually attributed to their involvement in reproductive conflict. As a result, these proteins are thought to often be involved in the formation of postmating-prezygotic incompatibilities between species. The *Drosophila dunni* subgroup consists of a dozen recently diverged species found across the Caribbean islands with varying levels of hybrid incompatibility. We performed experimental crosses between species in the *dunni* group and see some evidence of hybrid incompatibilities. We also find evidence of reduced survival following hybrid mating, likely due to postmatings-zygotic incompatibilities. We assessed rates of evolution between these species genomes and find evidence of rapid evolution and divergence of some reproductive proteins, specifically the seminal fluid proteins. This work suggests the rapid evolution of seminal fluid proteins may be associated with postmating-prezygotic isolation, which acts as a barrier for gene flow between even the most closely related species.

Keywords: *Drosophila*; hybrid incompatibilities; genome evolution; seminal fluid proteins

Introduction

Numerous groups of recently diverged species have been used to study speciation across multicellular taxa (Coyne and Orr 1989; McKinnon and Rundle 2002; Glor et al. 2005; Kitano et al. 2009; Brekke and Good 2014). These studies find an array of complex relationships between species caused by varying levels of divergence across their genomes, including premating isolation and reinforcement, postmating-prezygotic isolation and postzygotic isolation (Coyne and Orr 2004; Orr 2004; Presgraves 2007; Matute et al. 2010; Moyle and Nakazato 2010; Payseur and Rieseberg 2016). Recently diverged species with incomplete reproductive barriers prove to be more useful for understanding how new species evolve (Coyne and Orr 1989; Butlin et al. 2012). These species groups can be used in QTL studies to identify loci that contribute to the reduced fitness of hybrids (Howard et al. 2002; Noor et al. 2007; Kitano et al. 2009), or to identify genes that may be involved in the early stages of reproductive isolation, such as those causing incompatibilities in the heterogametic sex (known as Haldane’s Rule when incompatibilities in the heterogametic sex are in excess) (Haldane 1922; Coyne and Orr 1989; Orr 1995; Gavrilets and Waxman 2002; Coyne and Orr 2004; Phadnis and Orr 2009; Orr 1997).

Several studies have also highlighted that proteins transmitted in the seminal fluid to the female reproductive tract may be a major cause of postmating-prezygotic isolation (Bono et al. 2015; Ahmed-Braimah et al. 2017; 2020; Rowe et al. 2020). Further, previous work has highlighted that post-mating-prezygotic barriers are a major cause of incompatibilities in incompletely reproductively isolated species (Gavrilets and Waxman 2002; Coyne and Orr 2004; Andres et al. 2008; Goubrïére and Mallet 2010; Larson et al. 2012; 2013; Ahmed-Braimah 2016; Turissini et al. 2018; Castillo and Moyle 2019; Matute et al. 2020) and form more rapidly than other types of incompatibilities (Turissini et al. 2018).

Barriers to hybridization have also been examined in different *Drosophila* species groups, finding varying levels of divergence, and in some cases the mechanisms for isolation between species (Patterson 1947; Grant 1983; Coyne and Orr 1989; Presgraves 2007; Miller et al. 2010; Matute and Ayroles 2014; Ahmed-Braimah 2016; Turissini et al. 2018; Matute et al. 2020). Some studies, focusing on the effects of heterospecific matings on females, have found drastic changes in the females, including the swelling of the reproductive tract (Patterson 1947) and the activation of stress response pathways (Bono et al. 2011; Ahmed-Braimah et al. 2020). This is likely due to antagonistic interactions between male seminal fluid proteins and the heterospecific female tract (Knowles and Markow 2001; Bono et al. 2011; Ahmed-Braimah et al. 2017), and such pressure may drive the evolution of reinforcement (Coyne and Orr 2004; Turissini et al. 2018).

The *Drosophila dunni* subgroup is found within the cardini group in the *Drosophila* subgenus (Supplementary Figure 1) (Heed 1962). This species group diverged across the Caribbean islands,
creating endemic populations each on a different island or set of islands (Heed 1962; Hollocher et al. 2000; Wilder and Hollocher 2003). Despite their geographic isolation from each other, species are still able to hybridize (to varying levels of success) and are a useful species group for understanding several traits, such as the evolution of pigmentation or reproductive isolation (Stalker and Streisinger 1953; Hollocher et al. 2000; Wilder and Hollocher 2003). In some crosses, these hybrid offspring show evidence of Haldane’s rule (Haldane 1922; Orr 1997), with crosses producing only female offspring, or sterile male offspring (Heed 1962).

Here we performed experimental crosses in the dunnii group and found that in some crosses, heterospecific matings reduce female survival compared to conspecific matings, potentially caused by an insemination reaction-like effect (Patterson 1947). Using a combination of long-read and short-read sequencing, we assembled the genomes of four species in the Drosophila subgenus (Zhou et al. 2012; Zhou and Bachtrog 2015; Gramates et al. 2017; Hill et al. 2019). We also estimated levels of divergence between species and find several structural differences in reproduction genes which may be associated with incompatibilities. Finally, we estimated rates of evolution across these genomes and identify several pathways of groups of genes of interest diverging between species (particularly between D. nigrodunni and D. arawakana), including rapid divergence in immune pathways and in seminal fluid proteins.

Materials and methods
Drosophila stocks, husbandry and inbreeding
We obtained stocks for D. arawakana (stock number: 15182-2260.00), D. dunnii (stock number: 15182-2291.00), D. nigrodunni (stock number: 15182-2311.00) and D. similis (stock number: 15182-2321.00) from the National Drosophila Species Stock Center. Importantly, we note that there is reason to suspect that our D. similis line may actually be a subspecies of D. dunnii that is mislabeled at the Stock Center. First, after acceptance of the manuscript, Hope Hollacher (personal communication), who did some of the pioneering work with these species, let us know that she suspected mislabeling. Second, previous phylogenetic analysis of the four species would suggest D. similis should be more divergent from D. dunnii than it appears in this study (Brisson et al. 2006). However, we maintain our original labeling as D. similis throughout. Each species was maintained on standard instant fly food (Formula 4-24, Carolina Biological Supply Company, Burlington, NC) in an incubator at 23 °C. Before experiments, we inbred for three generations. Specifically, we established 10 separate crosses between individual pairs of a male and female for each species and chose a single successful cross per generation. We then repeated this for two further generations. We then randomly chose one of the successful inbred vials of flies to work with for each species for the remainder of the experiments described.

Experimental crosses within and between species
We performed initial crosses in all pairwise combinations of species, for both directions of the cross, as well as within species crosses, to confirm previous assessments of between species viability (Heed 1962; Wilder and Hollocher 2003).

For each species we cleared vials of adults at 9:00 AM US central time and then collected any emerged adults in 3-hour intervals, separating collected flies by sex. We then used these virgin flies to mate all species in pairwise combinations individually in 3 replicates. For each replicate we mated 10 males with 10 females (all aged 2–3 days) for 5 days (Wilder and Hollocher 2003; Cenzi De Ré et al. 2010). Following the 5-day mating period and removal of the parents, we collected offspring every day for 30 days. After aging virgin F1 offspring for 3 days, we mated 10 F1 virgin offspring to 10 flies of the opposite sex from their paternal species, to assess the fertility of the F1 flies. As D. arawakana was infected with Wolbachia, we sought to cure all species of any bacteria which may affect crosses. We created sublines of each species raised on food containing tetracycline-hydrochloride (0.05 mg/ml) for three generations. We then extracted DNA from females of each strain and tested for Wolbachia using PCR (wsp-81F (5’-TGGTCCATAAGTGATGAAGAAAC-3’), wsp-691R (5’-AAAAATTAAACGCTACTCCA-3’), under the following cycling conditions: 94 °C for 4 minutes, followed by 30 cycles of 40 seconds at 94 °C, 40 seconds at 55 °C, 60 seconds at 72 °C and a final extension step of 72 °C for 10 minutes) (Zhou et al. 1998). We then repeated experimental crosses, as described above, with the tetracycline cured strains.

