

# *tartan* underlies the evolution of *Drosophila* male genital morphology

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Male genital structures are among the most rapidly evolving morphological traits and are often the only features that can distinguish closely related species. This process is thought to be driven by sexual selection and may reinforce species separation. However, while the genetic bases of many phenotypic differences have been identified, we still lack knowledge about the genes underlying evolutionary differences in male genital organs and organ size more generally. The claspers (surstyli) are periphallal structures that play an important role in copulation in insects. Here, we show that divergence in clasper size and bristle number between *Drosophila mauritiana* and *Drosophila simulans* is caused by evolutionary changes in *tartan* (*trn*), which encodes a transmembrane leucine-rich repeat domain protein that mediates cell–cell interactions and affinity. There are no fixed amino acid differences in *trn* between *D. mauritiana* and *D. simulans*, but differences in the expression of this gene in developing genitalia suggest that cis-regulatory changes in *trn* underlie the evolution of clasper morphology in these species. Finally, analyses of reciprocal hemizygotes that are genetically identical, except for the species from which the functional allele of *trn* originates, determined that the *trn* allele of *D. mauritiana* specifies larger claspers with more bristles than the allele of *D. simulans*. Therefore, we have identified a gene underlying evolutionary change in the size of a male genital organ, which will help to better understand not only the rapid diversification of these structures, but also the regulation and evolution of organ size more broadly.

evolution | development | genetics | *Drosophila* | genitalia

The morphology of male genitalia can differ dramatically even between very closely related animal species (1). In *Drosophila mauritiana* males, for example, the size, shape, and bristle morphology of the claspers (surstyli), posterior lobes (epandrial posterior lobes), and anal plates (cerci) are strikingly different from those of its sister species *Drosophila simulans* and *Drosophila sechellia* (Fig. 1). Moreover, these differences have evolved in only the last 240,000 y since these species last shared a common ancestor (2) (Fig. 14).

As in other animal groups (1, 3–5), interspecific differences in the morphology of genital structures are thought to have been driven by sexual selection (6). However, the mechanisms [female choice, sperm competition, or sexual antagonism (5)], and their contribution to reproductive isolation between populations and species, have been difficult to address and resolve theoretically (7–9) and experimentally (10, 11). Genetic manipulation of the evolved loci would allow us to test directly the effect of male genital divergence on mating behavior and reproductive fitness and, therefore, facilitate the empirical study of these questions (12, 13). Although quantitative mapping studies of morphological differences in male genitalia between species of the *D. simulans* clade were first carried out more than three decades ago (14–21), the genetic bases of male genital divergence between these species has remained elusive. This is due, at least in part, to the large number of loci found to contribute to variation in size and shape of these structures (18, 19, 21).

The claspers are periphallal structures with an essential role in grasping and proprioception of the female and in securing genital coupling (12, 22–27). Previously, we found that multiple loci contribute to divergence in clasper size and bristle number between *D. simulans* and *D. mauritiana* (19). Here, we report the identification of one of these loci, *tartan* (*trn*). In addition, mapping and functional experiments strongly suggest that cis-regulatory changes in this gene underlie differences in clasper morphology between these two species.

## Results and Discussion

Previously, we identified two regions on the left arm of chromosome 3 that contribute to differences in clasper size and bristle number between *D. mauritiana* and *D. simulans* (19). Here, we have generated recombinant introgression lines (ILs) between the *D. mauritiana* D1 (*Dmau* D1) and *D. simulans* w<sup>501</sup> (*Dsim* w<sup>501</sup>)

## Significance

The morphology of male genitalia evolves rapidly, probably driven by sexual selection. However, little is known about the genes underlying genitalia differences between species. Identifying these genes is key to understanding how sexual selection acts to produce rapid phenotypic change. We have found that the gene *tartan* underlies differences between male *Drosophila mauritiana* and *Drosophila simulans* in the size and bristle number of the claspers—genital projections that grasp the female during copulation. Moreover, since *tartan* encodes a protein that is involved in cell interactions, this may represent an alternative developmental mechanism for morphological change. Therefore, our study provides insights into the genetic and developmental bases for the rapid evolution of male genitalia and organ size more generally.

