Evolution of Mechanisms that Control Mating in *Drosophila* Males

Graphical Abstract

- **Diverged ~3 million years ago**
  - *D. melanogaster*
  - *D. simulans*
  - **Ppk25**
  - Courting conspecifics
  - Courting other fly species
  - Eating bitter tastants
  - **Gr32a**

**Highlights**

- Gr32a and Gr33a do not inhibit interspecies or intermale mating by male *D. simulans*
- Gr32a and Gr33a inhibit feeding of bitter tastants by *D. simulans*
- Ppk25 promotes mating with conspecific females by male *D. simulans*
- Pathways that promote or inhibit mating have evolved differentially in *D. simulans*

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**In Brief**

Mechanisms that inhibit interspecies mating are critical to reproductive isolation of species. Ahmed et al. show that Gr32a, a chemoreceptor that inhibits interspecies courtship by *D. melanogaster* males, does not inhibit this behavior in the closely related *D. simulans*, indicating rapid evolution of peripheral sensory mechanisms that preclude interspecies breeding.
Evolution of Mechanisms that Control Mating in Drosophila Males

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INTRODUCTION

A species can be defined as a set of organisms that share a gene pool and breed with one another (Darwin, 1860; Dobzhansky, 1937; Mayr, 1988). The lack of interspecies breeding preserves advantages conferred by species-specific allele combinations (Mayr, 1988; Mayr and Dobzhansky, 1945; Orr, 2005; Orr et al., 2004), and mechanisms that preclude interbreeding must evolve rapidly to facilitate reproductive isolation between closely related species (Coyne and Orr, 1989; Mendelson, 2003). Individuals from closely related species rarely attempt to mate, suggesting that neural pathways underlying behavioral barriers to interbreeding must also evolve rapidly. How such neural pathways evolve is poorly understood.

Drosophilids provide a facile model for studies on how neural pathways have evolved. There are ~1,500 drosophilid species, many of which co-exist in overlapping habitats (Jezovit et al., 2017; Markow, 2015). They engage in species-typical stereotyped courtship rituals, and many genetic and neural pathways that regulate courtship of D. melanogaster are well defined (Bastock and Manning, 1955; Clowney et al., 2015; Demir and Dickson, 2005; Gill, 1963; Greenspan and Ferveur, 2000; Hall, 1978, 1994; Hotta and Benzer, 1976; Kalman et al., 2015; Kohatsu et al., 2011; Lin et al., 2016; Manoli et al., 2005; Pavlou and Goodwin, 2013; Ryner et al., 1996; Spieth, 1952; Thistle et al., 2012; Tootoonian et al., 2012). We previously demonstrated that sensory neurons expressing the gustatory chemoreceptor Gr32a are necessary to inhibit courtship displays toward the closely related D. simulans, which last shared an ancestor with D. melanogaster ~3 million to 5 million years ago (mya) (David et al., 2007; Tamura et al., 2004). D. simulans and D. melanogaster co-exist globally (reviewed in Jezovit et al., 2017) and are very similar in behavior and appearance (Sturtevant, 1919, 1920). Here we have examined how the Gr32a chemosensory pathway has evolved to inhibit interspecies courtship in D. simulans.

SUMMARY

Genetically wired neural mechanisms inhibit mating between species because even naive animals rarely mate with other species. These mechanisms can evolve through changes in expression or function of key genes in sensory pathways or central circuits. Gr32a is a gustatory chemoreceptor that, in D. melanogaster, is essential to inhibit interspecies courtship and sense quinine. Similar to D. melanogaster, we find that D. simulans Gr32a is expressed in foreleg tarsi, sensorimotor appendages that inhibit interspecies courtship, and it is required to sense quinine. Nevertheless, Gr32a is not required to inhibit interspecies mating by D. simulans males. However, and similar to its function in D. melanogaster, Pppk25, a member of the Pickpocket family, promotes conspecific courtship in D. simulans. Together, we have identified distinct evolutionary mechanisms underlying chemosensory control of taste and courtship in closely related Drosophila species.

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We found that tarsiless males (Fan et al., 2013; Manning, 1959; Spieth, 1952). Similarly, D. virilis males courted females (Fan et al., 2013; Manning, 1959; Spieth, 1952), and surgical extirpation of tarsi very early in courtship. This tapping restricts courtship of D. melanogaster males to court potential mates with their foreleg tarsi, and surgical extirpation of tarsi enables males to court conspecific females (Figure 1A). Tarsiless males also tap potential mates with foreleg tarsi (Manning, 1959; Spieth, 1952), and surgical extirpation of these tarsi enables D. simulans males to court D. melanogaster females (Fan et al., 2013; Manning, 1959; Spieth, 1952). We found that tarsiless D. simulans males also courted D. virilis females, a distantly related drosophilid species. The Chemosensory Pathway that Inhibits Interspecies Courtship Is Conserved

D. melanogaster males tap potential mates with their foreleg tarsi very early in courtship. This tapping restricts courtship to conspecífics because males lacking foreleg tarsi court conspecifics as well as other drosophilid species (Figure 1A) (Fan et al., 2013; Manning, 1959; Spieth, 1952). Similarly, D. simulans males also tap potential mates with foreleg tarsi (Manning, 1959; Spieth, 1952), and surgical extirpation of these tarsi enables D. simulans males to court D. melanogaster females (Fan et al., 2013; Manning, 1959; Spieth, 1952). We found that tarsiless D. simulans males also courted D. virilis females, a distantly related drosophilid (shared last common ancestor ~40 mya), and conspecific males (Figures 1B–1E and S1A). Tarsiless D. simulans males, like their D. melanogaster counterparts (Fan et al., 2013), also courted conspecific females (Figures 1B and 1C). Such conspecific courtship was performed by the tarsiless males at reduced intensity, likely because of reduced effectiveness in pursuing females or from loss of tarsal neurons that promote courtship. However, loss of tarsi did not lead to overall reduction in locomotor activity during conspecific courtship (Figure S1I); tarsiless males did show a small increase in locomotor activity when paired with D. melanogaster females (Figure S1J), most likely because they persisted in courting the females despite being rejected. Regardless, tarsiless D. simulans males, similar to their D. melanogaster counterparts, courted other species.

Gr32a Expression Is Conserved in D. simulans Foreleg Tarsi

Gr32a is expressed in sensory neurons in distal foreleg tarsi of D. melanogaster (Koganezawa et al., 2010; Miyamoto and Amrein, 2008; Moon et al., 2009; Scott et al., 2001; Thistle et al., 2012; Thorne et al., 2004), and it is required to detect contact-dependent cues on other species and to inhibit interspecies courtship (Fan et al., 2013) (Figure 2A). The genome of D. simulans encodes an ortholog of Gr32a (Drosophila 12 Genomes Consortium et al., 2007) (with four coding exons in both species and 97.8% identity in the encoded protein; Data S1), and we wondered whether this gene is expressed in foreleg tarsi of this species. The ~3.8 kb of D. melanogaster genomic DNA 5’ of the start codon is sufficient to drive reporter expression in subsets of neurons in...
chemosensory organs known to express Gr32a (Scott et al., 2001; Wang et al., 2004). Similar stretches of genomic DNA are also sufficient to drive reporter expression of other Grs (Weiss et al., 2011), indicating a conserved regulatory logic of expression for this gene family in *D. melanogaster*. We subcloned ∼3.8 kb of genomic DNA upstream of the *D. simulans* Gr32a start codon and used it to drive GAL4 expression (Gr32asim-GAL4) in transgenic *D. simulans* and *D. melanogaster* flies (Figure 2B). Transgene expression was visualized via the fluorescent reporter citrine (Inagaki et al., 2014) (Figures 2C and 2D). We observed citrine expression in three or four neurons in T4–T5 tarsal segments of *D. simulans* and *D. melanogaster*, demonstrating that regulatory sequences in the *D. simulans* Gr32a locus drive reporter expression in foreleg tarsi of both species (Figures 2C, 2D, and 2G). Moreover, the projections of Gr32a sensory neurons in the subesophageal zone (SEZ) appeared similar between the two species (Figures S1K and S1L), indicative of a shared peripheral expression pattern (Wang et al., 2004).