We assayed female survival for D. arawakana, D. dunnii, D. nigrodunni and D. similis in unmated flies and following mating, in both uncured and tetracycline cured flies. We considered a cross to be conspecific if we mated within species (e.g. D. dunnii × D. dunnii) and a cross to be heterospecific if we mated with the most closely related species where fertile hybrids were found in previous crosses (e.g. D. dunnii × D. similis and D. arawakana × D. nigrodunni). For these crosses we established 5-15 vials of 10 males and 10 females of the given species (with no males when measuring virgin females), all aged 2-3 days. We then recorded the survival of females every day (checking at 10:00 AM US central time) for 30 days, flipping the flies onto new food every 3-4 days and removing males after the first 5 days. We fit a survival curve across the total data for each cross-type using SurvMiner (Kassambabara et al. 2017) in R (Team 2013) and used Cox’s Proportional Hazards Model to identify significant differences in survival between sets of crosses. For the initial crosses we used the following model:

\[
\text{Survival (days post mating)} \sim \text{female species} \times \text{male species (if any)} + \text{vial.}
\]

We set the reference level as the conspecific cross (e.g. D. arawakana × D. arawakana) and looked for significant differences from these for interaction terms to determine if unmated females (e.g. D. arawakana × not mated) or heterospecifically crossed females (e.g. D. arawakana × D. nigrodunni) show significant differences from the conspecific cross. To consider the effect of Wolbachia infection on these crosses, we repeated these initial crosses alongside the same crosses with Wolbachia cured flies (cured as described above) and Cox’s Proportional Hazards Model was used to determine the effect of Wolbachia on survival, and to test for differences in survival between sets of crosses after accounting for Wolbachia:

\[
\text{Survival (days post mating)} \sim \text{female species} \times \text{male species (if any)} + \text{Wolbachia infection} + \text{vial.}
\]
Postmating dissection of the female reproductive tract
We collected virgin males and females for tetracycline-cured D. arawakana and D. nigrodunni as described above and aged them 2–3 days. We then established conspecific and heterospecific experimental crosses for 6 replicates of 10 males and 10 females at 10:00 AM US central time, as well as virgin control females for 6 replicates of 10 females. Following 24 hours of cohabitation, for 3 replicates of each cross, we separated the females for each cross and dissected the reproductive tract. Based on previous work describing the insemination reaction (Patterson 1947; Grant 1983; Markow and Ankney 1988), we scored the reproductive tract for each female, identifying if the female had mated (by the presence of sperm), if the reproductive tract appeared to be swollen (relative to the unmated virgin females) or if the reproductive tract was destroyed or damaged (alongside a swollen tract, if possible to tell). We repeated this scoring for the remaining 3 replicates of each cross 24 hours later (48 hours total). We then compared conspecific and heterospecific crosses for rates of mating and rates of swelling in the reproductive tract.

Genome sequencing, assembly and annotation
We extracted DNA following the protocol described in (Chakraborty et al. 2016) for D. arawakana, D. dunni, D. nigrodunni and D. similis females. We prepared the D. dunni and D. nigrodunni DNA as a sequencing library using the Oxford Nanopore Technologies Rapid 48-hour (SQK-RAD002) protocol, which we then sequenced separately using a MinION (Oxford Nanopore Technologies, Oxford, UK) (Jain et al. 2016a) (Supplementary Table 1). We also prepared the D. arawakana, D. dunni, D. nigrodunni and D. similis samples as Illumina libraries with a 300bp insert size which we sequenced on an Illumina HiSeq4000 to produce 150bp paired-end reads (Supplementary Table 1). We removed Illumina adapters using Sickle (Joshi and Fass 2011) and trimmed the Illumina sequences using Scythe (Buffalo 2018). For the two MinION genomes, bases were called post hoc using the built-in read_fast5_basecaller.exe program with options: –f FLOMIN106 –k SQK-RAD002 –r-t 4 (Jain et al. 2016b). For D. dunni, raw reads were assembled using Minimap2 and Miniasm (parameters: -x ava -o nt -t 8) (Li 2016). We then polished using Racon with Oxford Nanopore Technology reads for three iterations and Pilon with Illumina fragment library reads for three iterations (Walker et al. 2014). For the D. nigrodunni genome, we first used wtdbg2 to assemble the genome (parameters: -t 4 -L 1000) (Ruan and Li 2020). We then created a second assembly using Minimap2. For each, we ran Racon and Pilon for three iterations as described for D. dunni, then merged the two D. nigrodunni assemblies using Quickmerge (Liu and Yang 2013). Following this, we polished this merged genome using Pilon for four more iterations. Both assemblies were benchmarked using BUSCO (v 3.0.2) and the Diptera database (Simão et al. 2015).

For D. similis, we mapped data to the D. dunni genome before Pilon polishing and polished the D. dunni genome using D. similis data in Pilon for three iterations, to insert D. similis variants into the genome. Following these three iterations of polishing, we mapped D. similis data to this genome using BWA (Li and Durbin 2009) and SAMtools (Li et al. 2009), and called variants using Picard (http://broadinstitute.github.io/picard) and GATK Haplotypecaller (McKenna et al. 2010; DePristo et al. 2011). We then used BCfTools (Narasingham et al. 2016) to filter these variants, removing calls below a quality threshold of 200 and inserted them into the polished genome. This was repeated for two more iterations to create a D. similis alternate genome. The same pipeline was followed for D. arawakana mapped to the D. nigrodunni genome.

We used the D. innubila transcriptome (NCBI project PRJNA524688) (Hill et al. 2019) as well as protein databases from D. innubila, D. virilis, D. melanogaster, and M. domestica in MAKER2 (Holt and Yandell 2011) to annotate each genome, including using RepeatModeler (Smit and Hubley 2008) in an attempt to correctly assign repetitive regions and retraining a HMM using SNAP following each iteration (Johnson et al. 2008). This was repeated for three iterations to generate a GFF file containing gene evidence generated by MAKER2 (Holt and Yandell 2011).

Finally, we identified orthologous genomic regions pairwise for each of the four species examined here to each other and to the D. innubila genome using progressiveMauve (Darling et al. 2004). We visualized orthologous regions using ciricos (Zhang et al. 2013). We attempted to confirm any apparent structural differences based on progressiveMauve by mapping short reads for each species to a different genome and calling copy number differences using Delly (Rausch et al. 2012) and dudeML (Hill and Unckless 2019), taking the consensus of the two tools, but favoring the absence of a copy number variant when we found discrepancies between the two tools.

Assessing the repetitive content across the dunni group
For each genome, we identified the repetitive content de novo using RepeatModeler to call the repeats (engine = NCBI) (Smit and Hubley 2013–2015) and RepeatMasker (-gff -gcac -s) to identify the repetitive regions (Smit and Hubley 2013–2015). We also used dnaPipeTE (genome coverage = 1, sample number = 2, cpu = 4, genome size = 168000000) (Goubert et al. 2015) to identify the repetitive content in the short-read data for each species, which we used to produce a second map of reference genome repetitive regions using RepeatMasker. For both sets of repeat content assemblies we identified which TE families were shared between species and which were unique to species using blastn (e-value < 10e–5, hsps = 1, alignments = 1). We then identified what proportion of the genome each TE family constituted across species using RepeatMasker (Smit and Hubley 2013–2015).