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The authors declare no conflict of interest.

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Data deposition: Raw fastq files for the RNA sequencing data reported in this paper have been deposited at DNA Data Bank of Japan under accession nos. [DRA006755](#) and [DRA006758](#) for *D. mauritiana* and *D. simulans*, respectively.

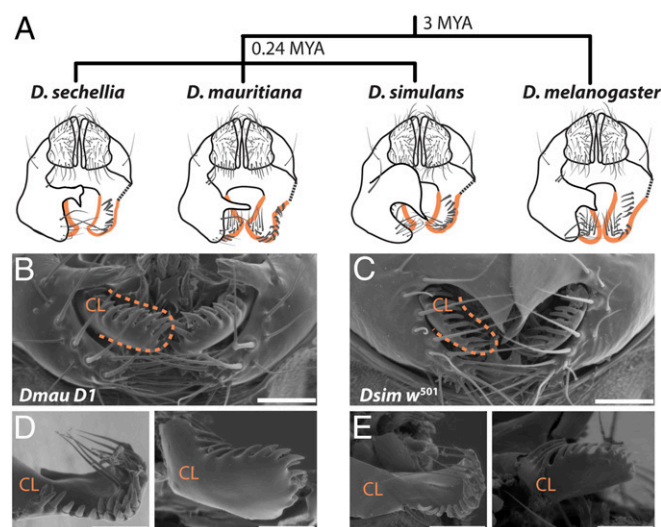
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**Fig. 1.** Divergence in periphallal structures in the *D. simulans* clade and its relationship to the outgroup *D. melanogaster* (2). (A) Schematic representation of the male analia and external genitalia (posterior view). Posterior lobes are illustrated as dissected away on the right-hand side, in order to facilitate visualization of the claspers (outlined in orange), which are typically covered by the posterior lobes. While the shape and size of the posterior lobes is species-specific, the claspers and anal plates are very similar between *D. simulans* and *D. sechellia*, which are smaller and have less bristles than those of *D. mauritiana* and *D. melanogaster*. In addition, the clasper bristles of *D. mauritiana* are shorter and thicker than those of the other three species (19, 20, 48). (B–E) Scanning electron micrographs of external male genitalia (B and C) and dissected claspers (D and E) of *Dmau D1* and *Dsim w<sup>501</sup>*, respectively. (Scale bars, 50 μm.)

strains (Dataset S1) to increase the resolution of one of these regions, C2, from ~3.5 Mb (24) to 177 kb. This interval explains about 16.3% of the difference in clasper size (and 37.9% of clasper bristle number) between the two parental strains (Fig. 2 and Datasets S2 *a* and *b*). The claspers of lines that are homozygous for introgressed *D. mauritiana* DNA in C2 are significantly larger than those of natural strains of *D. simulans* ( $P < 0.001$ , SI Appendix, Fig. S1). The change in clasper size caused by differences in C2 is therefore outside the range of variation in clasper size in *D. simulans*, suggesting that C2 underlies inter-specific divergence between *D. mauritiana* and *D. simulans* and not merely intraspecific polymorphism in clasper size in either or both of these species.

C2 contains eight protein-coding genes with orthologs in *Drosophila melanogaster*. RNA-Sequencing (RNA-Seq) data suggests that only one of these genes, *trn*, is expressed in the terminalia of *D. simulans* and *D. mauritiana* when the difference in clasper morphology develops between these two species (Dataset S3 and SI Appendix, Fig. S3). However, if the causative gene has a spatially restricted pattern of expression it may not have been detected in the RNA-Seq. Therefore, we knocked down the expression of all genes in the candidate region (with the exception of *CG34429*, for which there was no available UAS line) using RNA interference (RNAi) in *D. melanogaster* to test if these positional candidates are involved in clasper development (Dataset S4). In addition, we knocked down *CG11279* and *capricious* (*caps*), a gene closely related to *trn* and that functionally overlaps with *trn* in some contexts (28–34). *CG11279* and *caps* flank C2, but their cis-regulatory sequences may still be within this region (Fig. 24). We found that while knockdown of *trn* significantly reduced the size of the claspers (Dataset S4 and SI Appendix, Fig. S2), RNAi against the other nine genes tested, including *caps*, had no effect on clasper morphology in *D. melanogaster* (Dataset S4). Note that *trn* RNAi had no effect

