Ancient balancing selection at \textit{tan} underlies female colour dimorphism in \textit{Drosophila erecta}

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Dimorphic traits are ubiquitous in nature, but the evolutionary factors leading to dimorphism are largely unclear. We investigate a potential case of sexual mimicry in \textit{Drosophila erecta}, in which females show contrasting resemblance to males. We map the genetic basis of this sex-limited colour dimorphism to a region containing the gene \textit{tan}. We find a striking signal of ancient balancing selection at the ‘male-specific enhancer’ of \textit{tan}, with exceptionally high sequence divergence between light and dark alleles, suggesting that this dimorphism has been adaptively maintained for millions of years. Using transgenic reporter assays, we confirm that these enhancer alleles encode expression differences that are predicted to generate this pigmentation dimorphism. These results are compatible with the theoretical prediction that divergent phenotypes maintained by selection can evolve simple genetic architectures.
n developing his multifactorial theory of inheritance, R.A. Fisher\(^1\) showed that genetic variation at just a few loci, together with environmental variance, could lead to continuous variation in a phenotypic trait. And yet, a surprising number of traits in natural populations shows variation that is dichotomous, rather than continuous (for example, sexes, social castes, colour morphs, left–right asymmetry), and these dimorphisms can have either a complex or simple genetic architecture\(^2\)–\(^4\). The role of selection in maintaining dimorphic traits has had a long history in evolutionary biology\(^5\). However, the mechanisms leading to the origin of distinct morphs are largely unclear\(^6\)–\(^8\). Theory predicts that long-term frequency-dependent disruptive selection (FDDS) on a continuous polygenic trait, in which disruptive selection eliminates intermediate phenotypes, whereas frequency-dependent selection maintains extreme phenotypes, can lead to the evolution of dimorphic traits with simple genetic architectures\(^6\), but empirical support for this model is lacking\(^9\),\(^10\). 

*Drosophila* pigmentation offers unique opportunities to dissect the evolution of genetic architectures, in part due to its well-understood biochemical pathway, as well as the presence of both complex and monogenic systems in related species\(^11\). Notably, a number of *Drosophila* species have evolved an intriguing form of colour dimorphism, in which some females resemble males while others are visually distinct. A prime example of this female-limited colour dimorphism (FLCD) is found in *D. erecta*, a member of the melanogaster species subgroup. *D. melanogaster* reflects the ancestral condition of this clade, wherein males consistently have dark posterior abdomen, whereas females have a continuously varying abdominal pigmentation (Fig. 1a) that is dichotomous rather than continuous (for example, sexes, social castes, colour morphs, left–right asymmetry), and these dimorphisms can have either a complex or simple genetic architecture\(^2\)–\(^4\). The role of selection in maintaining dimorphic traits has had a long history in evolutionary biology\(^5\). However, the mechanisms leading to the origin of distinct morphs are largely unclear\(^6\)–\(^8\). Theory predicts that long-term frequency-dependent disruptive selection (FDDS) on a continuous polygenic trait, in which disruptive selection eliminates intermediate phenotypes, whereas frequency-dependent selection maintains extreme phenotypes, can lead to the evolution of dimorphic traits with simple genetic architectures\(^6\), but empirical support for this model is lacking\(^9\),\(^10\).

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The FLCD found in *D. erecta* and other *Drosophila* species is reminiscent of a classic system of sexual mimicry described in some damselfly species, in which male-mimicking females are believed to benefit from avoiding harassment by males when population densities are high\(^14\),\(^15\). Concordantly, Paytan\(^16\) found that evidence for frequency-dependent mating behaviour: when light females of *D. erecta* were common, dark females mated less. Further, it seems plausible that the cost of mating for females may be higher in *D. erecta* than in its non-FLCD relatives, in light of the species’ larger and serrated male phallus and the corresponding protective plates found in females\(^17\). Hence, one hypothesis for FLCD is that male-mimicking females may avoid costly rematings, as has been suggested for multiple damselfly species\(^14\),\(^15\).

