**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 periphallic 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.

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**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 *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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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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Datasets S2 and SI Appendix and RNA-Sequencing (RNA-Seq) data (Dataset S2) illustrate 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–D) Scanning electron micrographs of external male genitalia (B and C) and dissected claspers (D and E) of Dmau D1 and Dsim w501, respectively. (Scale bars, 50 μm.)

Fig. 1. Divergence in periphallic 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–D) Scanning electron micrographs of external male genitalia (B and C) and dissected claspers (D and E) of Dmau D1 and Dsim w501, 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 interspecific 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. 2A). 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, causing mis-specified 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 w501 (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 w501 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 pupariation 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 w501 (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 trnmax allele relative to the trnmin 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 trnmax (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 w501 and ILs) than those containing trnmin (Dsim w501 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 w501 (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 trnmax but in 74% of IL43 males, i.e., males with trnmin) (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...
High-resolution mapping of differences in clasper morphology between *Dsim w*^501^ 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*^501^, *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 *CG11279* 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*^501^ (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*^501^ (gray) or *Dmau D1* (orange) regions for C2. Boxes indicate the range, upper and lower quartiles, and median for each sample. (Scale bar, 20 μm.)
never detected at the base of the developing claspers (Fig. 3 A and B and SI Appendix, Fig. S44).

Although our expression analyses of developing claspers suggest that cis-regulatory changes in \( \text{trn} \) are likely to contribute to differences in clasper morphology between \( D. \text{mauritiana} \) and \( D. \text{simulans} \), we also found a total of 22 nucleotide differences in the coding sequence of \( \text{trn} \) between our mapped strains, \( D. \text{mauritiana} \) and \( D. \text{simulans} \) \( w^{501} \). 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 \( D. \text{simulans} \) and \( D. \text{mauritiana} \) with different combinations of amino acids at these three sites suggests that none of these substitutions is sufficient to explain the contribution of \( \text{trn} \) 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 \( \text{trn} \) contributes to the difference in clasper morphology between \( D. \text{mauritiana} \) and \( D. \text{simulans} \), we used CRISPR/Cas9 to make null alleles of \( D. \text{simulans} \) \( \text{trn} \) (in \( D. \text{simulans} \) \( w^{501} \)) and \( D. \text{mauritiana} \) \( \text{trn} \) (in IL43, see Fig. 2 and SI Appendix, Fig. S6). We then generated reciprocal hemizygotes for \( \text{trn} \), i.e., genetically identical male flies that differ only in whether they have a functional copy of \( \text{trn} \) from \( D. \text{mauritiana} \) or \( D. \text{simulans} \) (Fig. 4A) (39). Comparison of the claspers between male reciprocal hemizygotes of \( \text{trn} \) shows that flies with a functional \( D. \text{mauritiana} \) \( \text{trn} \) allele have significantly larger claspers \( (P < 0.05) \) with more bristles \( (P < 0.001) \) than those with a functional \( D. \text{simulans} \) \( \text{trn} \) allele (Fig. 4B and Dataset S2c). This confirms that, consistent with the effects of the introgressions containing \( \text{trn} \) (Fig. 2), \( D. \text{mauritiana} \) \( \text{trn} \) has evolved to confer larger claspers than \( D. \text{simulans} \).

We have found that \( \text{trn} \) 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, \( \text{trn} \) 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 \( \text{trn} \) expression in \( Drosophila \) are able to alter clasper size. Therefore, changes in cell affinity caused by variation in the temporal and/or spatial
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

Introgenression Mapping and Phenotyping. We increased the resolution of the previously predicted C2 region by generating recombinants between intertrogession line D11.01 and Dsim w^{901} (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 periphalic 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 biologic replicates of RNA-Seq libraries were generated from abdominal tip tissue dissected from 20 to 30 males for Dsim w^{901} and Dmau D1 as well as from whole genome data for 10 strains of each species submitted to the SRA database by the University of Rochester (Dataset S8). Dataset S6. 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, SRX688609, SRX688610, SRX688612, SRX688710, SRX688712; D. simulans lines: SRX497551, SRX497574, SRX497553, SRX497563, SRX497558, 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^{901}, and D. melanogaster w^{1118} 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 hAEP 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 3×P3-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 F1 males carrying the mutation (i.e., hemizygous for trn allele).

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