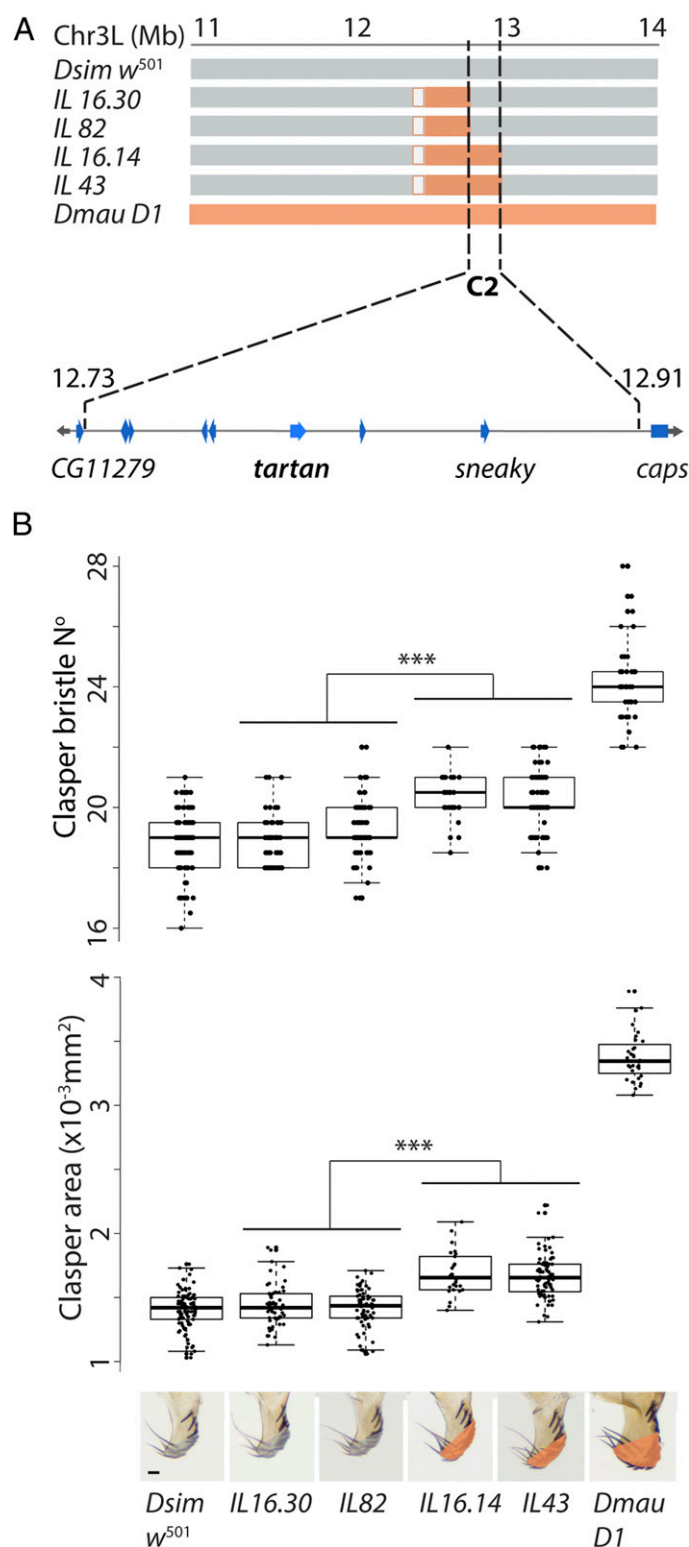
on the posterior lobes consistent with region C2 only affecting the claspers (Dataset S4).

*trn* encodes a leucine-rich repeat transmembrane protein (28, 30, 32, 33, 35, 36), and it is thought that its main function is to confer differences in affinity between cells and mediate their correct allocation to compartments in developing tissues such as the nervous system, trachea, eyes, wings, and legs (28, 30, 32, 35, 37, 38). Intriguingly, changes in *trn* expression can affect the allocation of cells between compartments, cause misspecification of compartmental boundaries, and even result in invasive movements of cells across such boundaries (33, 35, 38).