In this paper, we use introgression mapping to localize the genetic basis of *D. erecta* FLCD to a single locus on the X chromosome containing the melanin synthesis enzyme gene *tan*. Based on the genetic divergence estimates between morphs, we detect a strong signal of ancient balancing selection on the previously identified ‘male-specific enhancer’ of this gene. We confirm using transgenic reporters that alleles at this enhancer encode expression differences in females that are predicted to replicate their pigmentation differences. Our results support a hypothesis in which long-term balancing selection on female colour morphs yielded a simple genetic architecture involving the modification of a sexually dimorphic enhancer.

**Results**

**Introgression mapping of FLCD in *D. erecta***. Whereas multiple genes affect continuous pigmentation in related species such as *D. melanogaster*\(^18\)–\(^21\), we found FLCD in *D. erecta* to be caused by a single X-linked locus, in agreement with previous studies and observations\(^16\). When we crossed the dominant allele conferring dark female pigmentation from an inbred dark-female strain into an inbred light-female strain, all F1 flies were dark indistinguishable from their dark parents, indicating complete dominance of the dark allele. Phenotypes of F2 flies did not deviate from the 1:1 ratio expected from a single X-linked locus (680 and 645 dark and light females, respectively; \(X^2, P = 0.34\)). To identify the causative locus, we introgressed the dark allele into a light background through a series of backcrosses and performed genomic sequence analysis on seventh offspring of this introgression heterozygous for the dark allele (line BC7). After 12 generations, we generated an introgression line homozygous for the dark allele from four dark homozygous parents (line NN) and sequenced its genome as well. The dark pigmentation of these flies did not differ from the parental dark line (pigmentation scores: 18.45 ± 0.24; Mann–Whitney \(U\)-test \(P = 0.20\); Supplementary Fig. 1). We then estimated the proportion of alleles from the dark parent in both BC7 and NN lines (Supplementary Fig. 2). Since this ancestry proportion at the FLCD locus is expected to be 50% and 100% in the BC7 and NN lines, respectively, the average score of the FLCD locus should be near 75%. Only ten neighbouring windows comprising a 1-Mb interval on chromosome X conformed to this expectation. The 100-kb window with the highest ancestry metric contained the known pigmentation gene *tan* (Fig. 2a), an enzyme that helps govern the transition between light and dark pigment precursors\(^22\).

**Sequence divergence between morphs at the mapped region**. Examining parental strain genome sequences around the *tan* locus, we identified a ~0.5-kb interval with extreme sequence divergence between the female-light and female-dark alleles (Fig. 2b). This window corresponds to the ‘male-specific
Figure 2 | The FLCD locus maps to the tan male-specific enhancer (t_MSE) region in D. erecta. (a) Genetic mapping signal indicated by ancestry proportion of the dark parental line averaged between heterozygous dark 7th backcross generation and homozygous dark introgression line. Each dot corresponds to a 100-kb window, and each heterozygous dark 7th backcross generation and homozygous dark ancestry proportion of the dark parental line averaged between species, D. santomea (t_MSE) region in D. erecta (D. erecta t_MSE), which contributed to the loss of male abdominal pigmentation in another melanogaster subgroup species, D. santomea23, and has been localized to a ~150-bp region24. At the t_MSE, the divergence between D. erecta genomes was several times higher than normal (Fig. 2b). Comparing the t_MSE region to the rest of the X chromosome, the Hudson–Kreitman– Aguadé (HKA)-like test25 confirmed a substantial excess of within-species polymorphism relative to expectations based on divergence between D. erecta and its relative D. orena (P < 7 × 10⁻¹⁹; Supplementary Fig. 3 and Supplementary Table 1).