Phylogenetic relationship of the dunni group to the rest of the Drosophila genus
We next sought to estimate the consensus species tree to better place the dunni group in the Drosophila phylogeny. We chose to assemble a consensus multigene phylogeny as different genes have differing evolutionary histories and so the tree of a single gene may not best reflect the actual species (Mendes and Hahn 2016), we randomly sampled 100 conserved Drosophila genes which have orthologs in the human genome to support their conservation across large evolutionary distances for better estimating a phylogeny (genes chosen based on the list of orthologous Drosophila genes on FlyBase) (Gramates et al. 2017). We then extracted the coding sequences of these genes from our four focal species, as well as from several other Drosophila species found from Flybase genome database (Gramates et al. 2017) and the NCBI genomes database (Zhou et al. 2012; Hamilton et al. 2014; Palmieri et al. 2014; Zhou and Bachtrog 2015; Kitts et al. 2016, Hill et al. 2019). We aligned each group of orthologous genes separately using MAFFT (-auto) (Katoh et al. 2002) and created a multiple gene super-tree based on the consensus of each gene tree, following 100 bootstraps with PhyML (-b 100 -N 100 -GTR - gamma 8) (Le and Gascuel 2008; Guindon et al. 2010). We also
generated gene trees for each of the 100 genes independently, following the same protocol. In this case 66 of the 100 trees gave the same topology of the dunni group as the total tree, whereas 7 trees had distinct topologies and 27 trees gave the topology of D. similis as an outgroup to the other three species, with D. dunni a sister to the D. nigrodunni-D. arawakana complex.

Estimating rates of evolution across the dunni group

For each gene in the genomes of our four focal species, we identified orthology to each other and to genes in D. innubila using blastp (e-value < 0.00001, hsp = 1 alignment = 1) (Altschul et al. 1990). We aligned each set of orthologs using PRANK to generate a codon alignment and gene-tree (Löytynoja 2014), as subtle differences between the species tree and gene trees can result in false estimates of divergence (Mendes and Hahn 2016). We then estimated rates of both non-synonymous and synonymous substitutions using codeML (Yang 2007). Specifically, we estimated synonymous divergence (dS), non-synonymous divergence (dN) and the proportion of the two values (dN/dS). We estimated specific rates of evolution along each branch of the dunni group and leading into the dunni group using D. innubila as an outgroup using a free-ratios model (model 1), and compared this to a model for uniform evolution across all branches (model 0) (Yang 2007), choosing the best fitting model using a likelihood ratio test (p < 0.05). Finally, we also estimated rates of evolution across the entire dunni group phylogeny using codeML (sites models M7 and M8) (Yang 2007), choosing the best fitting model using a likelihood ratio test (p < 0.05). For all sets we removed genes that had saturated dS (dS > 1) which could result in a miscalculation of dN and dN/dS, removing 290 ortholog groups in total.

Using the estimated rates of evolution, we then compared the rates of evolution of all genes on the same branch, as well as the rate of evolution of the same gene between branches. For genes of similar levels of synonymous divergence (dS, windows of 0.001 dS, e.g. all genes within 0.001 dS of each other) we found the 97.5th upper percentile for dN/dS on each branch and across the total phylogeny. For our analyses we chose to focus on the upper and lower 97.5th percentiles as these values should be higher or lower than 2 standard deviation from the mean, given our normally distributed results (Shapiro–Wilk test P > 0.169). For the closely related species pairs (D. nigrodunni and D. arawakana, D. dunni and D. similis) we compared measures of dN/dS between species and found the 97.5th upper percentile for dN/dS per species per window of dN/dS for the paired species (0.001, sliding 0.001).

We then took outlier genes (e.g. genes above the 97.5th percentile in each category) and looked for enrichments in gene ontology categories compared to non-outlier genes using GOrilla (Eden et al. 2009). For GO categories of interest, such as those enriched for duplications or for high levels of dN/dS, we compared dN/dS of genes in these categories to the nearby genomic background. For each gene we extracted nearby genes (within 100kbp up or downstream on the same chromosome), of similar divergence levels on each branch (within 0.01 dS), we found the difference in dN/dS between the median of the background genes and the focal gene. We used a Wilcoxon-Rank Sum test to identify GO categories on each branch with significantly higher (or lower) dN/dS than the background.

Using the annotations of all species and D. innubila, we identified genes with more than one copy in one species, relative to all other species. We confirmed this by estimating copy numbers of genes in each species using short read information and dudeML (following the tutorial pipeline for N = 1) with the short read information mapped to the genome of the sister species (Hill and Unckless 2019). We then used GOrilla (Eden et al. 2009) to identify Gene ontology categories that are enriched for duplicates on specific branches, which we confirmed using PANTHER (Thomas et al. 2003).

Statistics

We used R for all statistics in this analysis (Team 2013), and ggplot2 for data visualization and figure production (Wickham 2016). False-discovery rate (FDR) multiple testing correction was performed in cases when necessary using R (R-Core-Team 2013).

Results

The Drosophila dunni group shows varying levels of hybrid incompatibility

We first surveyed the reproductive divergence between species in the D. dunni group. As shown previously (Heed 1962; Wilder and Hollocher 2003), we found that these species have varying levels of hybrid incompatibilities, with some crosses producing viable offspring (Figure 1, e.g. D. dunni x D. similis) and others producing sterile offspring (Figure 1, e.g. D. arawakana x D. dunni) or no offspring (e.g. D. nigrodunni x D. similis). In keeping with Haldane’s rule (Haldane 1922), some produce sterile males, or no males at all (Figure 1 and Supplementary Table 2, e.g. D. nigrodunni x D. arawakana), whereas no crosses produced only males or only fertile males. Despite divergence on levels comparable to the D. melanogaster subgroup (Supplementary Figure 1 and Supplementary Table 3), there are no characterized inversions between species (Stalker and Streisinger 1953; Cordeiro et al. 2014), allowing differences across the species group to be

![Figure 1](image-url)

Figure 1 Points show the number of offspring produced in three replicate crosses of 10 females of each species when crossed to males of different species. Boxes are separated by the paternal strain used in each cross. Points are colored based on the maternal strain used in each cross. Point shape shows the state of fertility of F1 offspring, either both fertile (circle), both sterile (triangle) or only females fertile (square). D. ara = D. arawakana, D. dun = D. dunni, D. nig = D. nigrodunni, D. sim = D. similis. Although we performed all pairwise heterospecific crosses, only crosses which produced offspring are shown on the plot, so fewer than 3 crosses are shown in some cases.
investigated with a higher resolution than the D. melanogaster group allows. Given the variety in levels of divergence and isolation between species (Figure 1, Supplementary Figure 1), we next examined the patterns of divergence between species. Our focus is on the two hybrid crosses which produce some fertile hybrid offspring, such as with D. nigrodunni and D. arawakana, in which one direction of the heterospecific cross produces only female offspring (Figure 1).

**Drosophila arawakana males reduce the lifespan of D. nigrodunni females**

Females and males within a species are able to coevolve: if males evolve reproductive strategies (including seminal fluid proteins) that harm their mates (Chapman 2001; Ravi Ram and Woflner 2005; Ahmed-Braimah et al. 2017), females can then evolve new ways to tolerate those strategies (Haerty et al. 2007). This is not true between species, so we expect that a history of reproductive conflict within species might be evident in heterospecific crosses (Phadnis and Orr 2009). Females mating with heterospecific males may be unequipped to deal with the mating strategies those males have evolved. Such a mismatch could result in reduced survival after mating, or increased stress response in females (Ahmed-Braimah et al. 2020). To search for evidence of sex bias factors causing reduced postmating survival, we established crosses between pairs of species produced some fertile offspring (D. nigrodunni ♀ x D. arawakana ♂, D. arawakana ♀ x D. nigrodunni ♂, D. similis ♀ x D. dunni ♂, D. dunni ♀ x D. similis ♂, Supplementary Table 2). We also established matched crosses within species, and a matched control of virgin (unmated) females. For each cross we recorded the survival of females following 5 days of mating.