Our RNA-Seq data indicates that *trn* is more highly expressed in *D. simulans* during early terminalia development but is subsequently up-regulated in *D. mauritiana* at a later stage (Dataset S3). However, these data correspond to the sum of all of the expression domains of *trn* throughout the terminalia at each of these stages and may conceal more subtle localized expression differences between these species in specific tissues like the developing claspers. Therefore, we investigated the spatial pattern of *trn* expression throughout terminalia development using mRNA in situ hybridization (ISH) in *Dmau D1* and *Dsim w<sup>501</sup>* (Fig. 3 A–C and SI Appendix, Fig. S3). Concomitantly, we observed a 4-h difference in the timing of terminalia development between the two strains used (Fig. 3 A–C and SI Appendix, Fig. S3). We found that during early pupal stages *trn* is more highly expressed in *Dsim w<sup>501</sup>* compared to *Dmau D1* at the center of the terminalia, from where the internal genital structures will develop, which may explain the overall higher expression of *trn* in *D. simulans* at 30 h after puparium formation (hAPF) according to the RNA-Seq data (Fig. 3 A and B and Dataset S3). However, during later stages, the expression of *trn* is detected in a wider domain and persists for longer at the base of the developing claspers of *Dmau D1* compared to *Dsim w<sup>501</sup>* (Fig. 3 A and B). This is consistent with higher expression of *trn* in *D. mauritiana* detected in the RNA-Seq data at ~50 hAPF (Dataset S3). These results are also consistent with the RNAi results in *D. melanogaster* where knockdown of *trn* results in the loss of *trn* expression at the base of the claspers (SI Appendix, Fig. S2B) and the development of smaller claspers (SI Appendix, Fig. S2A). Together, these results suggest that the higher and/or more persistent expression of the *trn<sup>mau</sup>* allele relative to the *trn<sup>sim</sup>* allele in the developing claspers is at least partially responsible for the larger claspers in *D. mauritiana*.

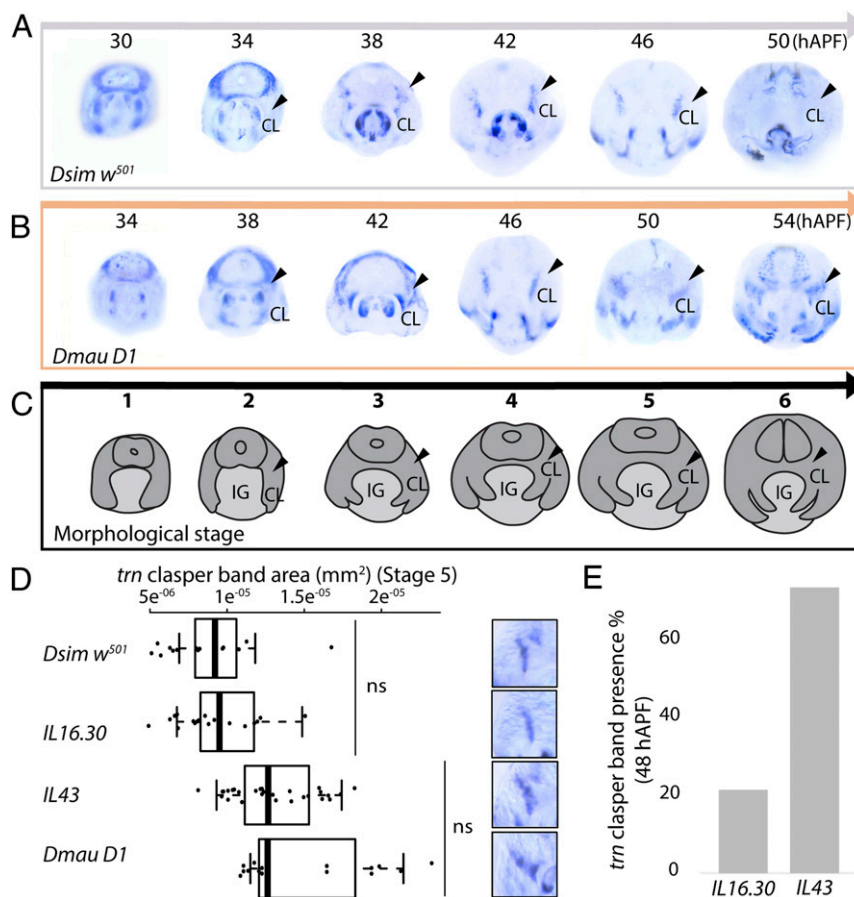
Quantitative analysis of *trn* ISH confirmed that males containing *trn<sup>mau</sup>* (*Dmau D1* and *IL43*) exhibit a larger expression domain at the base of the developing claspers at stage 5 (50 hAPF for *Dmau D1* and 46 hAPF for *Dsim w<sup>501</sup>* and *ILs*) than those containing *trn<sup>sim</sup>* (*Dsim w<sup>501</sup>* and *IL16.30*) (Fig. 3D and Dataset S5a). Moreover, although at stage 6 *IL43* and *IL16.30* seem to recapitulate the pattern observed in *Dsim w<sup>501</sup>* (i.e., *trn* expression no longer detected), we found that just before this, between stages 5 and 6 (48 hAPF in these *ILs* and *D. simulans*, SI Appendix, Fig. S3), there was variability in the presence of *trn* expression at the base of the developing claspers: expression was observed in 21% of *IL16.30* males (i.e., males with *trn<sup>sim</sup>*) but in 74% of *IL43* males (i.e., males with *trn<sup>mau</sup>*) (Fig. 3E and Dataset S5b). These data further support the hypothesis that spatial and/or temporal divergence in the expression of *trn* underlies differences in clasper size between *D. simulans* and *D. mauritiana*.

