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Co-evolving wing spots and mating displays are genetically separable traits in *Drosophila*

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The evolution of sexual traits often involves correlated changes in morphology and behavior. For example, in *Drosophila*, divergent mating displays are often accompanied by divergent pigment patterns. To better understand how such traits co-evolve, we investigated the genetic basis of correlated divergence in wing pigmentation and mating display between the sibling species *Drosophila elegans* and *Drosophila gunungcola*. *Drosophila elegans* males have an area of black pigment on their wings known as a wing spot and appear to display this spot to females by extending their wings laterally during courtship. By contrast, *D. gunungcola* lost both of these traits. Using Multiplexed Shotgun Genotyping (MSG), we identified a ~440 kb region on the X chromosome that behaves like a genetic switch controlling the presence or absence of male-specific wing spots. This region includes the candidate gene *optomotor-blind* (*omb*), which plays a critical role in patterning the *Drosophila* wing. The genetic basis of divergent wing display is more complex, with at least two loci on the X chromosome and two loci on autosomes contributing to its evolution. Introgressing the X-linked region affecting wing spot development from *D. gunungcola* into *D. elegans* reduced pigmentation in the wing spots but did not affect the wing display, indicating that these are genetically separable traits. Consistent with this observation, broader sampling of wild *D. gunungcola* populations confirmed that the wing spot and wing display are evolving independently: some *D. gunungcola* males performed wing displays similar to *D. elegans* despite lacking wing spots. These data suggest that correlated selection pressures rather than physical linkage or pleiotropy are responsible for the coevolution of these morphological and behavioral traits. They also suggest that the change in morphology evolved prior to the change in behavior.

KEY WORDS: Correlated traits, courtship behavior, *Drosophila*, optomotor-blind, pigmentation.

Animals often use colorful morphological structures to communicate with prospective mates during courtship (McKinnon and Pierotti 2010). In vertebrates and invertebrates, pigmented bodies or wings often evolve together with specific components of courtship behavior that animals use to display their colorful anatomy (Loxton 1979; Endler 1991; Sinervo et al. 2000; White et al. 2015). These correlated differences evolve both within and between populations, frequently distinguishing males from females or closely related species (Gray and McKinnon 2007; McKinnon and Pierotti 2010). In the handful of case studies examining the genetic basis of such co-evolving traits, linkage mapping and genome-wide association studies (GWAS) have shown that loci affecting pigmentation patterning tend to map to the same region of the genome as loci affecting variation in

mating behaviors (Lindholm and Breden 2002; Kronforst et al. 2006; Yeh et al. 2006; Thomas et al. 2008; Küpper et al. 2016; Lamichhaney et al. 2016; Merrill et al. 2019; reviewed in McKinnon and Pierotti 2010). That is, physical linkage of genetic variants often appears to underlie phenotypic correlations between mating behavior and pigmentation. Although linked loci tend to explain much of the variation observed for both traits, it remains unclear in all of these cases which genes underlie the observed correlations in phenotypic differences. A key challenge, therefore, is determining how frequently these patterns of genomic architecture underlie correlated evolution and whether a single pleiotropic locus or separate linked loci are involved.

Disentangling whether pleiotropic or physically linked loci underlie patterns of correlated evolution between pigmentation and mating behavior is important for understanding how natural selection generates differences between sexes and species. If two beneficial traits are genetically correlated due to separate, physically linked loci, theory predicts that natural or sexual selection (e.g., through predation or female choice) will act to minimize recombination between the causal loci (Charlesworth and Charlesworth 1976). It has been hypothesized that one solution to this problem might involve the evolution of chromosomal inversions that suppress recombination between two or more linked loci (Kirkpatrick and Barton 2006), and in multiple cases linked loci contributing to correlated evolution have been shown to lie within inversions (Thomas et al. 2008; Küpper et al. 2016; Lamichhaney et al. 2016). Alternatively, mutations at a single pleiotropic gene could cause correlated components of pigmentation and mating behavior to evolve simultaneously. Although such mutations have not yet been identified for pigmentation and behavior, they have been found for other co-evolving traits (e.g., Chang et al. 2009; Kent et al. 2009; McLean et al. 2011; Duveau and Félix, 2012; Endler et al. 2018; Nagy et al. 2018). Distinguishing between these genetic modes of phenotypic evolution requires, in part, high-resolution mapping of correlated traits.