In all cases, and consistent with studies in D. melanogaster (Chapman et al. 1993; Wigby and Chapman 2005), unmated flies generally survive longer than mated females, though not always significantly (Figure 2, Cox’s Proportional Hazards Ratio = 1.42, z = 3.868, P = 0.00011). The heterospecific crosses showed no difference from the conspecific crosses for D. similis and D. dunni (Figure 2, Cox Hazards Ratio = 0.931, Ratio z = -0.488, P = 0.62545), though D. similis heterospecifically mated females lived longer than conspecifically mated females (Figure 2, Cox Hazards Ratio = 1.35, z = 2.153, P = 0.03134). In contrast, when D. nigrodunni females are crossed to D. arawakana males, females have significantly decreased survival compared to conspecific crosses and virgin females (Figure 2, Cox Hazards Ratio = 0.489, 0.00

**Figure 2** Survival of females postmating. The proportion of surviving females over time after mating, for each species used in each cross, compared to virgin female survival. Boxes show the survival of females separated by their species, and colored based on the females being unmated (virgin, red), conspecific crossed (yellow, crossed to a male of their own species), heterospecific crossed (blue, crossed to a male of a different species). In the case of heterospecific crosses, D. arauakana is only crossed to D. nigrodunni and D. dunni is only crossed to D. similis. The shaded regions of each line represent the standard error of the survival curve.
As the D. arawakana strain examined was infected with Wolbachia and the D. nigrodunni was not, we cured all strains of bacteria using tetracycline-hydrochloride and repeated the survival assays. All females in this second block have reduced survival compared to the original survival assay, (Supplementary Figure 2, Cox Hazards Ratio = 0.783, $z = -5.654$, $P = 1.56e-08$), suggesting a difference in the two experiments that could be attributed to tetracycline-hydrochloride exposure or a simple block effect. In the tetracycline-hydrochloride exposed flies, we again found reduced survival in the D. nigrodunni ♂ × D. arawakana ♀ cross compared to the conspecific crosses (Supplementary Figure 3, Cox Hazards Ratio = 0.671, $z = -3.815$, $P = 0.000136$), refuting the idea that Wolbachia is causing the postmating lethality observed.

Swelling in the reproductive tract may be associated with the reduced female survival and reduced number of hybrid offspring

Other studies have highlighted a reaction between the seminal fluid of one species with the environment of the female reproductive tract in the other species, called the insemination reaction (Patterson 1947; Grant 1983; Markow and Ankney 1988). In the hours following mating, the reproductive tract swells, and, in some cases, this results in damage to the reproductive tract (Patterson 1947). Given the reduced survival of D. nigrodunni females following mating with D. araukana males and the reduced number of hybrid offspring, we hypothesized that an incompatibility between the diverged seminal fluid proteins and the heterospecific reproductive tract could cause the swelling of the reproductive tract which reduces female survival and the chance of producing hybrid offspring (Patterson 1947; Markow and Ankney 1988; Knowles and Markow 2001; Markow and O’Grady 2008). This swelling may be caused by a reaction between factors in the seminal fluid and the reproductive tract of the female Drosophila, because of the toxic nature of several components in the seminal fluid (Patterson 1947; Markow and Ankney 1988; Knowles and Markow 2001; Markow and O’Grady 2008, Bono et al. 2011).

We established experimental crosses within and between D. araukana and D. nigrodunni. Then, 24 and 48 hours after crossing we dissected the females to identify whether sperm was present in the female reproductive tract (Figure 3A and B), and score for abnormal reproductive tract consistent with the insemination reaction (Figure 3C and D). Interestingly, there were no significant differences between the number of mated females 24 and 48 hours after establishing crosses (Logistic regression: sperm presence ~ collection date: $z = 1.285$, $P = 0.198873$), but we did score significantly fewer mated females in heterospecific crosses versus conspecific crosses, suggesting that fewer successful matings occurred in hybrid pairs and some form of premating isolation between these geographically separated species (Logistic regression: sperm presence ~ cross type: $z = -2.948$, $P = 0.00319$). In several mated females when compared to virgin females, we found a swelling of the reproductive tract consistent with the insemination reaction (Figure 3C). Exclusively in several heterospecifically crossed females, we also saw damaged and destroyed reproductive tracts (Figure 3D). We found a significant excess of swollen/damaged tracts in heterospecifically mated D. nigrodunni compared to conspecific controls (Figure 3E, Logistic regression: swollen tract ~ D. nigrodunni cross type: $z = 4.723$, $P = 2.32e-06$). We found swollen tracts in D. araukana females and found no difference between heterospecific and conspecific females (Figure 3E, Logistic regression: swollen tract ~ D. araukana cross type: $z = 0.493$, $P = 0.622162$). This swelling in the conspecific crosses may be caused by toxic factors in the seminal fluid of D. araukana, as seen in other Drosophila subgenus species (Markow and Ankney 1988; Knowles and Markow 2001; Markow and O’Grady 2008).

Genes involved in copulation and immune defense have high rates of divergence between species

We reasoned that these incompatibilities between species could be caused by a divergence in reproduction proteins. Previous work has suggested that females may be susceptible to severe insemination reactions following hybrid matings (Markow and Ankney 1988; Knowles and Markow 2001). Specifically, that there is an arms race between sexes to block/unblock the female reproductive tract and that females of other species have not evolved to suppress these reactions (Markow and Ankney 1988; Knowles and Markow 2001; Markow and O’Grady 2008). Based on this, we...
sought to examine the levels of divergence and identify rapidly evolving genes between species which could be involved in this intersexual conflict. We sequenced, assembled and annotated the genomes of each species involved in our survey (see Materials and Methods), producing two high quality genomes with high synteny to each other and to D. innubila (Supplementary Tables 1 and 4 and Supplementary Figure 4A), and two assemblies derived from these de novo assemblies. The two de novo assemblies had high BUSCO scores (D. dunnii scored 93.9% complete, 79 fragmented and 93 missing out of 2799 total; D. nigrodunni scored 97.3%: 2721 complete, 37 fragmented and 41 missing out of 2799 total). Consistent with previous findings that failed to detect inversions within the dunnii group (Cordeiro et al. 2014), we found no large structural rearrangements between genomes, and no evidence of fixed inversions between species in the dunnii group (Heed 1962, Cordeiro et al. 2014), though we do find several inversions between the next closest whole genome available, D. innubila on Muller elements B, C and D (D. nigrodunni shown in Supplementary Figure 4B). We annotated the dunnii group genomes using a transcriptome from D. innubila in MAKER (Holt and Yandell 2011) and found between 10752 and 11581 genes in the assembled genomes of each species, most of which show orthology to previously identified genes in D. virilis, D. melanoogaster or D. innubila (Supplementary Table 5) (Hill et al. 2019).

When examining the repetitive content of each species, we found an expansion of Helitrons and long terminal repeat retrotransposons (LTRs) along the D. dunnii/D. similis branch, resulting in higher transposable element (TE) content in these two species compared to D. nigrodunni/D. arawakana (Supplementary Figure 5). We also found species-specific expansions of satellites, particularly in D. arawakana and D. nigrodunni, where ~4% of the genome appears to be satellite sequences exclusive to that species (Supplementary Figure 5).