We also carried out ISH for *CG11279* and *caps* (which are both also expressed in the terminalia, Dataset S3) and *CG34429* (which we were unable to knock down in *D. melanogaster*). This showed that, unlike *trn*, these genes are either not expressed in the developing genitalia or at least not in a pattern consistent with a role in clasper development and evolution (SI Appendix, Fig. S4). For example, although *caps* expression in the male genitalia is generally similar to that of *trn*, *caps* transcripts were



**Fig. 2.** High-resolution mapping of differences in clasper morphology between *Dsim w*<sup>501</sup> and *Dmau D1*. (A) Introgression line breakpoints on chromosome arm 3L define the 177-kb region C2 (gray, orange, and white boxes indicate DNA regions from *Dsim w*<sup>501</sup>, *Dmau D1*, or not verified, respectively). Coordinates are given in megabases with respect to the *D. simulans* genome (Flybase R2.02). This region contains eight protein coding genes including *trn* and is flanked by *CG1279* and *caps*. (B) Introgression lines containing region C2 from *Dmau D1* (*IL43* and *IL16.14*) contribute 37.9% of the difference in bristles (*Upper graph*) and 16.3% of the clasper size difference (*Lower graph*) of this strain compared to *Dsim w*<sup>501</sup> (*Dataset S2a*). *IL43* and *IL16.14* differed significantly from *IL16.30* and *IL82* in clasper bristle number and in clasper area ( $P < 0.001$ ). Asterisks indicate significance comparisons where  $P < 0.001$  (*Dataset S2c*). Shading in the pictures underneath the *Lower graph* indicates the area measured at the distal end of the claspers in lines containing *Dsim w*<sup>501</sup> (gray) or *Dmau D1* (orange) regions for C2. Boxes indicate the range, upper and lower quartiles, and median for each sample. (Scale bar, 20  $\mu$ m.)