Population genetic analysis of the t_MSE-containing locus. The exceptional differentiation between light and dark D. erecta t_MSE sequences suggests an ancient divergence time between these alleles, and is most consistent with a history of long-term balancing selection. Consistent with this explanation, when we sequenced a 1-kb region containing the t_MSE from 36 wild-caught D. erecta males, alleles clustered into two haplogroups similar to our light and dark strain t_MSE sequences (Fig. 3a and Supplementary Note 1). These haplogroups were present at intermediate frequencies in both Cameroon and Gabon (Fig. 3b), and no differentiation was observed between sequences from these populations (FST = 0.02). Since most variation was found between haplogroups rather than within them, FST between haplogroups showed very high levels of genetic differentiation (Fig. 3c). In contrast, flanking loci 5–10 kb away had low differentiation between t_MSE haplogroups, and HKA analysis confirmed a powerful excess of polymorphism at the t_MSE-containing locus relative to the flanking loci (G², P = 4.99 × 10⁻⁴ and 1.50 × 10⁻⁵ for up- and downstream loci, respectively; Supplementary Table 2 and Supplementary Notes 2 and 3). Notably among the 135 polymorphic sites of the t_MSE-containing locus, 76 nucleotide differences were fixed between the light and dark D. erecta alleles, one-third of which (that is, 26) fell within the t_MSE sequence (Supplementary Fig. 4).

Functional analysis of alleles at the t_MSE-containing locus. To investigate the link between t_MSE alleles and tan expression, we generated reporter constructs in which the t_MSE region of dark D. erecta, light D. erecta or D. orena was placed upstream of green fluorescent protein (GFP). Each construct was inserted into the same 51D site of the D. melanogaster genome using the FHC3I integrase system28. In transgenic flies, the level of GFP fluorescence in a relevant tissue then permits an assessment of the regulatory element’s activity. We found that GFP in the abdomen, consistent with the light female pigmentation of the source strains. A consistent match between t_MSE reporter activity and tan protein staining has been reported across a wide range of Drosophila species24. However, additional experiments will be needed to confirm that an
respectively. The SNP between the light and dark haplogroups, for the sexually dimorphic29. Although the exact binding sites of Bab1 in insects31. Aside from the damselfly case referenced above, described in at least two other groups of non-drosophilid dimorphism. The precise dissection of the genetic basis of this sexual colour by the transcription factors Hox genes D. erecta in and Bab2 are still unknown, it is possible that the dark haplotype still unknown but mapping indicates that it is different from species with monogenic FLCD (D. santomea), the FLCD locus is leading to the evolution of female-like light males23 as well as in other sexually monomorphic Drosophila species24.

In the abdomen of D. melanogaster, tan is upregulated in males by the Hox genes Abd-A and Abd-B24 and suppressed in females by the transcription factors bab1 and bab2 whose expressions are sexually dimorphic29. Although the exact binding sites of Bab1 and Bab2 are still unknown, it is possible that the dark haplotype in D. erecta involves the loss of such sites. In another Drosophila species with monogenic FLCD (D. kikkawai), the FLCD locus is still unknown but mapping indicates that it is different from tan or bab250, highlighting the complexity of this trait. Future investigations using recent advances in Drosophila molecular biology techniques as well as other species with FLCD will help the precise dissection of the genetic basis of this sexual colour dimorphism.

Although quite rare, female-limited colour variation has been described in at least two other groups of non-drosophilid insects31. Aside from the damselfly case referenced above, certain butterflies have both mimetic and non-mimetic female morphs, which vary in frequency geographically due to spatially varying selective pressures32–34. These morphs are associated with ~400 kb chromosomal rearrangements in Heliconius butterflies3. In Papilio polytes, complex pigmentation variation correlates with ~130 kb inversion-associated alleles of doublesex4,35, a component of the sexual differentiation pathway. In our study, the genetic tools and knowledge of Drosophila allowed us to localize the genetic basis of a sex-limited dimorphic trait to a regulatory element of tan, indicating the strong potential of this system for further insights regarding the mechanisms of sex-specific evolution and the origin of dimorphic traits.