We next tested whether the ∼3.8 kb regulatory DNA sequence from these two species drives expression in the same tarsal neurons. We generated *D. melanogaster* flies harboring GAL4 under control of conspecific ∼3.8 kb DNA sequence 5′ of Gr32a such that this transgene (Gr32anet-GAL4) was inserted into the same landing site that we had used for Gr32asim-GAL4 (Figures 2B, 2D, and 2F). Importantly, Gr32anet-GAL4 regulated reporter expression in *D. melanogaster* foreleg tarsi, as described previously for other GAL4 alleles of Gr32a (Fan et al., 2013; Miyamoto and Amrein, 2008; Moon et al., 2009; Scott et al., 2001). In *D. melanogaster* flies bearing both Gr32asim-GAL4 and Gr32anet-GAL4, we observed a similar number of citrine+ foreleg tarsal neurons compared with flies bearing these GAL4 drivers individually (Figure S1C). Together, these data are consistent with the notion that the upstream regulatory region of Gr32a in

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**Figure 2. A Regulatory Region in the Gr32a Locus Is Functionally Conserved**

(A) We sought to determine whether, similar to *D. melanogaster*, Gr32a was expressed in *D. simulans* foreleg tarsi.
(B) Schematic of transgenic constructs using a DNA sequence 5′ of Gr32a start codon from *D. simulans* (orange) and *D. melanogaster* (blue) to drive GAL4 expression. Sequence identity in this region between the two species is noted by solid orange color.
(C–F) Gr32asim-GAL4 (C and D) and Gr32anet-GAL4 (E and F) each drive comparable citrine expression in distal tarsal segments T4 and T5 in both *D. simulans* (C and E) and *D. melanogaster* (D and F) male forelegs.
(G) Quantification of data shown in histological panels (C–F). Mean ± SEM. Each circle denotes the number of citrine+ cells per male foreleg tarsi per genotype. n = 11–18 per genotype. Scale bar, 50 μm. See also Table S1 and Figure S1.
Figure 3. Gr32a Is Not Required to Inhibit Interspecies Courtship but Is Essential for Quinine Sensing in D. simulans

(A) We tested whether, similar to D. melanogaster males, Gr32a inhibits interspecies courtship by D. simulans males.

(B and C) WT and Gr32a mutant D. simulans males court conspecific but not D. melanogaster or D. virilis females.

(D and E) WT and Gr32a mutant D. simulans males show similar low levels of courtship toward conspecific males.

(F) We tested whether, similar to D. melanogaster, Gr32a inhibits feeding on quinine-containing food in D. simulans.

(G) Schematic of feeding assay for starved D. simulans given choice of colored food containing sucrose or sucrose and quinine. Flies with blue, red, purple, or no food dye colored abdomens were enumerated after exposure to food for 90 min.

(legend continued on next page)
the two species is functionally conserved and sufficient to drive expression in the same foreleg tarsi neurons of *D. melanogaster*.

We next tested whether the ~3.8 kb genomic DNA 5’ of *D. melanogaster* Gr32a start codon would drive expression in foreleg tarsal neurons of *D. simulans*. We inserted Gr32a<sup>mel-GAL4</sup> into the landing site we used to generate *D. simulans* flies bearing Gr32a<sup>sim-GAL4</sup> (Figures 2B and 2E). We observed reporter expression in three or four neurons restricted to T4-T5 tarsal segments of *D. simulans* in a pattern mirroring that observed in *D. simulans* bearing Gr32a<sup>sim-GAL4</sup> (Figures 2C, 2E, and 2G). Given that all GAL4 and UAS transgenes we built in *D. simulans* were inserted into a single landing site that afforded us reliable and non-leaky expression, we could not directly test whether the same neurons were labeled by Gr32a<sup>mel-GAL4</sup> and Gr32a<sup>sim-GAL4</sup> in this species. Nevertheless, our findings strongly suggest that similar cis and trans regulatory features regulate Gr32a expression in foreleg tarsi of the two species.

We find that the ~3.8 kb of regulatory genomic DNA is conserved in multiple insects (mean nucleotide conservation phyloP score = 1.4; see Figures S1D–S1F). Coding exons for another gene (*D. melanogaster* CG6201) contribute to this sequence similarity, but some of the most conserved blocks of sequence are intergenic regions (Figure S1E). Overall, nucleotide substitutions have occurred in this region at 42.5% the rate of *D. simulans* sequences in the first coding exon (Figures S1H and S1I). Within *D. melanogaster* and *D. simulans*, ~95% of the DNA sequence is identical across this ~3.8 kb region. To examine sequence differences at single-nucleotide resolution, we tested each position for a faster or slower rate of DNA substitutions in *D. melanogaster* than expected, given the rate in *D. simulans* and 25 other insects. We also conducted the comparable test for *D. simulans*. This analysis revealed that few bases in the ~3.8 kb region are evolving faster than expected (>99% bases with phyloP score > -2; Figures S1G and S1H). Because the ~3.8 kb region is highly conserved and *D. melanogaster* and *D. simulans* diverged from a common ancestor only recently, it was difficult to detect whether this stretch of DNA is evolving slower than expected subsequent to speciation from this shared ancestor. Together, our findings show that this ~3.8 kb region is conserved in sequence and function in *D. melanogaster* and *D. simulans* such that it is sufficient to drive expression in neurons of foreleg tarsi.

Gr32a and Gr33a Are Essential to Inhibit Interspecies Courtship in *D. simulans* Males

We tested whether Gr32a was essential to inhibit interspecies courtship in *D. simulans* males (Figure 3A). We targeted distinct sequences in the first coding exon of *D. simulans* Gr32a to generate three different mutant alleles via the CRISPR/Cas9 system (Figures S2A–S2C). Two of the alleles (Gr32a<sup>1170</sup> and Gr32a<sup>1256</sup>) are predicted to lead to 10 and 26 bp deletions in the first coding exon that result in a frameshift and premature stop codon; these likely encode a non-functional Gr32a chemoreceptor protein (Figures S2C and S2D). The third allele (Gr32a<sup>1141</sup>) has a 141 bp deletion that is predicted to eliminate 47 amino acids from the predicted N-terminal intracellular domain of this chemoreceptor (Figures S2B–S2D, S2F, and S2G). We next tested *D. simulans* males homozygous mutant for these Gr32a alleles for courtship displays toward conspecics and members of other species. We observed that each of the three mutants courted conspecific females similar to WT controls (Figures 3B and 3C). Moreover, these mutants did not increase courtship toward conspecific males or *D. melanogaster*, *D. yakuba*, or *D. virilis* females (Figures S3B–S3E). Our findings indicate a divergence in behavioral function of Gr32a between *D. simulans* and *D. melanogaster*, a conclusion consistent with previous sequence analyses showing that bitter-sensing Grs such as Gr32a may be evolving rapidly (Gardiner et al., 2009; McBride et al., 2007). In summary, Gr32a mutant *D. simulans* males do not show elevated courtship toward other species, a finding in sharp contrast to Gr32a-null *D. melanogaster* males, which court other species avidly (Fan et al., 2013).