In the Oriental *Drosophila melanogaster* species group, male-specific wing spots are phylogenetically correlated with mating displays (Kopp and True 2002; Fig. 1A). Species with wing spots perform elaborate wing display dances during courtship, extending their wings laterally, turning their dorsal wing surfaces toward the female, and waving them up and down; species without wing spots lack display behavior (Kopp and True 2002; Fig. 1A,B). Correlated gains and losses of both traits have evolved repeatedly (Kopp and True 2002; Fig. 1A). For example, in *D. elegans* and *D. gunungcola*, sibling species from this group that are estimated to have diverged 2–2.8 million years ago (Prud'homme et al. 2006), *D. elegans* (Bock and Wheeler 1972) males possess wing spots and perform wing displays, whereas *D. gunungcola* (Sultana et al. 1999) males lost both traits (Kopp and True 2002; Prud'homme et al. 2006; Yeh et al. 2006; Fig. 1B; Videos 1 and 2). Previously, Yeh et al. (2006) and Yeh and True (2014) discovered that *D. elegans* and *D. gunungcola* can generate fertile F_1 hybrid female offspring in the lab and they performed interspecific crosses to study the genetic basis of wing spot and wing display divergence. Through quantitative trait locus (QTL) mapping, they showed that evolution of linked loci on the X chromosome contributed to divergence in both traits (Yeh et al. 2006; Yeh and True 2014). One QTL explaining wing spot size variation was linked to the pigmentation gene *yellow*, supporting the hypothesis that *yellow cis*-regulatory divergence contributes to wing pigmentation evolution (Wittkopp et al. 2002a; Gompel et al. 2005; Prud'homme et al. 2006). It remained unclear, however, whether the same or different loci on the X chromosome underlie correlated differences in wing spot and wing display between these species.

To distinguish between these possibilities, we re-examined the genetic basis of wing spots and wing display divergence between *D. elegans* and *D. gunungcola*. Specifically, we (1) generated recombinant backcross progeny segregating for both traits, (2) assembled chromosome-length scaffolds of *D. elegans*, (3) used Multiplexed Shotgun Genotyping (MSG) (Andolfatto et al. 2011) to estimate recombination crossover positions across the genome, (4) generated quantitative measures of both wing spots and wing display behavior to estimate the effect size of loci contributing to divergence, and (5) generated advanced, recombinant introgressions on the X chromosome in an attempt to separate quantitative trait loci (QTL) underlying wing spots and wing display behavior. These experiments showed that a single locus on the X chromosome behaves like a genetic switch for wing spot divergence. Males with their wing spots turned off via introgression, however, performed wing displays like *D. elegans* males, indicating that the two traits are genetically separable. These findings suggest that wing spot and wing display behavior might have originally diverged independently. Consistent with this hypothesis, newly collected *D. gunungcola* strains from Indonesia appear to completely lack wing spots but retain the ability to perform wing displays. This observation suggests that the loss of wing spots occurred prior to the loss of wing display in the reference strain of *D. gunungcola* used in this study and in prior work.

Materials and Methods

FLY STOCKS

The *D. elegans HK* (Hong Kong) and *D. gunungcola SK* (Sukarami) lines used in this study were a gift from John True (Stony Brook University). Species stocks were kept on a 12 h light-dark cycle at 23°C on a University of Michigan "R food" diet containing molasses (http://lab-express.com/flyfoodsupplies.htm#rfood) (Wirtz and Semey