Our study provides a rare example of the genetic basis of an ancient balanced polymorphism with clear morphological consequences. Most known examples include genes involved in immunity interactions or mate recognition such as major histocompatibility complexes in vertebrates36, blood groups in primates37, self-incompatibility in plants38 and mating-types in fungi39. Balancing selection can involve different mechanisms such as heterozygous advantage, spatially or temporally variable selective pressures, or frequency-dependent selection. In the case of D. erecta FLCD, it is not clear why ecological factors would maintain discrete pigmentation morphs at similar frequencies in different populations, and preserve them for millions of years. Payant16 conducted extensive experiments on factors maintaining D. erecta FLCD in the laboratory. She observed a mating preference for light females when the frequency of the light allele was between 0.5 and 0.7. Although further study is called for, frequency-dependent sexual selection thus represents a plausible explanation for FLCD, especially given the morphological similarity of our case with FLCD in damselflies, a prime model for frequency-dependent sexual selection14,15,40.

The monogenic nature of FLCD and the frequency-dependent mating results cited above suggest that this trait could be expression change at tan is responsible for FLCD, and to identify the causative nucleotide(s).

**Discussion**

The above results motivate the hypothesis that the dark-female t_MSE allele of D. erecta evolved by extending the activity of an otherwise male-specific enhancer into females as well. Increasing tan expression in the female abdomen is predicted to increase production of melanic pigments22, leading to the male-like pigmentation observed in females of dark D. erecta strains. This dark allele may therefore represent a loss of sexual dimorphism at the molecular and phenotypic levels, even as it creates a novel dimorphism among females. Curiously, this same cis-regulatory element of tan has also underlain the loss of sexual dimorphism in the related species D. santomea leading to the evolution of female-like light males23 as well as in other sexually monomorphic Drosophila species24.

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The monogenic nature of FLCD and the frequency-dependent mating results cited above suggest that this trait could be
consistent with theoretical predictions for FDSS, which can lead to the evolution of dimorphic traits with simple genetic architectures\textsuperscript{7}. Disruptive selection would explain the lack of intermediate phenotypes found in nature, whereas evidence from this work is consistent with a role for balancing selection in maintaining *D. erecta* FLCD, potentially due to frequency-dependent sexual selection. FDSS should increase the effect of one or a few loci relative to all others. In agreement with this model, other *Drosophila* species have more modest and continuous pigmentation variation because of several genes including *tan*\textsuperscript{18–20,41}, whereas in the *D. erecta* lineage *tan*’s role increased to shape a discrete colour dimorphism. These results reflect important steps towards understanding the evolutionary and genetic mechanisms that give rise to dimorphic traits and sex-specific variation in nature.

**Methods**

**Laboratory strains and pigmentation phenotyping.** We generated several isofemale lines from a mass culture of *D. erecta* collected from Gabon in 2006 and where both colour morphs segregate with no intermediate phenotypes. We then selected two lines that were homozygous either for the dark or light allele. We scored female pigmentation on the last two abdominal tergites from 0 (no pigmentation) to 10 (completely pigmented)\textsuperscript{12} for 10 females from the two parental lines as well as from a mass culture of a French population of *D. erecta* collected from Prunay in 2011 and a laboratory line of *D. erecta* (strain no. 14021-0245.01 at Drosophila Stock Center, UCSD). Scores were summed over the two tergites and the R software package (http://www.r-project.org) was used to generate histograms illustrating the phenotypic distribution of each line. All lines were raised on standard *Drosophila* medium at 21 °C.