We identified orthologous genes across species using BLAST (Altschul et al. 1990) with D. innubila as an outgroup when possible. For each group of orthologous genes, we identified the proportion of synonymous (dS) substitutions and amino acid changing, nonsynonymous substitutions (dN) (per possible synonymous or nonsynonymous substitution, respectively) occurring on each branch of the phylogeny using codeML (branch-based approach) (Yang 2007). We also estimated these substitution rates across the entire dunnii group phylogeny (Figure 4A, sites-based approach, M7 and M8) (Yang 2007). This allowed us to calculate dN/dS to identify genes showing signatures of rapid or unconstrained evolution on any branch of the phylogeny, or across the entire tree (Figure 4A). For the dN/dS estimates on each branch, we identified genes in the upper 97.5th percentile for dN/dS in windows of 0.01 dS. dN/dS in D. nigrodunni is significantly correlated with dN/dS in D. arawakana (Figure 4B), as well as in all other pairwise species comparisons (Figure 4, Pearson’s correlation coefficient = 0.844, t = 7.3774, df = 7569, P = 1.786e–13). We find several of the same proteins (or proteins in the same functional group) are rapidly evolving across the entire phylogeny (Figure 4A and Supplementary Table 6). Primarily, copulation proteins (specifically a subset of these, seminal fluid proteins) are overrepresented among the most rapidly evolving genes on every branch of the dunnii group phylogeny (Supplementary Table 6, p < 0.05 after FDR multiple testing correction). Although not significant outliers, we also find that immune recognition proteins, antiviral RNA and piRNA pathways are also rapidly evolving in some species, consistent with arms races between the species and their parasites (Supplementary Table 6).

Rapidly evolving genes may provide clues into the selective forces acting on species since their divergence. For the main species pairs of interest (e.g. D. nigrodunni and D. arawakana) we identified genes in the upper 97.5th percentile for windows of dN/dS in the other species, to find genes rapidly evolving in one species but not the other (Figure 4B). As expected, copulation-associated proteins were in the upper 97.5th percentile for both species, whereas genes in the Toll immune pathway are rapidly evolving in D. arawakana but not D. nigrodunni, conversely the JAK-STAT immune pathway is rapidly evolving in D. nigrodunni but not D. arawakana (Supplementary Table 6, Figure 4B). These results suggest each species may differ in their

**Figure 4** Rates of evolution across the Drosophila dunnii phylogeny, showing non-synonymous divergence versus synonymous divergence across (A) the whole phylogeny and (B) comparing the proportion of non-synonymous to synonymous divergence between D. nigrodunni and D. arawakana. JAK-STAT, Toll and seminal fluid proteins are highlighted due to their enrichments in one or the other species.
pathogen pressure, resulting in context dependent immune evolution, as seen elsewhere in the Drosophila subgenus (Obbard et al. 2009; Hill et al. 2019).

We sought to confirm the rapid evolution of reproductive pathways and immune pathways after controlling for the background rate of evolution. We found the difference between dN/dS for each immune and reproductive gene and genes at neighboring loci on the chromosome (within 100kbp), of similar levels of divergence (+- 0.01 dS). We found significantly elevated rates of evolution of antiviral proteins, copulation proteins and a subset of these, seminal fluid proteins, across the entire phylogeny (Figure 5, one-sided T-test mu = 0, P = 0.0434). We also found a significant correlation between the rates of evolution on the D. arawakana and D. nigrodunni branches for antiviral genes (Pearson’s correlation = 0.795, t = 2.163, P = 0.0288), immune recognition genes (Pearson’s correlation = 0.877, t = 5.791, P = 0.000175) and piRNA genes (Pearson’s correlation = 0.659, t = 3.506, P = 0.00292). The highest average rate of evolution in our survey occurred in seminal fluid proteins on the D. nigrodunni and D. arawakana branches (Figure 5, one-sided t-test, mu = 0, p < 0.05). Consistent with previous results before controlling for background evolution, we find elevated rates of evolution of the Toll signaling pathway in D. arawakana, and JAK-STAT in D. nigrodunni (Figure 5). Interestingly, when comparing the specific genes rapidly evolving between D. nigrodunni and D. arawakana, copulation and seminal fluid genes are mostly evolving at different rates between species (Figure 5), whereas the other rapidly evolving genes are consistent between species (Figures 4B and 5). Consistent with this, we did not find a correlation between measures of dN/dS in D. arawakana and D. nigrodunni copulation genes (Pearson’s correlation = 0.187, t = 1.417, P = 0.162), seminal fluid proteins (Pearson’s correlation = 0.0341, t = 0.224, P = 0.823), JAK-STAT (Pearson’s correlation = 0.183, t = 0.625, P = 0.545) or Toll-signaling proteins (Pearson’s correlation = 0.450, t = 1.334, P = 0.224). This could suggest that distinct copulation/seminal fluid proteins (and immune pathways) are important in each species, as different proteins are rapidly evolving in the different species (Figure 5), and may even suggest (though unlikely) a molecular divergence in the roles these proteins are playing in each species (Haerty et al. 2007).

Using orthology to D. innubila, we also identified duplications relative to these two species in each duni group genome, and specific to each species. Consistent with the estimates in rates of evolution, we found enrichments of duplications in cell motility and copulation (specifically premating behavior) across the entire phylogeny (Supplementary Figure 6 and Supplementary Table 7). We also found enrichments of duplications in Toll signaling genes in D. arawakana (P = 0.000569, enrichment = 5.44). Overall this suggests that the pathways showing elevated levels of nucleotide divergence (namely Toll signalling and copulation genes) also have more copy number variation between species than expected.

**Figure 5** Difference of dN/dS between focal genes in specific functional categories and their nearby background genes, focusing on copulation genes and immune genes. Different copulation proteins and seminal fluid proteins (SFP, a subset of copulation proteins) are rapidly evolving between D. nigrodunni and D. arawakana. These gene categories are assigned based on FlyBase gene ontology list and refer to genes which have previously been shown to play a role in reproduction. A selection of genes in each category is labeled by name in each plot. Please note the different scales on each axis and plot. Plots are labeled with a * if we find a positive correlation between the two axes (P < 0.05).
Drosophila species have served as prominent models in genetics research, including in understanding the divergence between populations and the evolution of species (Coyne and Orr 1989; Orr 2005). This is facilitated by the extensive genetic tools available in the species group to identify the genetic basis of reproductive isolation, both prezygotic and postzygotic (Orr 2005; Phadnis and Orr 2009; Hales et al. 2015; Turissini et al. 2018). Many islands contain endemic species of Drosophila with differing levels of isolation. For example, the island endemics in the D. simulans complex (Cabot et al. 1994; Kliman et al. 2000; Matute and Ayyroles 2014), with D. mauritiana, D. simulans and D. sechellia have served as a rich system for understanding reproductive isolation (Cabot et al. 1994; Kliman et al. 2000). Like the D. simulans complex, the D. dunni species subgroup has radiated across a chain of islands (Heed 1962), though with easier to define species relationships than is seen in the simulans subcomplex (Cabot et al. 1994; Kliman et al. 2000; Matute et al. 2014). Due to the recent radiation of this group, many species pairs in the dunni subgroup produce offspring (Stalker and Streisinger 1953; Heed 1962), some of which are fertile, and so provide a potentially useful model system for dissecting the genetics of reproductive isolation.

Here, we assessed the extent of hybrid incompatibilities between species of the dunni subgroup, focusing on postmating-prezygotic incompatibilities. We then sequenced and assembled the species genomes to identify highly divergent and rapidly evolving genes which could be involved in these incompatibilities. Between D. nigrodunni and D. arauwakana, we found elevated divergence of several immune system pathways, as well as divergence in genes involved in copulation. This rapid evolution of copulation genes (primarily seminal fluid proteins) fits with the reduced survival of females following insemination by a heterospecific male. Consistent with the divergence in the seminal fluid proteins, we found evidence of swelling of the reproductive tract (Knowles and Markow 2001), and a decrease in hybrid mating compared to within species, suggesting both pre and postmating prezygotic incompatibilities. We also found several instances of postzygotic incompatibilities between species, including sterility and male inviability (Figure 1).

Most of the striking differences appear when comparing D. nigrodunni and D. araukowana (Figures 1–5). This pair is slightly less diverged than other pairings within the group (Supplementary Figure 1 and Supplementary Table 3) and are allopatrically separated (Heed 1962; Wilder and Hollocher 2003), allowing for the neutral accumulation of substitutions with a reduced chance of introgression (Coyne and Orr 1989; 2004). Due to this reduced divergence and reduced incidence of incompatibilities (Orr 1995; Welch 2004), we may have caught this species pair at the opportune time where these hybrid incompatible effects are visible (unlike the D. dunni and D. simulans pair which are less diverged, Supplementary Table 3), whereas other species pairs are too far diverged (Figure 1).