**Fig. 3.** The spatial and temporal expression of *trn* differs in the developing claspers of *D. simulans* and *D. mauritiana*. Expression shown at 4-h intervals hAPF in *Dsim w<sup>501</sup>* (A) and *Dmau D1* (B). (C) Illustration of the developing structures at each morphological stage (SI Appendix, Fig. S3). Black arrowheads indicate expression at the base of the developing claspers. CL, clasper; IG, internal genitalia. (D) Analysis of *trn* expression domain at the base of the developing clasper at stage 5. *trn<sup>sim</sup>* males, *Dsim w<sup>501</sup>* and IL 16.30, exhibit significantly smaller expression domains than *trn<sup>mau</sup>* males, IL 43 and *Dmau D1* (all comparisons in *trn* expression domain between lines are significant [ $P < 0.001$ ], except for those indicated by ns (nonsignificant), see also Dataset S5b). Boxes show the range, upper and lower quartiles, and the median for each sample. Representative *trn* expression at the base of the claspers is shown on the right-hand side. (E) The proportion of males with *trn* expression at the base of the clasper at 48 hAPF (between stages 5 and 6) in IL 16.30 and IL 43. IL43 males exhibit on average 51.9% more *trn* expression at the base of the claspers than IL 16.30 males (Dataset S5c).

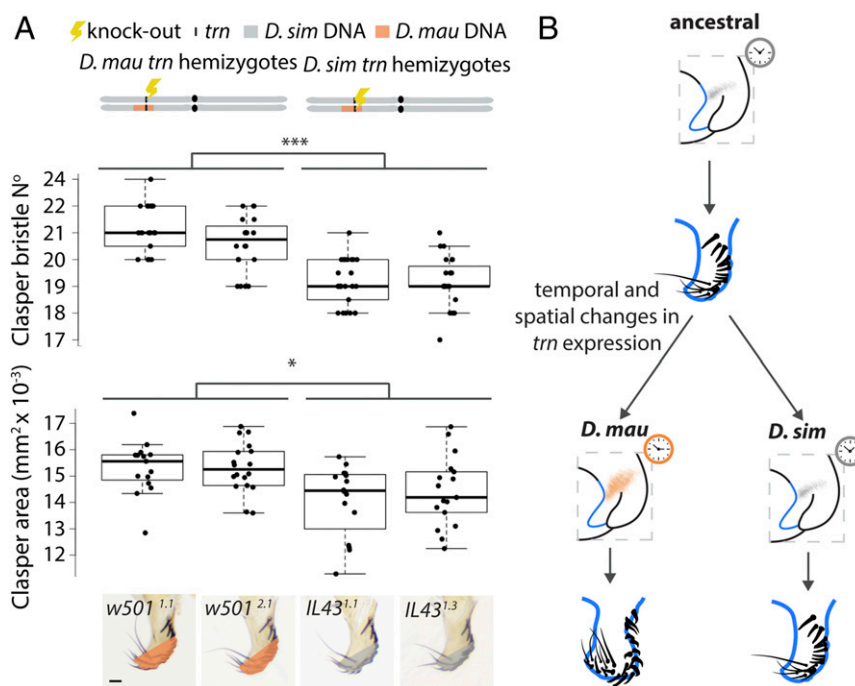
never detected at the base of the developing claspers (Fig. 3 A and B and SI Appendix, Fig. S4A).

Although our expression analyses of developing claspers suggest that cis-regulatory changes in *trn* are likely to contribute to differences in clasper morphology between *D. mauritiana* and *D. simulans*, we also found a total of 22 nucleotide differences in the coding sequence of *trn* between our mapped strains, *Dmau D1* and *Dsim w<sup>501</sup>*. Only three of these differences are non-synonymous and none is fixed between the two species (Dataset S6). In addition, a comparison of clasper size between strains of *D. simulans* and *D. mauritiana* with different combinations of amino acids at these three sites suggests that none of these substitutions is sufficient to explain the contribution of *trn* to the difference in clasper size between the species (SI Appendix, Fig. S5 and Dataset S2e). However, although the clasper size of the two mapped strains is also well within the range of their species (SI Appendix, Fig. S1 and Dataset S2d), we cannot rule out that one or more of these three amino acid substitutions may contribute to the difference in clasper size between the two strains used in this study.