**Generation of introgression lines.** The light *D. erecta* line was submitted to inbreeding, then a dark allele of a single male was introgressed into the light line for 12 successive backcrosses. For each backcross, 20 virgin, heterozygous dark females were crossed with 20 males from the parental light strain. After 12 generations, several male and virgin female pairs were established and their F\textsubscript{2} progeny was checked for the presence of recessive light homozygotes. Four dark homozygous pairs were thus identified and pooled to generate the introgression line (NN) homozygous for the dark allele.

**Preparation of genome libraries.** We sequenced the genomes of pooled samples (30 flies) for the parental dark and light lines, dark heterozygous flies from the introgression line (NN) and the laboratory line of *D. arenata*. Genomic DNA was obtained using chloroform extraction and ethanol precipitation. DNA was then fragmented using Bioruptor sonicator (Diagenode), and paired-end libraries with ~300 bp inserts were prepared using NEBNext DNA Library Prep Reagent Set for Illumina (New England Biolabs, no. E6000L). Concentration and quality of libraries were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). They were then sequenced at UW-Madison Biotechnology Center on the Illumina HiSeq 2000 platform with 100 bp read lengths.

**Alignment of genomic reads.** For each genome, reads were mapped to the *D. erecta* reference genome r.1.3 obtained from Flybase (http://www.flybase.org) using default parameters in BWA ver. 0.6.2-r126 (ref. 42), and the BAM files were remapped using Stampy ver. 1.0.21 (ref. 43). Reads were then filtered for a mapping quality of 20 and for proper pairs with samtools ver. 0.1.18 (ref. 44). BAM files were cleaned by removing unmapped reads and sorted by coordinate, and PCR...
Duplicates were marked using Picard ver. 1.109 (http://picard.sourceforge.net). Alignment around indels was then improved using GATK ver. 3.2 (ref 45.46). The average coverage per genome was about × 17 and × 30 for the parental light and dark lines, respectively, and × 44 and × 80 for BC7 and NN introgression lines, respectively, and × 21 for D. arena.

**Genome mapping.** The PoPoolation2 ver. 1.201 software package47 was used to generate a synchronized map file for the five genomes aligned to the D. erecta reference. The FLCD locus was identified by estimating the dark strain ancestry proportion in BC7 and NN introgression lines. For each diploid single-nucleotide polymorphism (SNP) with a minimum count of ten in all lines, this quantity was calculated as the proportion of sequence reads carrying the allele that is fixed in the dark parental line and absent in the light parental line. Dark strain ancestry proportion was averaged across SNPs within 100 kb windows. As a quality filter, window counts within 100 kb windows with fewer than 0.01 fixed difference per 1,000 sites between parental lines were excluded as potentially identical-by-descent between these strains. Genetic distances (Dxy) were then compared between the two parental lines of D. erecta at 100 kb windows that conformed to the expected average ancestry score of 75% and centred on 10, using a perl script (available upon request from the first author). For each diachile SNP, with a minimum sequencing coverage of ≥ 2, Dxy was estimated from the formula:

\[ D_{xy} = p_1 q_2 + p_2 q_1 \]

where p is the frequency of the allele similar to the allele in the reference D. erecta genome, and q is the frequency of the alternative allele in each line. Average Dxy at each SNP ± 250 SNPs (that is, sliding windows of 501 SNPs) was then estimated.

**HKA-like test.** To test for a chromosome-wide signal for balancing selection, we estimated the number of polymorphic SNPs (that is, SNPs with Dxy > 0.50 between the light and dark parental lines) and divergent SNPs (that is, SNPs with Dxy = 1) between both lines and D. arena along the X chromosome, that is, scaffolds 4,614 and 4,690 (ref. 48). For each SNP, the observed numbers of polymorphic and divergent SNPs at the SNP ± 50 SNPs (that is, sliding windows of 101 SNPs) were then compared with their expected numbers estimated from the chromosome-wide pattern using the HKA-like test45. Significance levels for deviation from expectations for each SNP window were estimated using z2 test at 1 degree of freedom as implemented in R.