The functional annotation of the more diverged genes may also provide us with clues as to how these species are diverging. For example, we identified divergence in the copy number for male mating behavior genes between D. arauwakana and D. nigrodunni (Supplementary Figure 6), and female mating behavior genes are enriched in the upper 97.5th percentile for dN/dS across the whole dunni group phylogeny (Supplementary Table 6, enrichment = 15.44, P = 0.000113). These accumulating differences may cause a divergence in premating behavior, resulting in the reduced rate of hybrid matings scored (Figure 3).

We also see no difference in the proportion of hybrid matings after 24 hours and 48 hours, suggesting that in these cases, if a female has rejected all males, she may not change her mind later, supporting the evolution of some pre-mating isolation between species, despite isolation (Coyne and Orr 2004; Gourbière and Mallet 2010; Turissini et al. 2018). Hybridization between island-endemic flies separated by ~500 kilometers of ocean may be unlikely (Coyne et al. 1982), but selection against hybridization between our focal species and other dunni group species may have led to the evolution of reinforcement against heterospecific mating (Gourbière and Mallet 2010; Turissini et al. 2018).

Alternatively, this pre-mating isolation could have evolved due to the neutral divergence between the two species (Orr and Turelli 2001; Turissini et al. 2018). We also found seminal fluid proteins are rapidly diverging between species (Figures 4 and 5) and found an increased incidence of swollen and deformed reproductive tracts, consistent with an insemination reaction-like effect and a toxic incompatibility between the seminal fluid proteins and their environment (Figures 2 and 3) (Markow and Ankeny 1988; Knowles and Markow 2001). Previous work has highlighted that male expressed genes with the fastest rates of evolution are more likely to contribute to paternal gametic incompatibility (Ahmed-Braimah et al. 2017), which may also be the case here. In fact, studies in other species have also identified postmating-prezygotic incompatibilities as a major cause of isolation between species, even in cases with some gene flow (Gavrilets and Waxman 2002; Gavrilets 2003; Larson et al. 2012; 2013; Ahmed-Braimah 2016; Turissini et al. 2018). A recent study identified the upregulation of the JAK-STAT pathway (a stress response pathway) in Drosophila females following heterospecific mating, likely due to the negative effects of the accessory gland proteins (Ahmed-Braimah et al. 2020). The rapid evolution of JAK-STAT proteins in D. nigrodunni may also be due to this species requiring a well-adapted stress response pathway, given its negative reaction to heterospecific matings (Figures 1–3).

Focusing on specific copulation proteins, we found several proteins are rapidly evolving in all species (Supplementary Table 6). Painless, a transporter protein, is in the upper 97.5th percentile for dN/dS in all four species analyzed. Painless plays a role in the pre-mating sexual receptivity of females, and incompatible hybrid interactions with this protein may explain the reduced success in hybrid mating (Sakai et al. 2009). Similarly, lingerer is also rapidly evolving in D. arauwakana and D. nigrodunni, a protein thought to act as a controller for copulatory organs during courtship and so may affect hybrid mating success (Kunijoishi et al. 2002). We also found several seminal fluid associated proteins are evolving exclusively in one species, such as Esterase-6 and Muscleblind in D. arauwakana or Lectin-64Cb in D. nigrodunni (Figure 5 and Supplementary Table 6). Inferred through mutant phenotypes, these proteins appear to regulate female receptivity post-mating in D. melanogaster (Chapman 2001; Bloch Qazi et al. 2003; Ravi Ram and Wolfner 2005). Though the insemination reaction is not seen in D. melanogaster (Alonso-Pimentel et al. 1994), it is not unreasonable to assume these proteins may play a role in it in other species and could cause the postmating phenotypes we see here (including the toxic effects in hybrid crosses, Figure 3).

There are several potential possible causes for the rapid evolution of copulation proteins seen in this study. The first, as mentioned above, is due to a reproductive conflict between males and females (Markow and Ankeny 1988; Knowles and Markow 2001). As the two sexes benefit from different outcomes of reproduction, each sex will in turn evolve methods to prevent or suppress the reproductive goals of the other sex, resulting in an arms
race scenario (Knowles and Markow 2001). Consistent with this, we found rapid evolution of the seminal fluid proteins, the main tool in male sexual conflict (Figure 4) and seminal fluid induced swelling of the female reproductive tract (Figure 3) (Alonso-Pimentel et al. 1994; Ravi Ram and Wollner 2005). The second possible cause of divergence here is reinforcement (Gourbière and Mallet 2010). The reduced hybrid mating coupled with post-mating isolation seen between several species may imply that reinforcement may drive divergence, though the species studied here are allopatric, so such reinforcement would have to have evolved prior to geographic isolation (Heed 1962). Relaxed selection could also be occurring if sexual selection is less important in these species (Dapper and Wade 2020). We would expect reduced polymorphism and elevated non-synonymous substitutions weighted by non-synonymous polymorphism if an arms race was occurring between species (McDonald and Kreitman 1991; Messer and Petrov 2013), but elevated polymorphism if relaxed selection was occurring (Dapper and Wade 2020). Unfortunately, polymorphism information is not currently available for our focal species to help determine the main force driving evolution.

Several of the functional gene categories identified in this study as highly divergent between species are also promising regions for future study, particularly when focusing on immune evolution. Our findings are consistent with other studies that find immune proteins are more rapidly evolving than background genes (Sackton et al. 2007; Obbard et al. 2009; Shultz and Sackton 2019), consistent with an arms-race between the host and its pathogens. However, in the species studied here, we find several cases of species-specific rapid evolution of an immune pathway, such as the rapid evolution of JAK-STAT in D. nigrodunni, specifically the genes Diedel, lingeer and Socs36E (Figures 4 and 5). As the general stress response pathway, the JAK-STAT pathway is activated following mating in several Drosophila (Hoffmann 2003; Ahmed-Braimah et al. 2020), suggesting it may be involved in reproductive conflict in the dunni group, resulting in its rapid adaptation. Alternatively, as immune pathways are constantly evolving in response to their pathogens, this difference could be explained by differences in immune pathogens in this species group (Sackton et al. 2007; Unckless et al. 2016; Hill et al. 2019). Hypothetically, the lack of any substantive natural Gram-negative bacterial pathogens in D. dunni would result in a lack of rapid evolution (and divergence) in the IMD pathway, the immune pathway associated with resisting infection by Gram-negative bacteria. Although a lack of fungal or Gram-positive bacterial pathogens in D. nigrodunni could result in the lack of evolution of the Toll pathway, but rampant evolution in D. arawakana (Figures 4 and 5).

The repetitive content also appears to be diverging rapidly across this species complex (Supplementary Figure 5). This is commonly seen between diverging species, given the elevated mutation rate/transposition of selfish factors compared to the rest of the genome (Kofler et al. 2012; 2015; Adrion et al. 2019), and has been implicated in the formation of hybrid incompatibilities for several species (Satyaki et al. 2014). Consistent with this we found several species specific TE families in the dunni complex. However, we did not find a significant excess of dysgenic ovaries in hybrid females compared to normal females (Fisher’s exact test p-value > 0.05 for all cases). Several cases of hybrid incompatibilities caused by differences in TE content results in sterility caused by maternally inherited factors over paternally inherited (as is usually seen). This may be due to the absence of maternally loaded silencing RNAs against specific TEs (Bingham et al. 2002; Aravin et al. 2007; Brennecke et al. 2008). If this were the case, we would expect the hybrid sterility to be in the opposite direction to what we observe, with sterile females (Figure 1, Supplementary Figure 5) (Kidwell et al. 1977), and so do not expect the hybrid incompatibilities seen here to be caused by repetitive content. However, this is a simplistic view of the effects of transposon activity on hybrid fertility, given the complex hybrid dysgenesis cases seen in D. virilis (Petrov et al. 1995; Evgen’ev et al. 1997; Erwin et al. 2015), and even the complex cases of tolerance to dysgenesis seen in the supposedly simple case in D. melanogaster (Kelleher et al. 2018), so may require further study to fully understand if TEs play a role in the divergence of the dunni complex.