Gr33a is co-expressed with Gr32a in foreleg tarsi in *D. melanogaster*, and it is required to inhibit intermale but not interspecies courtship in males of this species (Fan et al., 2013; Moon et al., 2009). Gr33a is also encoded in the *D. simulans* genome (Drosophila 12 Genomes Consortium et al., 2007), and we wondered if this chemoreceptor had evolved to inhibit interspecies courtship in this species. Using CRISPR/Cas9, we generated two mutant alleles of Gr33a, one with a 10 bp deletion (Gr33a<sup>1256</sup>) that leads to a frameshift and premature stop codon and the other encompassing an in-frame deletion (96 bp, Gr33a<sup>1286</sup>)(Figures S3A–S3D). Male *D. simulans* mutant for each of these alleles courted conspecific females similar to WT controls and did not increase courtship toward conspecific males or *D. melanogaster*, *D. yakuba*, or *D. virilis* females (Figures S3E–S3H). Together, our results indicate that chemosensory receptor-mediated inhibition of courtship toward reproducitively-futile targets (conspecific males and members of other species) has diverged between the closely related *D. melanogaster* and *D. simulans*.

Both Gr32a and Gr33a Are Required in *D. simulans* to Detect Quinine

In *D. melanogaster*, Gr32a and Gr33a are also essential for a behavioral aversion to quinine, a bitter tastant (Lee et al., 2010; Jordt and Julius, 2002; Prieto-Godino et al., 2017; Wisotzky et al., 2011). Given the divergence of behavioral function of
Gr32a between D. melanogaster and D. simulans, we wondered if Gr32a and Gr33a were required in D. simulans for a response to quinine (Figures 3F and S3I). We tested this in a feeding preference assay in which starved flies were offered a choice between food containing a low concentration of sugar (1 mM sucrose) or a high concentration of sugar (5 mM sucrose) spiked with quinine (0.5 mM) (Montell, 2009; Moon et al., 2009; Tanimura et al., 1982) (Figure 3G). WT D. simulans preferred feeding on the low concentration of sugar, whereas flies mutant for either Gr32a or Gr33a showed reduced preference for feeding on sugar alone (Figures 3H and S3J). Although all mutant lines showed a loss of preference for feeding on sugar alone, there was some variability in the phenotypes observed for the different alleles. Such variability likely resulted from subtle differences in the assay conditions or genetic background; consistent with this notion, there was no statistical difference in behavior between flies bearing the largest and smallest deletions for both genes. It is possible that all D. simulans Gr32a and Gr33a mutations we have generated disrupt sensing quinine but not chemosensory cues from other species, a notion that could be tested when deficiencies spanning Gr32a and Gr33a become available in this species. Our present findings show that quinine sensing via Gr32a and Gr33a is conserved between D. melanogaster and D. simulans.

Ppk25 Promotes Conspecific Courtship in D. simulans Males

Our findings show that chemosensory receptor mechanisms that inhibit courtship of reproductively futile targets in D. melanogaster are not used in D. simulans. We wondered whether genetic loci that promote courtship had also differentiated between these two species. Many loci promote courtship of D. melanogaster males toward conspecific females (reviewed in Dickson, 2008; Yamamoto and Koganemura, 2013). We chose to test the function of the Ppk25 pickpocket ion channel subunit that is expressed in foreleg tarsi chemosensory neurons and appears to exclusively promote courtship in D. melanogaster (Figure 4A) (Clowney et al., 2015; Kaliman et al., 2015; Lin et al., 2005; Starostina et al., 2012; Vijayan et al., 2014). We generated two alleles of Ppk25 in D. simulans via CRISPR/Cas9, a 2 bp insertion and a 4 bp deletion in the first coding exon, that are predicted to lead to frameshifts and premature stop codons and are likely to be null mutations (Figures S4A–S4D). D. melanogaster Ppk25 is required for male courtship in the dark (Boll and Noll, 2002; Jezovit et al., 2017; Kohatsu and Yamamoto, 2015; Krstic et al. 2009; Lin et al., 2005; Spieth, 1974). Unlike D. melanogaster, D. simulans males court conspecific females vigorously only under bright illumination (Grossfeld, 1971; Jezovit et al., 2017) (Figures S4E and S4F). Furthermore, this requirement for bright illumination in D. simulans overrides courtship disinhibition following tarsectomy (Figure S4G). We tested whether Ppk25 modulated courtship by D. simulans males in bright light or dark conditions. D. simulans males mutant for Ppk25 showed reduced courtship of conspecific females in the dark (Figures 4B and 4C). These mutants also showed subtle, but significant, reduction in courtship under bright illumination, suggesting a more stringent requirement for Ppk25 in courtship in this species (Figures 4D and 4E). D. simulans males mutant for Ppk25 did not display elevated courtship to other drosophilids (Figures S4H and S4I), indicating that it does not function in this species to inhibit interspecies courtship. In fact, we found that compared with WT males, Ppk25 mutant D. simulans showed reduced courtship of D. yakuba females (Figures S4H and S4I). In summary, Ppk25 functions in both D. melanogaster and D. simulans to promote WT courtship displays.

DISCUSSION

Changes in morphological or other traits across evolution continue to be vigorously investigated (Carroll, 2008). We have examined whether the Gr32a+ chemosensory pathway that inhibits interspecies courtship in D. melanogaster functions similarly in D. simulans. We find that although D. simulans Gr32a is expressed in foreleg tarsi, similar to its counterpart in D. melanogaster, it is not required to inhibit interspecies courtship. It is possible that Gr32a neurons in foreleg tarsi still function to inhibit this behavior, a notion we attempted to address experimentally by inactivating Gr32a+ neurons. However, it was technically challenging to generate the requisite reagents required (Kir2.1, tetanus toxin light chain, shibirets) (Luo et al., 2008) in this species, despite numerous attempts. D. simulans males sense aversive cues on the cuticle of D. melanogaster females (Billeter et al., 2009; Coyne et al., 1994; Ferveur, 2005; Jallon, 1984). Given that Gr32a is not essential for this function, what chemoreceptors might be used to detect such repellents in D. simulans? It is possible that in this species, Gr32a and Gr33a function redundantly to inhibit interspecies courtship, a hypothesis difficult to test directly because these loci are only 1 Mb apart in the genome. Regardless, our findings still demonstrate a divergence in the function of Gr32a between D. melanogaster and D. simulans. The gustatory and ionotropic chemoreceptor families contain many members, and our results are also consistent with the idea that a different chemoreceptor(s) functions to inhibit interspecies courtship by D. simulans males (Joseph and Carlson, 2015). Although changes in centrally located courtship circuits may confer species-specific phenomonal responses (Seeholzer et al., 2018), our results show that there is divergence in chemoreceptor-mediated suppression of interspecific courtship between D. melanogaster and D. simulans (Figure 4F). In other words, our findings show that these closely related species use distinct peripheral chemosensory pathways to suppress interspecific courtship.