**Single fly DNA extraction.** DNA was extracted from 19 and 4 ethanol-preserved male specimens of D. erecta collected from Mt. Bafut (Cameroon) and Mboumi, la Lopez and Libreville (Gabon), respectively. The Gabonese population was completed by sequencing 8 males from isofemale lines collected in 2000 and 5 males from the mass culture collected in 2006. Each fly was ground in 49 μl of squishing buffer and 1 μl proteinase K and incubated for 20–30 min at 37°C followed by 2 min at 95°C.

**PCR amplification.** We amplified the tan regulatory region (scaffold_4690:5994939..5995995 of the reference genome rel. 1.3) using ereF1 (5'-CGCTACGGATCCAGTGGTTA-3') and ereR1 (5'-GGCTACGGATCCAGTGGTTA-3') primers. These primers amplified the entire ~1 kb interval harbouring the regulatory region. Given the low DNA quality of some of the specimens, we amplified the t_MSE region for those specimens using primers ereF1 (5'-TCCGCTCACTTGGATTGATT-3') and ereR1 (5'-AGGCGAGCTCGTCTGCTA-3') using primers ereF1 (5'-AGGCGAGCTCGTCTGCTA-3') and ereR1 (5'-AGGCGAGCTCGTCTGCTA-3'). PCR products were cloned into the S3aG vector55, and inserted into the dark parental line and absent in the light parental line. Dark strain ancestry proportion was calculated as the proportion of sequence reads carrying the allele that is fixed in the dark parental line and absent in the light parental line. In each diachile SNP, with a minimum sequencing coverage of ≥ 2, Dxy was estimated from the formula:

\[ D_{xy} = p_1 q_2 + p_2 q_1 \]

where p is the frequency of the allele similar to the allele in the reference D. erecta genome, and q is the frequency of the alternative allele in each line. Average Dxy at each SNP ± 250 SNPs (that is, sliding windows of 501 SNPs) was then estimated.

**Functional validation of the tan regulatory region.** A ~850-bp region including the t_MSE sequence (scaffold_4690:5995962..5996007 of the D. erecta reference genome rel. 1.3) was PCR amplified using forward (5'-TCCGCTCACTTGGATTGATT-3') and reverse primers (5'-TGGCGCTCACTTGGATTGATT-3') and inserted into the 51D site of D. melanogaster using PCR3 integrate-mediated site-specific transgenesis (Best Gene Inc.). Presence or absence of GFP expression of transgenic male and females were scored at eclosion under an Olympus SZX16 Stereo Microscope equipped with an Olympus DP71 microscope digital camera.