Conclusions

Overall, our findings suggest that the rapid divergence of reproductive genes may have led to incompatibilities between species in the dunni group, such as the insemination reaction associated with reduced female survival. We also found multiple areas for further investigation in the D. dunni group, either in immune evolution or continuing to investigate the speciation in this species group, suggesting promise in the future of research for this group.

Data availability

All data used in the analyses in this study are available at figshare (https://doi.org/10.25387/g3.13865258). Sequencing information and assembled genomes used in this study is available in the SRA BioProject PRJNA661260 (Supplementary Table 1 has details).

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Author contributions

TH was involved in experimental design, the genomic and bioinformatic analysis, experimental crosses and analysis of results, and writing the manuscript. HLRS was involved in experimental crosses between species and survival assays. RLU was involved in experimental design and writing the manuscript. 

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References
Adron JR, Begun DJ, Hahn MW. 2019. Patterns of transposable element variation and clinality in Drosophila. Mol Ecol. 28:1523–1536.
Ahmed-Braimah Y. 2016. Multiple genes cause postmating prezygotic reproductive isolation in the drosophila virilis group. G3 (Bethesda). 6:4067–4076.
Ahmed-Braimah YH, Unckless RL, Clark AG. 2017. Evolutionary dynamics of male reproductive genes in the Drosophila virilis subgroup. G3 (Bethesda). 7:3145–3155.
Ahmed-Braimah YH, Wolner MF, Clark AG. 2020. Differences in post-mating transcriptional responses between conspecific and heterospecific matings in Drosophila. Mol Biol Evol. msaa264:1–14.
Alonso-Pimentel H, Tolbert LP, Heed WB. 1994. Ultrastructural examination of the insemination reaction in Drosophila. Cell Tissue Res. 275:467–479.
Altshul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol. 215:403–410.
Andres JA, Maroja LS, Harrison RG. 2008. Searching for candidate speciation genes using a proteomic approach: seminal proteins in field crickets. Proc R Soc B. 275:1975–1983.
Aravin AA, Hannon GJ, Brennecke J. 2007. The piwi-piRNA pathway provides an adaptive defense in the transposon arms race. Science. 318:761–764.
Bingham PM, Kidwell MG, Rubin GM. 1982. The molecular basis of P-M hybrid dysgenesis: the role of the P element, a P-strain-specific transposon family. Cell. 29:995–1004.
Bloch Qazi MC, Heifetz Y, Wollner MF. 2003. The developments between gametogenesis and fertilization: ovulation and female sperm storage in drosophila melanogaster. Developmental Biology. 256:195–211.
Bono JM, Matzkin LM, Hoang K, Brandsmeier L. 2015. Molecular evolution of candidate genes involved in post-mating-prezygotic reproductive isolation. J Evol Biol. 28:403–414.
Bono JM, Matzkin LM, Kelleher ES, Markow TA. 2011. Postmatting transcriptional changes in reproductive tracts of con- and heterospecifically mated Drosophila mojavensis females. Proc Natl Acad Sci U S A. 108:7878–7883.
Brekke TD, Good JM. 2014. Parent-of-origin growth effects and the evolution of hybrid inviability in dwarf hamsters. Evolution. 68: 3134–3148.
Brennecke J, Malone CD, Aravin AA, Sachidanandam R, Stark A, et al. 2008. An epigenetic role for maternally inherited piRNAs in transposon silencing. Science (New York, N.Y.). 322:1387–1392.
Brisson JA, Wilder J, Hollocher H. 2006. Phylogenetic analysis of the Cardini group of drosophila with respect to changes in pigmenta- tion. Evol. 60:1228–1241.
Buffalo V. 2018. Scythe.
Butlin R, Debelle A, Kerth C, Snook RR, Beukeboom LW, et al. 2012. What do we need to know about speciation? Trends Ecol Evol. 27: 27–39.
Cabot L, Davis AW, Johnson NA, Wu C-I. 1994. Genetics of reproductive isolation in the Drosophila simulans clade: complex epistasis underlying hybrid male sterility. Genetics. 137:175–189.
Castillo DM, Moyle LC. 2019. Conspecific sperm precedence is reinforced, but postcopulatory sexual selection weakened, in sympatric populations of Drosophila. Proc R Soc B. 286:20182535.
Cenzi De Ré F, Loreto ELS, Robe LJ. 2010. Gene and species trees reveal mitochondrial and nuclear discordance in the Drosophila cardini group (Diptera: Drosophilidae). Invertebrate Biology. 129:353–367.
Chakraborty M, Baldwin-Brown J G, Long A D, Emerson J J. 2016. Contiguous and accurate de novo assembly of metazoan genomes with modest long read coverage. Nucleic Acids Res. 44:e147.
Chapman T. 2001. Seminal Fluid-mediated Fitness traits in Drosophila. Heredity. 87:511–521.
Chapman T, Hutchings J, Partridge L. 1993. No reduction in the cost of mating for Drosophila melanogaster females mating with spermless males. Proc R Soc. 253:211–217.
Cordeiro J, De Toni DC, de Souza da Silva G, da Silva Valente VL. 2014. Chromosomal evolution in the Drosophila cardini group (Diptera: Drosophilidae): photomaps and inversion analysis. Genetica. 142:461–472.
Coyne JA, Orr HA. 2004. Speciation.
Coyne JA, Boussey IA, Prout T, Bryant SH, Jones JS, et al. 1982. Long distance migration of Drosophila. Am Natur. 119:589–595.
Coyne JA, Orr HA. 1989. Patterns of speciation in Drosophila. Evolution. 43:362–381.
Dapper AL, Wade MJ. 2020. Relaxed selection and the rapid evolution of reproductive genes. Trends Genet. 36:640–649.
Darling ACE, Mau B, Blattner FR, Perna NT. 2004. Mauve: multiple alignment of conserved genomic sequence with rearrangements mauve: multiple alignment of conserved genomic sequence with rearrangements. Genome Res. 14:1394–1403.
DePristo MA, Banks E, Poplin R, Garimella KV, Maguire JR, et al. 2011. A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat Genet. 43:491–498.
Eden E, Navron R, Steinfeld I, Lipson D, Yakhini Z. 2009. GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. BMC Bioinformatics. 10:1–7.
Erwin AA, Galdos MA, Wickersheim ML, Harrison CC, Marr KD, et al. 2015. piRNAs are associated with diverse transgenerational effects on gene and transposon expression in a hybrid dysgenic syndrome of D. virilis. PLoS Genet. 11:e1005332–33.
Evgen’ev MB, Zelentsova H, Shostak N, Kozitsina M, Barskyi V, et al. 1997. Penelope, a new family of transposable elements and its possible role in hybrid dysgenesis in Drosophila virilis. Proc Natl Acad Sci USA. 94:196–201.
Frank SA. 1991. Divergence of meiotic drive-suppression systems as an explanation for sex-biased hybrid sterility and inviability. Evolution. 45:262–267.
Gavrilets S. 2003. Perspective: models of speciation: what have we learned in 40 years? Evol. 57:2197–2215.
Gavrilets S, Waxman D. 2002. Sympatric speciation by sexual conflict. PNAS. 99:10533–10538.
Glór RE, Losos JB, Larson A. 2005. Out of Cuba: overwater dispersal and speciation among lizards in the Anolis carolinensis subgroup. Mol Ecol. 14:2419–2432.
Goubert C, Modolo L, Vieira C, Moro CV, Mavingui P, et al. 2015. De novo assembly and annotation of the Asian tiger mosquito (Aedes albopictus) repeatome with dnaPipeTE from raw genomic reads and comparative analysis with the yellow fever mosquito (Aedes aegypti). Genome Biol Evol. 7:e1192–1205.
Gourbière S, Mallet J. 2010. Are species real? The shape of the species boundary with exponential failure, reinforcement, and the *missing* snowball. Evolution. Int J Organ Evolution. 64:1–24.”
Matute DR, Comeault AA, Earley E, Serrato-Capuchina A, Peede D. 2020. Rapid and Predictable Evolution of Admixed Populations Between Two *Drosophila* Species Pairs. Genetics. 214:211–230.