The divergence in chemoreceptor-mediated suppression of courtship between D. melanogaster and D. simulans does not reflect a global reorganization of molecular pathways that regulate courtship (Figure 4F). We find that similar to its role in D. melanogaster, Ppk25 is required to promote courtship toward conspecific females in D. simulans. Ppk25 is required to sense 7,11-heptacosadiene, an aphrodisiac cue, in D. melanogaster (Kaliman et al., 2015; Starostina et al., 2012); however, 7,11-heptacosadiene is an aversive cue for D. simulans males (Billeter et al., 2009), so it will be interesting to understand how Ppk25 functions in both species to promote conspecific courtship. Although 7,11-heptacosadiene serves as a cuticular attractant to D. melanogaster males, elimination of all cuticular pheromones in D. melanogaster females does not eliminate courtship...
by D. melanogaster males, and in fact, it disinhibits courtship by D. simulans males (Billeter et al., 2009; Coyne et al., 1994; Savarit et al., 1999). Thus cuticular attractants are not essential for courtship and anti-aphrodisiacs may guide avoidance of courtship with reproductively futile targets such as individuals of other species; together with our previous findings (Fan et al., 2013), our results show that Gr32a is essential for detection of such aversive compounds by D. melanogaster but not D. simulans males.

Both Gr32a and Gr33a are required for avoidance of quinine in D. melanogaster and D. simulans. Thus, the chemosensory functions of Gr32a and Gr33a in avoiding quinine and inhibiting courtship of reproductively futile targets are evolutionarily dissociable (Figure 4F). The same behavioral trait (tapping) and sensorimotor appendage (foreleg) inhibit courting of reproductively dead-end targets in D. melanogaster and D. simulans, but our studies show that the molecular mechanisms that preclude such courtship have diverged between these species. Previous work from our and other labs shows that different genetic pathways control distinct quantitative aspects of behavioral subroutines (Ding et al., 2016; Greenwood et al., 2013; Weber et al., 2013; Xu et al., 2012). Together, these findings demonstrate that modifications in genetic pathways can be used to gate a behavior or to implement quantitative changes in that behavior. We also find that although chemoreceptor mechanisms inhibiting...
interspecies courtship have differentiated between closely related species, a chemosensory pathway promoting courtship appears to have a similar positive valence in both species. It will be interesting to determine whether these courtship-promoting and courtship-inhibiting pathways evolve in a similar pattern across other drosophilid species. Alternatively (Jacob, 1977; Luo, 2015), our findings may reflect the idiosyncratic nature of selective forces that exploit mutations in apparently random pathways to effect evolutionary change. It should be possible to distinguish between these alternatives by studying mechanisms that regulate courtship in additional drosophilid species.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **CONTACT FOR REAGENT AND RESOURCE SHARING**
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
- **METHOD DETAILS**
  - Generating D. simulans Gr32a, Gr33a, or Ppk25 mutants
  - Generating D. simulans and D. melanogaster transgenic animals
  - Molecular analysis of Gr32a, Gr33a, and Ppk25 mutations in D. simulans
  - Histology
  - Courtship assays
  - Taste assay
  - Tests for Non-Neutral Evolution
  - Hydrophobicity plot
- **SUPPLEMENTAL QUANTIFICATION AND STATISTICAL ANALYSIS**

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.04.104.

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AUTHOR CONTRIBUTIONS

O.M.A. and N.M.S. designed the fly experiments. O.M.A., K.M.T., P.H.S., and J.P. conducted the fly experiments and analyzed behavioral data. A.A.–H. and K.S.P. conducted bioinformatics analyses of Gr32a ~3.8 kb regulatory region. S.P. helped with molecular analysis of Gr33a and Ppk25 mutants. G.W.D. provided invaluable advice, resources, and laboratory space for some of these experiments. J.-M.K. and D.L.S. provided reagents to generate transgenic D. simulans lines. O.M.A., A.A.–H., K.S.P., and N.M.S. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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## Star Methods

### Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| Quinine-HCl | SigmaAldrich | CAS: 6119-47-7 |
| Sucrose | JT Baker | CAS: 57-50-1 |
| Brilliant blue FCF | Wako Pure Chemical | CAS: 3844-45-9 |
| Sulforhodamine | SigmaAldrich | CAS: 3520-42-1 |
| **Critical Commercial Assays** | | |
| SuperScript III First-Strand Synthesis | Invitrogen, ThermoFisher | Cat # 18080051 |
| **Experimental Models: Organisms/Strains** | | |
| *D. simulans*: wildtype | Drosophila Species Stock Center (University of California, San Diego) | 14021-0251.001 |
| *D. simulans*: w^601 | Drosophila Species Stock Center (University of California, San Diego) | 14021-0251.195 |
| *D. yakuba*: wildtype | Drosophila Species Stock Center (University of California, San Diego) | 14021-0261.00 |
| *D. virilis*: wildtype | Drosophila Species Stock Center (University of California, San Diego) | 15010-1051.00 |
| *D. melanogaster*: Canton-S | Bloomington Drosophila Stock Center | RRID:BDSC_64349, |
| *D. simulans*: Gr32a^{110} | This paper | N/A |
| *D. simulans*: Gr32a^{126} | This paper | N/A |
| *D. simulans*: Gr32a^{174} | This paper | N/A |
| *D. simulans*: Gr33a^{110} | This paper | N/A |
| *D. simulans*: Gr33a^{196} | This paper | N/A |
| *D. simulans*: Ppk25^{1-2} | This paper | N/A |
| *D. simulans*: Ppk25^{44} | This paper | N/A |
| *D. simulans*: UAS-ReaChR::Citrine.sim986 | This paper | N/A |
| *D. melanogaster*: UAS-ReaChR::Citrine.VK05 | Bloomington Drosophila Stock Center | RRID:BDSC_53749 |
| *D. simulans*: Gr32a^{sim-GAL4.sim986} | This paper | N/A |
| *D. simulans*: Gr32a^{sim-GAL4.sim986} | This paper | N/A |
| *D. melanogaster*: Gr32a^{sim-GAL4.attP2} | This paper | N/A |
| *D. melanogaster*: Gr32a^{sim-GAL4.attP2} | This paper | N/A |
| **Oligonucleotides** | | |
| Primers: Amplifying Gr32a Regulatory Region | See Table S1 | N/A |
| CRISPR oligos: Targeting *D. simulans* Gr32a | See Table S1 | N/A |
| CRISPR oligos: Targeting *D. simulans* Gr33a | See Table S1 | N/A |
| CRISPR oligos: Targeting *D. simulans* Ppk25 | See Table S1 | N/A |
| Primers: RT-PCR of *D. simulans* Gr32a, Gr33a, Ppk25, and tubulin | See Table S1 | N/A |
| **Recombinant DNA** | | |
| pJFRC2(UAS-ReaChR::Citrine) | Inagaki et al., 2014 | N/A |
| pBPGrw | Pfeiffer et al., 2008 | RRID: Addgene_17574 |
| pDCC6 | Gokcezade et al., 2014 | RRID: Addgene_59985 |

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Nirao Shah (nirao@stanford.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

*D. simulans* (14021-0251.001), *w501 D. simulans* (14021-0251.195), *D. yakuba* (14021-0261.00), and *D. virilis* (15010-1051.00) were obtained from the Drosophila Species Stock Center at the University of California, San Diego. WT *D. melanogaster* were in the Canton-S background. *D. melanogaster* UAS-ReaChR::Citrine.VK05 was obtained from the Bloomington Drosophila Stock Center (#53749). Transgenic and CRISPR-mediated mutant flies were generated as described below.