**References.**

1. Fisher, R. A. The correlation between relations on the supposition of Mendelian inheritance. Phil. Trans. R. Soc. Edinburgh 52, 399–433 (1918).
2. Roff, D. A. The evolution of dimorphic traits: predicting the genetic correlation between environments. Genetics 136, 395–401 (1994).
3. Joron, M. et al. Chromosomal rearrangements maintain a polymorphic superegene controlling butterfly mimicry. Nature 477, 203–U102 (2011).
4. Kunte, K. et al. doublesex is a mimicry superegene. Nature 507, 229–232 (2014).
5. Ford, E. B. Polymorphism. Biol. Rev. 20, 73–88 (1945).
6. Mather, K. Polymorphism as an outcome of disruptive selection. Evolution 9, 52–61 (1955).
7. Kopp, M. & Hermisson, J. The evolution of genetic architecture under frequency-dependent disruptive selection. Evolution 60, 1537–1550 (2006).
8. Rueffler, C., Van Dooren, T. J. M., Leimar, O. & Abrams, P. A. Disruptive selection and then what? Trends Ecol. Evol. 21, 238–245 (2006).
9. Hansen, T. F. The evolution of genetic architecture. Annu. Rev. Evol. Ecol. Syst. 37, 123–157 (2006).
10. Schwander, T. & Leimar, O. Genes as leaders and followers in evolution. Trends Ecol. Evol. 26, 143–151 (2011).
11. Wittkopf, P. J., Carroll, S. B. & Kopp, A. Evolution in black and white: genetic control of pigment patterns in Drosophila. Trends Genet. 19, 495–504 (2003).
12. David, J. R., Capy, P. & Gauthier, J. P. Abdominal pigmentation and growth temperature in Drosophila melanogaster: similarities and differences in the norms of reaction of successive segments. J. Evol. Biol. 3, 429–445 (1990).
13. Ohsnishi, S. & Watanabe, T. K. Genetic analysis of color dimorphism in Drosophila montium subgroup. Jpn J. Genet. 60, 355–358 (1985).
14. Sirot, L. K., Brockmann, H. J., Marnis, C. & Muschtt, G. Maintenance of a female-limited polymorphism in Ischnura ramburii (Zygoptera: Coenagrionidae). Anim. Behav. 66, 763–775 (2003).
15. Gossum, H. V. et al. Reflectance spectra and mating patterns support intraspecific mimicry in the colour polymorphic damselfly Ischnura elegans. Ecol. Evol. 25, 139–154 (2010).
16. Payant, V. Le polymorphisme de coloration abdominale de Drosophila erecta est-il gouverné par un gène sélectivement neutre? Genet. Sel. Evol. 20, 37–50 (1988).
17. Yassin, A. & Orgogozo, V. Coevolution between male and female genitalia in the Drosophila melanogaster species subgroup. PLoS ONE 8, e57158 (2013).
18. Rebez, M., Pool, J. E., Kassner, V. A., Aquadro, C. F. & Carroll, S. B. Stepwise modification of a modular enhancer underlies adaptation in a Drosophila population. Science 326, 1663–1667 (2009).
19. Bickel, R. D., Kopp, A. & Nuzhdin, S. V. Composite effects of polymorphisms near multiple regulatory elements create a major-effect QTL. PLoS Genet. 7, e1001275 (2011).
20. Bastide, H. et al. A genome-wide, fine-scale map of natural pigmentation variation in Drosophila melanogaster. PLoS Genet. 10, e1003534 (2013).
21. Dembeck, L. M. et al. Genetic architecture of abdominal pigmentation in Drosophila melanogaster. PLoS Genet. 11, e1005163 (2015).
22. True, J. R. et al. Drosophila tan encodes a novel hydrolase required in pigmentation and vision. PLoS Genet. 1, e63 (2005).
23. Jeong, S. et al. The evolution of gene regulation underlies a morphological distance between two Drosophila BC7 isofemale sister species. Cell 132, 783–793 (2008).
24. Camino, E. M. et al. The evolutionary origination and diversification of a dimorphic gene regulatory network through parallel innovations in cis and trans. PLoS Genet. 11, e1003156 (2015).
25. Begun, D. J. et al. Population genomics: whole-genome analysis of polymorphism and divergence in Drosophila simulans. PLoS Biol. 5, e101 (2007).
26. Lee, W. H. & Watanabe, T. K. Evolutionary genetics of the Drosophila melanogaster subgroup. I. Phylogenetic relationships based on matings, hybrids and proteins. Jpn J. Genet. 62, 225–239 (1987).
27. Obbard, D. J. et al. Estimating divergence dates and substitution rates in the Drosophila phylogeny. Mol. Biol. Evol. 29, 3459–3473 (2012).