To confirm that sequence divergence in *trn* contributes to the difference in clasper morphology between *Dmau D1* and *Dsim w<sup>501</sup>*, we used CRISPR/Cas9 to make null alleles of *D. simulans trn* (in *Dsim w<sup>501</sup>*) and *D. mauritiana trn* (in IL43, see Fig. 2 and

SI Appendix, Fig. S6). We then generated reciprocal hemizygotes for *trn* i.e., genetically identical male flies that differ only in whether they have a functional copy of *trn* from *D. mauritiana* or *D. simulans* (Fig. 4A) (39). Comparison of the claspers between male reciprocal hemizygotes of *trn* shows that flies with a functional *D. mauritiana trn* allele have significantly larger claspers ( $P < 0.05$ ) with more bristles ( $P < 0.001$ ) than those with a functional *D. simulans trn* allele (Fig. 4B and Dataset S2c). This confirms that, consistent with the effects of the introgressions containing *trn* (Fig. 2), *D. mauritiana trn* has evolved to confer larger claspers than *D. simulans trn*.

We have found that *trn* is a gene that underlies the rapid evolution in the size of a male genital organ and more generally a gene that contributes to differences in animal organ size (e.g., refs. 40–42). Many examples of phenotypic evolution, including differences in genital bristles between other *Drosophila* species (43), have been found to be caused by changes in the expression of transcription factors (44). However, *trn* is a transmembrane protein that appears to mediate differences in cell-cell contact directly through its extracellular domain, directing cells toward their correct positions via cues that are currently unknown (33, 35). Our results suggest that differences in *trn* expression in *Drosophila* are able to alter clasper size. Therefore, changes in cell affinity caused by variation in the temporal and/or spatial



**Fig. 4.** Reciprocal hemizygotes of *trn* show that this locus contributes to evolutionary differences in male clasper morphology. (A) Schematic at the top illustrates the third chromosome of the reciprocal hemizygotes carrying a functional allele of *trn* from only *Dmau* D1 (Left) and *Dsim* *w*<sup>501</sup>. We found a significant difference in their clasper area ( $F_{(3, 61)} = 7.012$ ,  $P < 0.001$ ) and clasper bristle number ( $F_{(3, 83)} = 26.29$ ,  $P < 0.001$ ), shown in the boxplots underneath. Flies with a functional *trn* allele from *D. mauritiana* (*IL43*<sup>1.1</sup> and *IL43*<sup>1.3</sup>) have significantly larger claspers ( $*P < 0.05$ ) with more bristles ( $***P < 0.001$ ) than those with a functional *D. simulans* *trn* allele, *w501*<sup>1.1</sup> and *w501*<sup>2.1</sup> (Dataset S2d). Boxes show the range, upper and lower quartiles, and the median for each sample. (B) Evolutionary changes increased the spatial domain and temporal expression of *trn* during clasper development in *D. mauritiana* have led to larger claspers with more bristles in this species compared to *D. simulans*. Orange and gray shading indicate broad and narrow expression of *trn* at the base of the developing claspers in *D. mauritiana* and *D. simulans*, respectively. The correspondingly colored clocks indicate differences in the persistence of this expression domain. (Scale bar, 20  $\mu$ m.)

expression of transmembrane proteins that mediate cell interactions may represent another mechanism for the evolution of organ size. There is also some evidence, however, that *trn* could act as a ligand and may transduce signals, although its intracellular domain appears to be dispensable for most of its functions (28, 30, 33, 36). Therefore, further study of the function of *trn* and characterization of its role in organ size regulation and evolution is required.

## Materials and Methods

**Introgression Mapping and Phenotyping.** We increased the resolution of the previously predicted C2 region by generating recombinants between introgression line *D11.01* and *Dsim* *w*<sup>501</sup> (19) (SI Appendix, SI Materials and Methods). Flies were phenotyped and genotyped as described previously (19) using molecular markers (Dataset S7). All stocks and crosses were maintained on a standard cornmeal diet at 25 °C under a 12-h:12-h dark/light cycle unless otherwise stated. The periphallid structures and T1 legs were dissected, imaged, and measured as described in ref. 19 (SI Appendix, SI Materials and Methods and Dataset S2). All statistical analyses were conducted in R Studio.