METHOD DETAILS

Generating *D. simulans* Gr32a, Gr33a, or Ppk25 mutants

CRISPR guides were chosen from a list generated by flyCRISPR Optimal Target Finder (flycrispr.molbio.wisc.edu/tools). We targeted exon 1 of *D. simulans* Gr32a and Ppk25, and exon 2 of Gr33a. CRISPR oligos were annealed and ligated to plasmid pDCC6 [Addgene # 59985, (Gokcezade et al., 2014)] following restriction digest with *BbsI*. Sequences used to synthesize CRISPR oligos are provided in Table S1. Plasmids were injected at 100 ng/ul concentrations for each of 2 - 3 plasmids targeting a single gene. Animals were screened for mutations by PCR followed by 15% non-denaturing PAGE (Zhu et al., 2014) or directly by sequencing. Please see Table S3 for details on results of CRISPR injections for *D. simulans*. All CRISPR-generated mutant strains were backcrossed at least 5 times.
to WT *D. simulans* before testing for behavior in order to minimize effects of off-target mutations on phenotypes under study. Subsequent to this out-crossing to WT *D. simulans*, we mated heterozygous flies to obtain homozygous stocks for each allele. Given the absence of balancers in *D. simulans*, we verified genotypes at each generation by PCR analysis to generate homozygous stocks.

**Generating *D. simulans* and *D. melanogaster* transgenic animals**

To make Gr32a-GAL4 lines, we amplified the ~3.8 kb region upstream of the Gr32a start codon from *D. simulans* or *D. melanogaster* (primer sequences provided in Table S1) and subcloned it into pENTR/TOPO plasmid followed by Gateway-mediated subcloning into pBPGw. We then pHiC31-integrated each DNA construct into Chr III landing sites for each species, sim966 for *D. simulans* and attP2 for *D. melanogaster* (Groth et al., 2004; Knapp et al., 2015; Pfeiffer et al., 2010; Stern et al., 2017). pJFRC2(10xUAS-ReaChR::Citrine) plasmid (Inagaki et al., 2014) was provided by David Anderson, and it was used to generate the Citrine reporter in *D. simulans* using the landing site described above. Embryo injections were performed by Rainbow Transgenics (Camarillo, CA) or BestGene (Chino Hills, CA).

**Molecular analysis of Gr32a, Gr33a, and Ppk25 mutations in *D. simulans***

RNA was isolated from 10 WT or mutant *D. simulans* males (Trizol, ThermoFisher) and converted to cDNA using SuperScript III First-Strand Synthesis (Invitrogen, ThermoFisher). RT-PCR was performed using primers based on coding sequence (Table S1) that spanned exon-intron junctions of the respective locus (*Gr32a*, *Gr33a*, or *Ppk25*) to avoid amplifying products from genomic DNA. Use of these primers did not generate detectable product in no-RT controls. We subcloned and sequenced RT-PCR products from flies mutant for each allele of *Gr32a*, *Gr33a*, and *Ppk25*; we also directly sequenced RT-PCR products from flies mutant for each allele of *Gr32a* (except *Gr32a*326), *Gr33a*, and *Ppk25*. RNA isolation and the subsequent RT-PCR and sequencing were performed on 2-3 independent cohorts of WT and mutant flies. Sequence reads of subclones obtained from these RT-PCR studies and their alignment to the corresponding WT allele confirmed the presence of the expected mutation for each fly stock.

**Histology**

Tarsi were dissected in ice-cold PBS, fixed in fresh 4% paraformaldehyde at 22°C, washed 3x in PBT, and then mounted as described before (Fan et al., 2013). Samples were imaged using a Zeiss LSM700 (Z stacks) and processed in ImageJ.

**Courtship assays**

All courtship assays were performed at zeitgeber time 6-10 at 22°C, illuminated by a fluorescent ring lamp (22W) suspended 4 cm above the courtship chamber and recorded with a Sony camcorder (HDR-XR550V) (Fan et al., 2013). Experiments performed under dark conditions were illuminated by red LEDs and recorded as above in a dark room. Virgin flies were collected at eclosion and light entrained (12 hours L/D, 25C) for 5-7 days prior to testing. Experimental males were kept in isolation and tested with flies that were group-housed (~20 flies per vial) by species and sex. Foreleg tarsi were surgically removed at eclosion and males were tested as described above. We used *w*51 *D. simulans* as targets in male-male assays to distinguish them by eye color from test males. Behavioral assays were scored blind to genotype, using the MATLAB software ScoreVideo (Wu et al., 2009). We scored courtship as the period of time male flies spent chasing the stimulus fly, performing unilateral wing extension (courtship song), licking, abdominal bending (attempted copulation), or copulation. Courtship Index (CI) was calculated as the time spent by the male performing these behaviors, divided by the total assay time (15 minutes).

**Taste assay**

Preference assays were performed as described previously (Moon et al., 2009). 60-well plates were prepared the day prior to experiment and kept at 4°C. Dyes were diluted from stock solutions (Brilliant blue FCF and Sulforhodamine B, 12.5 mg/ml each) and resuspended in agarose, to which sucrose or sucrose spiked with quinine-HCl were subsequently added. Final concentrations were: agarose (1%), Brilliant blue FCF (0.125 mg/mL; Wako Pure Chemical), Sulforhodamine B (0.125 mg/mL; SigmaAldrich), sucrose (1 mM; JT Baker), and sucrose (5 mM) spiked with quinine (0.5 mM; SigmaAldrich). Substrate with sucrose or sucrose spiked with quinine were randomly colored blue or red and counterbalanced for all experiments. 3-4 day old male and female flies were flipped into fresh food for 2 days at 12-hour light/dark cycle at 25°C. Flies were then food deprived by flipping them into vials containing 1% agarose and placed in the dark for 24 hours. Flies were then briefly anesthetized with CO2 and loaded onto the 60-well plates (zeitgeber time 2-3), which were placed in the dark at 25°C for 90 min. Abdomens were scored as blue, red, purple (mixed eating), or no color. A Preference Index was calculated for each 60-well plate as follows: \( \frac{(N^B + 0.5*N^R)/(N^B + N^R + 0.5*N^P)}{(N^B + 0.5*N^R)}/(N^B + N^R + N^P) \) where \( N^B, N^R, \) and \( N^P \) = total # flies with blue, red, and purple abdomens, respectively. Each genotype was tested ≥ 6 times.