28. Bischko, J., Macla, R. K., Hediger, M., Karch, F. & Basler, K. An optimized transgenesis system for Drosophila using germ-line-specific F0/C31 integrases. Proc. Natl Acad. Sci. USA 104, 3312–3317 (2007).
29. Rogers, W. A. et al. Recurrent modification of a conserved is-y regulatory element underlies fruit fly pigment diversity. PLoS Genet. 9, e1003740 (2013).
30. Kopp, A. M. Metamorphs and phylogenetic replication: a systematic approach to the evolution of developmental pathways. Evol. Int. J. Org. Evol. 63, 2771–2789 (2009).
31. Wellenreuther, M., Svensson, E. I. & Hansson, B. Sexual selection and genetic trans-specific polymorphism at the mating type locus of the root decay fungus P. canadensis. Lepidoptera: Papilionidae. Evolution 50, 222–236 (1996).
32. Baxter, S. W. et al. Genomic hotspots for adaptation: the population genetics of Müllerian mimicry in the Heliconius melpomene clade. PLoS Genet. 6, e1000794 (2010).
33. Nishihara, T., Chou, P. M. et al. The Btl regulatory element underlies fruit fly pigmentation diversity. Current Biology 28, 179–187 (2018).
34. Scriber, J. M., Hagen, R. H. & Lederhouse, R. C. Genetics of mimicry in the tiger swallowtail butterflies, Papilio glaucus and P. canadensis (Lepidoptera: Papilionidae). Evolution 50, 456–470 (1996).
35. Roux, C. et al. Recent and ancient signature of balancing selection around the S-locus in Drosophila. Proc. Natl Acad. Sci. USA 109, 18493–18498 (2012).
36. Diepen, L. T. A., van Olson, Å., Ihrmark, K., Stenlid, J. & James, T. Y. Extensive trans-specific polymorphism at the mating type locus of the root decay fungus Heterobasidion. Mol. Biol. Evol. 30, 2286–2301 (2013).
37. Takahashi, Y., Kagawa, K., Svensson, E. I. & Kawata, M. Evolution of increased phenotypic diversity enhances population performance by reducing sexual harassment in damselflies. Nat. Commun. 5, 4468 (2014).
38. Wittkop, P. J. et al. Intraspecific polymorphism to interspecific divergence: genetics of pigmentation in Drosophila. Science 326, 540–544 (2009).
39. Koffler, R., Pandey, R. V. & Schlotterer, C. PoPoolation2: identifying differentiation between populations using sequencing of pooled DNA samples (Pool-Seq). Bioinformatics 27, 3435–3436 (2011).
40. Schaeffer, S. W. et al. Polymorphic chromosomal maps of 11 Drosophila species: the order of genomic scaffolds inferred from genetic and physical maps. Genetics 179, 1601–1655 (2008).
41. Librado, P. & Rozas, J. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452 (2009).
42. Hudson, R. R., Kreitman, M. & Aguadé, M. A test of neutral molecular evolution based on nucleotide data. Genetics 116, 153–159 (1987).
43. Edgar, R. C. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797 (2004).
44. Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729 (2013).
45. Drummond, A. J., Suchard, M. A., Xie, D. & Rambaut, A. Bayesian Phylogenetics with BEAUti and the BEAST 1.7. Mol. Biol. Evol. 29, 1969–1973 (2012).
46. Ordway, A. J., Hancuch, K. N., Johnson, W., Williams, T. M. & Rebeiz, M. The expansion of body coloration involves coordinated evolution in cis and trans within the pigmentation regulatory network of Drosophila pseudoobscura. Dev. Biol. 392, 431–440 (2014).
47. Williams, T. M. et al. The regulation and evolution of a genetic switch controlling sexually dimorphic traits in Drosophila. Cell 134, 610–623 (2008).

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Author contributions
A.Y. conceived the project, helped design the study, performed genome mapping, population genomics and phylogenetic analyses, produced and analysed transgenic flies, and drafted the manuscript with input from all authors. H.B. performed genome alignment and population genetics analyses and produced transgenic flies. H.C. conceived the functional analysis and produced the GFP reporter constructs. M.V. collected and taxonomically sorted wild-caught flies. J.R.D. conceived the project and conducted the introgression analysis. J.E.P. designed the analyses, directed the project and wrote the manuscript.

Additional information
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