**RNA Sequencing and Differential Expression Analysis.** Three independent biological replicates of RNA-Seq libraries were generated from abdominal tip tissue dissected from 20 to 30 males for *Dsim* *w*<sup>501</sup> and *Dmau* *w*<sup>501</sup> males at 30 and 50 hAPF (SI Appendix, Fig. S3). Indexed libraries were sent to Macrogen Japan for sequencing in a single lane of HiSeq4000 (Illumina), producing 100-bp paired-end reads. Raw fastq files are deposited at DDBJ under the accession no's. DRA006755 and DRA006758 for *D. mauritiana* and *D. simulans*, respectively. Genes were considered not expressed if reads per kilobase million (RPKM) was below 1.5. RNA-Seq analysis of genes in C2 as well as *CG11279* and *caps* is summarized in Dataset S3.

**RNAi Knockdown of C2 Candidate Genes.** We conducted RNAi knockdown of all of the genes within region C2 (with the exception *CG34429* for which there was no available UAS line) in *D. melanogaster* using UAS-RNAi lines from Vienna Drosophila Resource Center (VDRC, [www.vdrc.at](http://www.vdrc.at)) and from Bloomington Drosophila Stock Center (Dataset S8). UAS males of our candidate genes were crossed to NP6333-GAL4 driver female virgins (P(GawB) PenNP6333) (45) carrying the transgene UAS-Dicer-2 P(UAS-Dcr-2.D). Crosses for the RNAi were carried out at 25 °C. The genital morphology of the male knockdowns was compared to NP6333-GAL4, UAS-Dicer, and UAS-RNAi controls. Clasper bristle number and tibia length were measured for 16 individuals of each genotype. Differences in clasper bristle number and tibia size were assessed using a one-way ANOVA followed by a Tukey's test (Dataset S4). For raw phenotypic data, see Dataset S2a.

***trn* Sequence Analysis.** To assess the number of interspecific nucleotide substitutions in the coding sequence of *trn*, we used polymorphism data from Pool-seq data from 107 strains of *D. mauritiana* and from 50 strains of sub-Saharan *D. simulans* (46, 47) available at <http://www.popoolation.at/pgt/> as well as from whole genome data for 10 strains of each species submitted to the SRA database by the University of Rochester (*D. mauritiana* lines: SRX135546, SRX688576, SRX688581, SRX688583, SRX688588, SRX688609, SRX688610, SRX688612, SRX688710, SRX688712; *D. simulans* lines: SRX497551, SRX497574, SRX497553, SRX497563, SRX497558, SRX497564, SRX497559, SRX495510, SRX495507, SRX497557). The sequence analysis is summarized in Dataset S6.

**In Situ Hybridization.** We performed in situ hybridization to detect *trn*, *CG11279*, *CG34429*, and *caps* expression in the male terminalia of *Dmau* D1, *Dsim* *w*<sup>501</sup>, and *D. melanogaster* *w*<sup>1118</sup> at a range of developmental time-points (SI Appendix, Fig. S3).

**Quantification of Temporal and Spatial *trn* Expression.** To investigate potential differences in the *trn* expression domain between introgression lines used to map the C2 interval, in situ hybridizations were carried out at 46 hAPF in

*Dsim*  $w^{501}$ , *IL* 16.30, *IL* 43, and 50 hAPF in *Dmau* D1. It is at these time points that the largest differences in *trn* expression can be detected between the two parental species (Fig. 3 A and B). *Dsim*  $w^{501}$ , *IL* 16.30, and *IL* 43 are morphologically equivalent at these stages (SI Appendix, Fig. S3).

**Generation of Reciprocal Hemizygotes and Statistical Analysis.** We inserted 3xP3-DsRed to disrupt the reading frame of *trn* in *Dsim*  $w^{501}$  and *IL43* using CRISPR/Cas9 (SI Appendix, Fig. S6). Injections were carried by The University of Cambridge Department of Genetics Fly Facility. Transgenic *Dsim*  $w^{501}$  and *IL43* males heterozygous for the mutation were then crossed to noninjected

*IL43* and *D. simulans*  $w^{501}$  virgin females, respectively, to generate F<sub>1</sub> males carrying the mutation (i.e., hemizygous for *trn* allele).

See extended methodological details in SI Appendix, SI Materials and Methods.

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