**Tests for Non-Neutral Evolution**

Alignments of genomes from 27 insect species (23 drosophilids, housefly, mosquito, honeybee, and beetle) were generated for coordinates (dm6: chr2L:11,110,412-11,114,209) encompassing the *D. melanogaster* Gr32a ~3.8 kb regulatory sequence, and this alignment was subsequently downloaded from the Table Browser (UCSC Genome Browser, 2015 update) (Blanchette et al., 2004; Karolchik et al., 2004; Rosenbloom et al., 2015). PhyloP scores were computed for this region for three main tests: 1) a
basewise “all-branches” test for conserved or accelerated evolution in all species compared to a neutral model (one test per nucleotide), 2) a whole-region “all-branches” test for conserved evolution in all species compared to a neutral model (one test for the whole region), and 3) a basewise “subtree” test for conserved or accelerated evolution in the designated species (D. melanogaster or D. simulans) compared to the other species (one test per nucleotide for each designate species) (Pollard et al., 2010). PhyloP scores are negative log10 P values of a likelihood ratio test comparing two evolutionary models (alternate versus neutral or subtree versus subtree complement). Scores near “0” indicate the expected rate of evolution, while large scores indicate conservation (phyloP score > 2) or acceleration (phyloP score < −2). PhyloP scores were tallied across coding sequence, introns, UTRs, and intergenic regions (Siepel et al., 2005). The phylogenetic model for neutral evolution was based on 4-fold degenerate sites in the 27-species genomic alignment and also downloaded from the UCSC Genome Browser. PhyloP scores and R code are made available for reproducible workflow at https://github.com/aavilaherrera/flymating (Allaire et al., 2017; R Core Team, 2017; Xie, 2016) (https://cran.r-project.org/doc/FAQ/R-FAQ.html#Citing-R). This code uses bedtools and bedops (Neph et al., 2012; Quinlan and Hall, 2010).

Hydrophobicity plot

Hydrophobicity scores were generated with ProtScale (Artimo et al., 2012) using D. melanogaster or D. simulans Gr32a amino acid sequences as input. We used the Kyte and Doolittle hydrophobicity scale with a window size of 19 amino acids and uniform weights across all residues. The seven transmembrane domains were identified using HMMTOP (Tusnády and Simon, 1998, 2001) to predict the topology of Gr32a for both D. melanogaster and D. simulans.

QUANTIFICATION AND STATISTICAL ANALYSIS

We used Fisher’s exact test to analyze categorical data (e.g., percent assays with CI > 0.05) and we used the Bonferroni correction for multiple group comparisons as necessary. For other comparisons, we first tested whether data were normally distributed using a Lilliefors’ goodness-of-fit test using MATLAB. Data for Figure S1B were analyzed with a Student’s t test; data for all other figure panels were tested with a non-parametric test (Kolmogorov-Smirnov test for two groups or Kruskal-Wallis test). A Tukey’s post hoc test following multiple group comparisons was used to determine which groups differed significantly.
Supplemental Information

Evolution of Mechanisms that Control Mating in *Drosophila* Males

Osama M. Ahmed, Aram Avila-Herrera, Khin May Tun, Paula H. Serpa, Justin Peng, Srinivas Parthasarathy, Jon-Michael Knapp, David L. Stern, Graeme W. Davis, Katherine S. Pollard, and Nirao M. Shah
Figure S1, related to Figures 1 and 2

A

D. simulans
D. melanogaster
D. yakuba
D. virilis

Divergence Time (million years)

B

x D. yak ♀

D. simulans male:
- Control
- No Tarsi

C

Corresponding GAL4 alone
Corresponding GAL4
+ Gr32a<sup>sim</sup>-GAL4

D

~3.8 kb Regulatory Region
Gr32a Coding Sequence

27 insects
Alignment by Multiz

E

27 insects
Conservation by PhastCons

F

27 insects

G

D. melanogaster

H

D. simulans

I

x D. sim ♀

J

x D. mel ♀

D. simulans male:
- Control
- No Tarsi

K

SEZ

L

SEZ

Citrine

# of alignment columns

PhyloP score

# of Citrine+ Cells

# Mid-Line Crossings

PhyloP score

PhyloP score
Figure S1: A regulatory region upstream of Gr32a coding sequence is conserved across drosophilids. Related to Figures 1 and 2.

(A) Evolutionary relationship of the four Drosophila species used in this study.

(B) Foreleg tarsi do not inhibit D. simulans males from courting D. yakuba females.
Mean ± SEM; each circle denotes CI of a D. simulans male; n = 22 - 23/cohort; ***p<0.001.

(C) No difference in the number of Citrine+ cells in T4 and T5 foreleg segments of D. melanogaster males observed with either Gr32a^{mel}-GAL4 or Gr32a^{sim}-GAL4 alone or in combination. These findings indicate that the upstream regulatory sequence in Gr32a is functionally conserved between D. melanogaster and D. simulans; however, it is formally possible that the similarity in number of Citrine+ cells in D. melanogaster carrying one or both GAL4 alleles reflects effects of transvection in the presence of both GAL4 alleles rather than functional conservation. Mean ± SEM; each circle denotes Citrine+ cell count for a foreleg tarsum; n = 10 - 17/genotype.

(D) 27-insect alignment of the ~3.8 kb DNA element that drives Gr32a expression in D. melanogaster and D. simulans. mel, D. melanogaster (blue); sim, D. simulans (orange); sec, D. sechellia; yak, D. yakuba; ere, D. erecta; bia, D. biarmipes; suz, D. suzukii; ana, D. ananassae; bip, D. bipectinata; eug, D. eugracilis; ele, D. elegans; kik, D. kikkawai; tak, D. takahashii; rho, D. rhopaloa; fic, D. ficusphila; pse, D. pseudoobscura; per, D. persimilis; mir, D. miranda; wil, D. willistoni; vir, D. virilis; moj, D. mojavensis; alb, D. albomicans; gri, D. grimshawi; dom, Musca domestica; gab, Anopheles gambiae; mlf, Apis mellifera; cas, Tribolium castaneum.

(E) Track showing PhastCons conservation score across the region in (D). Pink bar indicates the intergenic region directly 5’ of the Gr32a start codon and 5’UTR, which contains several blocks of highly conserved sequence. Higher peaks indicate higher likelihood of bases being in a strongly conserved element.
(F) Distribution of nucleotide resolution phyloP conservation scores for the region shown in (D). Most bases in the region are evolving at the same or slower rate than 4-fold degenerate (4D) sites in the multiple sequence alignment of 27 insects. Large positive (> 2) or negative (< -2) scores indicate conservation or acceleration, respectively. Scores near “0” indicate a similar substitution rate to 4D sites.

(G, H) Distribution of phyloP scores for branch-specific evolutionary tests. Most bases in the ~3.8 kb region are likely evolving more slowly or as slowly as expected in D. melanogaster (G) and D. simulans (H) compared to the other 26 insects in the tree. Scores near “0” indicate a similar rate of DNA evolution in the designated species relative to all other species in the tree.

(I, J) Removing the foreleg tarsi of D. simulans males does not diminish locomotor activity during courtship assays. Mean ± SEM; each circle denotes # of midline crossings of a D. simulans male; n = 9 - 11/cohort; *p<0.05.