Matute DR, Gavin-Smyth J, Liu G. 2014. Variable post-zygotic isolation in *Drosophila melanogaster*/D. simulans hybrids. J Evol Biol. 27:1691–1705.

McDonald JH, Kreitman M. 1991. Adaptive protein evolution at the Adh locus in *Drosophila*. Nature. 351:652–654.

McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, et al. 2010. The genome analysis toolkit: a mapreduce framework for analyzing next-generation DNA sequencing data. Proc Int Conf Intellect Cap Knowledge Manage Organ Learn. 20:1297–1303.

McKinnon JS, Ruddle HD. 2002. Speciation in nature: the three spine stickleback model systems. Trends Ecol Evol. 17:480–488.

Mendes F, Hahn MW. 2016. Gene tree discordance causes apparent substitution rate variation. Syst Biol. 65:711–721.

Messer PW, Petrov DA. 2013. The McDonald-Kreitman test and its extensions under frequent adaptation: problems and solutions. Proc Natl Acad Sci USA. 110:8615–8620.

Miller WJ, Ehrman L, Schneider D. 2010. Infectious speciation revisited: impact of symbiont-depletion on female fitness and mating behavior of *Drosophila paulistorum*. PLoS Pathog. 6:e1001214.

Moyle LC, Nakazato T. 2010. Hybrid incompatibility "snowballs" between Solanum species. Science (New York, NY). 329:1521–1523.

Narasimhan V, Danecek P, Scally A, Sivachenko A, Cibulskis K, et al. 2010. The genome analysis toolkit: a mapreduce framework for analyzing next-generation DNA sequencing data. Proc Int Conf Intellect Cap Knowledge Manage Organ Learn. 20:1297–1303.

Patterson JT. 1947. The insemination reaction and its bearing on the problem of speciation in the Mulleri subgroup. Univ Tex Publ. 87:1521–1523.

Payseur BA, Rieseberg LH. 2016. A genomic perspective on hybridization and speciation. Mol Ecol. 25:2337–2360.

Petrov D, A, Schutzman JL, Hartl DL, Lozovskaya ER. 1995. Diverse transposable elements are mobilized in hybrid dysgenesis in *Drosophila virilis*. Proc Natl Acad Sci USA. 92:8050–8054.

Phadnis N, Orr HA. 2009. A single gene causes both male sterility and segregation distortion in *Drosophila* hybrids. Science. 323:376–379.

Pomiankowski A, Hurst LD. 1993. Genomic conflicts underlying Haldane’s rule. Genetics. 133:425–432.

Presgraves DC. 2007. Speciation genetics: epistasis, conflict and the origin of species. Curr Biol. 17:R125–127.

R Core Team. 2013. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

Rausch T, Zichner T, Schlattl A, Stutz AM, Benes V, et al. 2012. DELLY: structural variant discovery by integrated paired-end and split-read analysis. Bioinformatics. 28:1333–1339.

Ravi Ram K, Wolfer MF. 2005. Sustained post-mating response in *drosophila melanogaster* requires multiple seminal fluid proteins. PLoS Genet. e238.

Rowe M, Whittington E, Borziak K, Ravinet M, Eroukhmanoff F, et al. 2020. Molecular diversification of the seminal fluid proteome in a recently diverged passerine species pair. Mol Biol Evol. 37:488–506.

Ruan J, Li H. 2020. Fast and accurate long-read assembly with wtdbg2. Nat Methods. 17:155–158.

Sackton TB, Lazzaro BP, Schlenke TA, Evans JD, Hultmark D, et al. 2007. Dynamic evolution of the innate immune system in *Drosophila* Nat Genet. 39:1461–1468.

Sakai T, Kasuya J, Kitamoto T, Aigaki T. 2009. The *Drosophila* TRPA channel, painless, regulates sexual receptivity in virgin females. Genes Brain Behav. 8:546–557.

Satyaki PR, Cuykendall TN, Wei KH, Brideau NJ, Kwak H, et al. 2014. The Hmr and Lhr hybrid incompatibility genes suppress a broad range of heterochromatic repeats. PLoS Genet. 10:e1004240.

Shultz A, Sackton TB. 2019. Immune genes are hotspots of shared positive selection across birds and mammals. eLife. 8:e41815.

Simão FA, Waterhouse RM, Ioannidis P, Kriventseva EV, Zdobnov EM. 2015. BUSCO: Assessing genome assembly and annotation completeness with single-copy orthologs. Bioinformatics. 31:3210–3212.

Smit AFA, Hubley R. 2008. RepeatModeler Open-1.0. Smit AFA, Hubley R. Green, P. 2013–2015. RepeatMasker Open-4.0, pp. RepeatMasker.

Stalker HD, Streisinger D. 1953. Taxonomy and hybridization in the Cardini Group of *Drosophila*. 46:343–358.

Team RC. 2013. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

Thomas PD, Campbell MJ, Kejariwal A, Mi H, Karlak B, et al. 2003. PANTHER: a library of protein families and subfamilies indexed by function. Genome Res. 13:2129–2141.

Turissini DA, McGirr JA, Patel SS, David JR, Matute DR. 2018. The rate of evolution of postmating-prezygotic reproductive isolation in *drosophila*. Mol Biol Evol. 35:312–334.

Unckless RL, Howick VM, Lazzaro BP. 2016. Convergent balancing selection on an antimicrobial peptide in *Drosophila*. Curr Biol. 26:257–262.

Walker BJ, Abeel T, Shea T, Priest M, Abouelliel A, et al. 2014. Pilon: An integrated tool for comprehensive microbial variant detection and genome assembly improvement. PLoS ONE. e112963.9.

Welch JJ. 2004. Accumulating Dobzhansky-Muller incompatibilities: reconciling theory and data. Evol. 58:1145–1156.

Wickham H. 2016. *ggplot2*. Elegant Graphics for Data Analysis. New York: Springer-Verlag.
Wigby S, Chapman T. 2005. Sex peptide causes mating costs in female Drosophila melanogaster. Curr Biol. 15:316–321.

Wilder J. A, Hollocher H. 2003. Recent radiation of endemic Caribbean Drosophila of the dunni subgroup inferred from multilocus DNA sequence variation. Evol. 57:2566–2579.

Yang Z. 2007. PAML 4: phylogenetic analysis by maximum likelihood. Mol Biol Evol. 24:1586–1591.

Zhang H, Meltzer P. and Davis, S 2013. RCircos: an R package for Circos 2D track plots.

Zhou Q, Bachtrog D. 2015. Ancestral chromatin configuration constrains chromatin evolution on differentiating sex chromosomes in Drosophila. PLoS Genet. 11:e1005331–21.

Zhou Q, Zhu H-M, Huang Q-F, Zhao L, Zhang G-J, et al. 2012. Deciphering neo-sex and B chromosome evolution by the draft genome of [i]Drosophila albomicans[/i]. BMC Genomics. 13:109.

Zhou W, Rousset F, O’Neill S. 1998. Phylogeny and PCR-based classification of Wolbachia strains using wsp gene sequences. Proc R Soc Lond B. 265:509–515.

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