(K, L) Projection pattern of Gr32a neurons in the SEZ is similar between D. melanogaster and D. simulans males. n = 3 - 4 brains/genotype; scale bar = 50 µm.
Figure S2, related to Figure 3

**A**

![D. simulans Gr32a](image)

**B**

WT | Gr32a<sup>Δ10</sup> | Gr32a<sup>Δ26</sup> | Gr32a<sup>Δ141</sup>
---|---|---|---
1650bp | Gr32a | tubulin
500bp | 400bp

**C**

WT: CCGGATGCCCGTGTAAACCGGGATGCCTTCAGTAGAGATG/GATGCCCATATACGAACAGGTCTCCGACTATG
Δ10bp: CCGGATGCCCG----------GATG/GATGCCCATATACGAACAGGTCTCCGACTATG
Δ26bp: CCGGATGCCCG--------------------------GATG/GATGCCCATATACGAACAGGTCTCCGACTATG
Δ141bp: CCGGATGCCCG------------------------------//--------------------------ACTATG

**D**

WT: MSPNTVIEMPTQKAQSHPYPRPISPRPYPVVLTDASRFPSAPMPARSHDPVFEDIRTIGMPSVEMHHLCQRGAMIIPFLRTFAPYC
PMPVVLTDASRFPSAPMPARSHDPVFEDIRTIGMPSVEMHHLCQRGAMIIPFLRTFAPYC
Δ10bp: MSPNTVIEMPTQKAQSHPYPRPISPRPYPVVLTDASRFPSAPMPARSHDPVFEDIRTI
Δ26bp: MSPNTVIEMPTQKAQSHPYPRPISPRPYPVVLTDASRFPSAPMPARSHDPVFEDIRTI
Δ141bp: MSPNTVIEMPTQKAQSHPYPRPISPRPYPVVLTDASRFPSAPMPARSHDPVFEDIRTI

**E**

![Courtship Index](plot)

**F**

![Amino Acid Hydrophobicity](plot)

**G**

![Amino Acid Hydrophobicity](plot)
Figure S2: Generating Gr32a mutant *D. simulans* via CRISPR/Cas9. Related to Figure 3.

(A) Schematic of *D. simulans* Gr32a locus. Pink arrows, PCR primers; green triangles, CRISPR target sites; black rectangles, exons.

(B) RT-PCR products for Gr32a and tubulin in WT and Gr32a mutant *D. simulans*, using PCR primers shown in (A). DNA ladder shown in first lane.

(C) DNA sequence comparison of WT and mutant Gr32a alleles. PAM, Protospacer Adjacent Motif.

(D) Predicted amino acid sequence of WT and mutant *D. simulans* Gr32a. The predicted first transmembrane domain (TM1) is highlighted in gray in the WT protein. *, premature stop codon.

(E) No difference in courtship of *D. yakuba* females by WT and Gr32a mutant *D. simulans* males. Mean ± SEM; each circle represents CI of a male; n = 17 - 21/genotype.

(F) Predicted location of the seven transmembrane domains (black rectangles) in Gr32a based on plot shown in (G). The NH\textsubscript{2} terminal is predicted to be intracellular.

(G) Hydrophobicity plot of *D. simulans* and *D. melanogaster* Gr32a. Predicted transmembrane domains are shown by gray shading.

Please see Table S2.
Figure S3, related to Figure 3

A

D. simulans Gr33a

B

|     | WT   | Gr33a<sup>Δ10</sup> | Gr33a<sup>Δ96</sup> |
|-----|------|--------------------|--------------------|
|     | 1000bp | 850bp               | 500bp               |
|     | 800bp  | 400bp               |                    |

C

WT  CATTCCACTGAATCGCCAGCAATCGGAAACTAACTT//CTCTCATAAATCTTACCCACATTATTGGCCTCTG
Δ10bp CATTCCACTG----------AATCGGAAACTAACTT//CTCTCATAAATCTTACCCACATTATTGGCCTCTG
Δ96bp CATTCCACTGAATCGGAAACTAACTT//CTCTCATAAATCTTACCCACATTATTGGCCTCTG

D

WT  MIQIMNWFSHVIGILPLNRQLQSETNFILYAMCIVPYPVTLYACVFVACLTHICLGCFLDSNVCKLSHLFMHLGAFLY
Δ10bp MIQIMNWFSHVIGILPLNRHQTSYSTTP*-----------------------------GLCFLDSNVCKLSHLFMHLGAFLY
Δ96bp MIQIMNWFSHVIGILPLNRQLQSETNFILYAMCIVPYPVTLYACVFVACLTHICLGCFLDSNVCKLSHLFMHLGAFLY

E

|       | x D. sim ♀ | x D. mel ♀ | x D. yak ♀ | x D. vir ♀ |
|-------|------------|------------|------------|------------|
| Courtship Index (C.I.) | 1          | 0.8        | 0.8        | 1          |
|       | (20) (24) | (17) (13) | (17) (21) | (10) (10) |

G

|       | x D. sim♂ |
|-------|-----------|
| Courtship Index (C.I.) | 1 |
|       | (21) (14) (17) |

F

|       | C.I. > 0.05 | Fraction Assays |
|-------|-------------|-----------------|
|       | (20) (24) | (17) (13) (14) |

H

|       | C.I. > 0.05 | Fraction Assays |
|-------|-------------|-----------------|
|       | (17) (21) | (10) (10) |

I

D. melanogaster

Gr33a Feeding

J

D. simulans:

|       | Preference Index |
|-------|------------------|
|       | (10) (6) (10)    |

D. simulans:

|       | Gr33a<sup>Δ10</sup> | Gr33a<sup>Δ96</sup> |
|-------|-----------------------|----------------------|
|       | Control               | Gr33a<sup>Δ10</sup> |
|       |                        | Gr33a<sup>Δ96</sup> |
Figure S3: Gr33a is not required to inhibit interspecies courtship behavior of *D. simulans* males but does inhibit *D. simulans* from feeding on quinine. Related to Figure 3.

(A) Schematic of *D. simulans* Gr33a locus. Pink arrows, PCR primers; green triangles, CRISPR target sites; black rectangles, exons.

(B) RT-PCR products for Gr33a and tubulin in WT and *Gr33a* mutant *D. simulans*, using PCR primers shown in (A). Note that products from WT, *Gr33a*\(^{\Delta 10}\) and *Gr33a*\(^{\Delta 96}\) flies were run on the same gel, and lane between *Gr33a*\(^{\Delta 10}\) and *Gr33a*\(^{\Delta 96}\) has been cropped out for clarity of comparison. DNA ladder shown in first lane.

(C) DNA sequence comparison of WT and mutant *Gr33a* alleles.

(D) Predicted amino acid sequence of WT and mutant *D. simulans* Gr33a. *, premature stop codon.

(E, F) WT and *Gr33a* mutant *D. simulans* males court conspecifics at high levels and show similar low (*D. yakuba*) to minimal (*D. melanogaster* and *D. virilis*) levels of courtship toward females of other species.

(G, H) WT and Gr33a mutant *D. simulans* males show similar low levels of courtship toward conspecific males.

Mean ± SEM; each circle denotes CI of one male; n = 10 - 24/genotype.

(I) We tested whether, similar to *D. melanogaster*, Gr33a inhibits feeding on quinine-containing food in *D. simulans*.

(J) Significant decrease in preference by *Gr33a* mutant *D. simulans* for food containing only sucrose.

Mean ± SEM; each circle denotes Preference Index for one experiment; 90 ± 4 *D. simulans* of each genotype were used/experiment; n = 6 - 10 experiments/genotype; ***p<0.001.
Figure S4, related to Figure 4

A

D. simulans ppk25

B

WT

+2bp

Δ4bp

Ppk25

650bp

500bp

500bp

400bp

600bp

PAM

Protospacer

C

WT

+2bp

Δ4bp

Ppk25

650bp

500bp

500bp

400bp

600bp

PAM

Protospacer

D

WT

+2bp

Δ4bp

Ppk25

650bp

500bp

500bp

400bp

600bp

PAM

Protospacer

E

D. sim ♂

x D. sim ♀

F

D. mel ♂

x D. mel ♀

G

x D. sim ♀

(In Dark)

x D. mel ♀

(In Dark)

H

x D. mel ♀

x D. yak ♀

x D. vir ♀

D. simulans male:

Control

No Tarsi

I

C.I. > 0.05,

Fraction Assays

(14) (20) (20)

(18) (21) (24)

(6) (9) (12)
Figure S4: Ppk25 is not essential to inhibit interspecies courtship by *D. simulans* males.

Related to Figure 4.

(A) Schematic of *D. simulans* Ppk25 locus. Pink arrows, PCR primers; green triangles, CRISPR target sites; black rectangles, exons.

(B) RT-PCR products for Ppk25 and tubulin in WT and Ppk25 mutant *D. simulans*, using PCR primers shown in (A). DNA ladder shown in first lane.

(C) DNA sequence comparison of WT and mutant Ppk25 alleles.

(D) Predicted amino acid sequence of WT and mutant *D. simulans* Ppk25. *, premature stop codon.

(E, F) *D. simulans* and *D. melanogaster* males court conspecific females less under red light-only illumination.

(G) *D. simulans* males require foreleg tarsi for courtship under dark conditions.

(H, I) No difference between *D. simulans* males WT or mutant for Ppk25 in courtship of *D. melanogaster* and *D. virilis* females. Mutant males court *D. yakuba* females less than WT (G). Mean ± SEM; each circle denotes CI for one male; n = 6-24/cohort; **p<0.01; ***p<0.001.
Table S1. Related to Figures 2, S2, S3, and S4

| Name               | Experiment                                               | 5' to 3' Sequence                                   |
|-------------------|----------------------------------------------------------|----------------------------------------------------|
| sim32 fwd         | Amplifying ~3.8kb Gr32a regulatory region from *D. simulans* to generate Gr32a<sup>sim</sup>-GAL4 | GTCCCCTTGCGGTTGTTCCT                                 |
| sim32 rev         |                                                          | TTCAATTACCAAGTGTTCG                                 |
| mel32 fwd         | Amplifying ~3.8kb Gr32a regulatory region from *D. melanogaster* to generate Gr32a<sup>mel</sup>-GAL4 | AAGTGGTTTGCTTGGGAT                                 |
| mel32 rev         |                                                          | TTCAATTACCAAGTGTTCG                                 |
| CrisprGr32a A     |                                                          | CRISPR oligos targeting *D. simulans* Gr32a         |                                                                 |
| CrisprGr32a A rev |                                                          | CTTCGGAAGGCATCCCCGGTTAACA                            |
| CrisprGr32a B     |                                                          | AAACATGGAACAGGATGCTCC                              |
| CrisprGr32a B rev |                                                          | CTTCGTCGGAGACCTGTTTCGTATAT                           |
| CrisprGr32a C     |                                                          | AAACATATACGAACAGGCTCCGAC                            |
| CrisprGr32a C rev |                                                          | CTTCGTGTATACGTTCTTCGTAAG                            |
| CrisprGr33a A     |                                                          | CRISPR oligos targeting *D. simulans* Gr33a         |                                                                 |
| CrisprGr33a A rev |                                                          | CTTCGTCACGTAACTGCCCAGCAAT                            |
| CrisprGr33a B     |                                                          | AAACATGTAACAGGCATTCG                                |
| CrisprGr33a B rev |                                                          | CTTCGATAATCCTACCCACATT                              |
| CrisprGr33a C     |                                                          | AAACATGTAAGGTAAGATTAT                              |
| CrisprGr33a C rev |                                                          | CTTCGGCTGAGTCTTTATGCGCGAAA                         |
| CrisprPpk25 A     |                                                          | CRISPR oligos targeting *D. simulans* Ppk25         |                                                                 |
| CrisprPpk25 A rev |                                                          | CTTCGAGGGAGTCGGCCAAGCAAC                            |
| CrisprPpk25 B     |                                                          | AAACCTGACGTCCGACGACGAC                              |
| CrisprPpk25 B rev |                                                          | CTTCGATCGCCATCGCCGCA                                |
| Gr32a RTPCR fwd   | primers for RT-PCR of *D. simulans* Gr32a               | TAATCCACAATGCCAAGCAA                                |
| Gr32a RTPCR rev   |                                                          | AGGAACCTTATCAGATATTATC                              |
| Gr33a RTPCR fwd   | primers for RT-PCR of *D. simulans* Gr33a               | CCGAGTACGCGAATTCCA                                  |
| Gr33a RTPCR rev   |                                                          | TCGGATCGTATCGTATC                                  |
| Ppk25 RTPCR fwd   | primers for RT-PCR of *D. simulans* Ppk25              | ACATCGAATACGCAAGG                                  |
| Ppk25 RTPCR rev   |                                                          | ATCCAGTGTATGATTTATC                                 |
| primers for RT-PCR of *D. simulans* tubulin | CTTGCCTCGTGTAACACT |
|---------------------------------------------|---------------------|
| tubulin RTPCR fwd                            | GGATCCTGCTCCAGAACAG |

Table S1: List of oligos and primers used in this study. Related to Figures 2, S2, S3, and S4.
Table S2. Related to STAR Methods and Figure S2

| Predicted TM domains | Gr32a Amino Acid Range |  |
|----------------------|------------------------|--|
|                      | **D. melanogaster**    | **D. simulans** |
| TM1                  | 101-119                | 101-120          |
| TM2                  | 134-156                | 131-149          |
| TM3                  | 187-206                | 187-206          |
| TM4                  | 215-239                | 215-239          |
| TM5                  | 310-329                | 310-327          |
| TM6                  | 356-378                | 354-378          |
| TM7                  | 419-443                | 419-443          |
| TM, transmembrane    |                        |                 |

Table S2: Predicted transmembrane domains of Gr32a. Related to STAR Methods and Figure S2.
Table S3. Related to STAR Methods and Figures S2, S3, and S4.

| Locus | # CRISPR Guides Injected | # injected embryos | # larvae | # G0 flies | # G0 flies that yielded progeny (F1) | # G0 flies that yielded mutant F1 flies | # F1 flies bearing distinct indel mutations | Homozygous Stocks used in study |
|-------|--------------------------|--------------------|----------|------------|--------------------------------------|----------------------------------------|------------------------------------------|-------------------------------|
| Gr32a | 3                        | 300                | 40       | 15         | 7                                    | 3                                      | 7                                        | 3                             |
|       | 2                        | 300                | 15       | 10         | 5                                    | 1                                      | 3                                        | 0                             |
| Gr33a | 3                        | 519                | 298      | 78         | 26                                   | 3                                      | 7                                        | 2                             |
| Ppk25 | 2                        | 510                | 185      | 86         | 44                                   | 2                                      | 4                                        | 2                             |

Table S3: *D. simulans* pDCC6 CRISPR injection counts. Related to STAR Methods and Figures S2, S3, and S4.