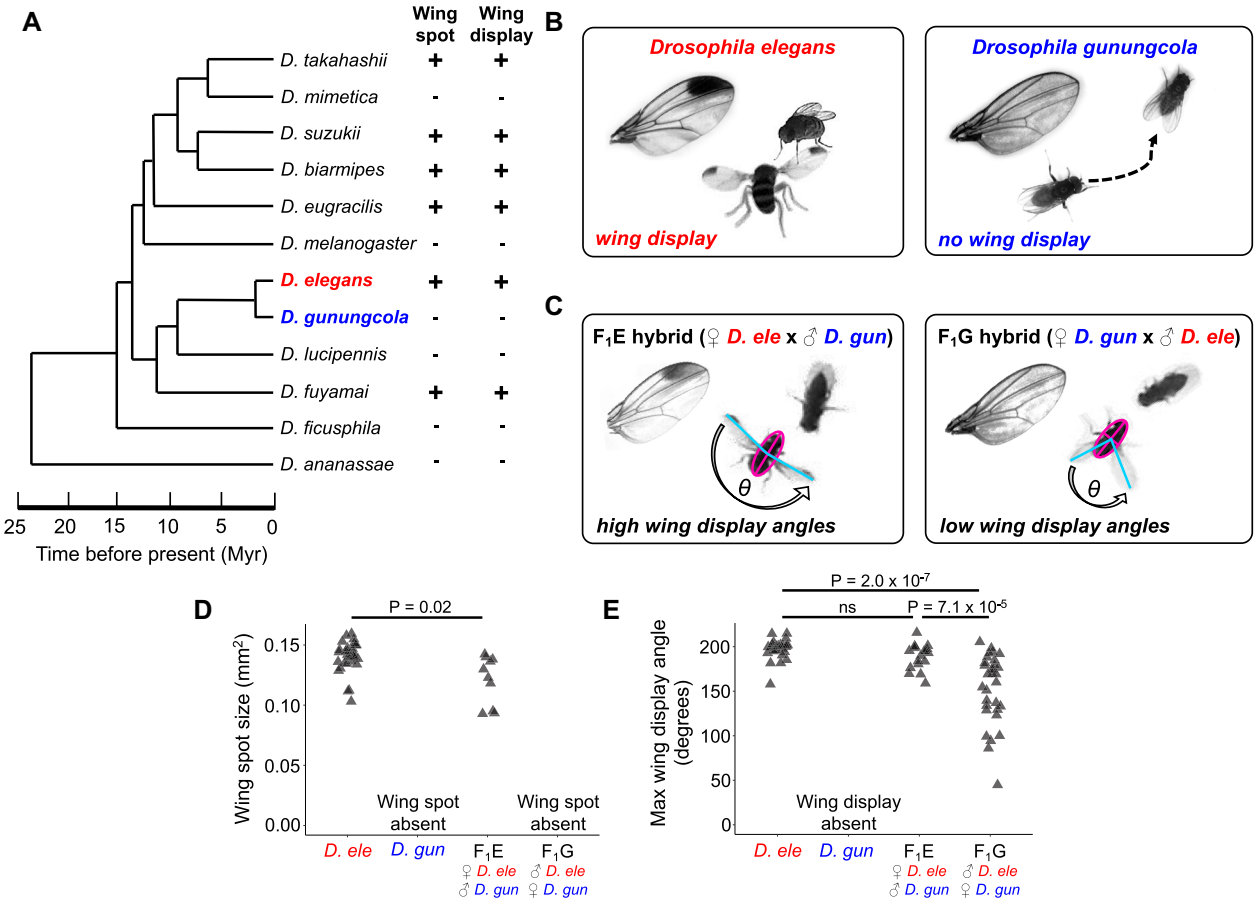

Figure 1. Wing pigmentation and wing display behavior in *D. elegans*, *D. gunungcola*, and F_1 hybrids. (A) Phylogeny of the "Oriental" *Drosophila melanogaster* species group adapted from Kopp and True (2002) and Prud'homme et al. (2006). Plus (+) signs indicate species possess wing spots and/or wing displays, and minus (–) signs indicates wing spots and/or wing displays are absent. (B) Males in *D. elegans* (left) possess wing spots and perform bilateral wing display behaviors in front of females during courtship (Video 1). Wing spots and wing displays are absent in *D. gunungcola* males (right) (Video 2). (C) F_1 hybrid males inheriting their X chromosome from *D. elegans* mothers (F_1E, left) possess wing spots and perform wing display behavior like *D. elegans* (Video 3). F_1 hybrid males inheriting their X chromosome from *D. gunungcola* mothers (F_1G, right) are spotless and perform wing displays with low bilateral wing angles (Video 4). (D) Quantification of wing spot size (see Methods) in male *D. elegans* and F_1E. Wing spots are larger in *D. elegans* than F_1E (Student's *t*-test; $t = -2.8057$; $df = 11.43$; $P = 0.017$; two-tailed). (E) Quantification of maximum bilateral wing display angles during courtship (see Methods) in male *D. elegans* and F_1 hybrids. F_1G hybrids showed lower maximum wing display angles than *D. elegans* and F_1E hybrids (one-way ANOVA: $F_{2,71} = 20.92$; $P < 7.18 \times 10^{-8}$; post hoc Tukey HSD was significant between *D. elegans* and F_1G: $P < 2.0 \times 10^{-7}$ and between F_1E and F_1G: $P < 7.1 \times 10^{-5}$). Gray triangles represent individual replicates.

1982). Maintaining these species on R food at high densities (50–100 flies per vial) allowed for the parental population to build up to thousands of flies to collect hundreds of virgins for interspecific crosses (see below). Neither *D. elegans* nor *D. gunungcola* pupate on the sides of the vial, so adults were flipped out when third instar L3 larvae developed and Fisherbrand filter paper (cat# 09-790-2A) was added to the food to create pupation space.

GENERATING HYBRID PROGENY

Virgin males and females of *D. elegans HK* and *D. gunungcola SK* (the same lines used previously in Yeh et al. 2006; Yeh and True 2014) were isolated upon eclosion and stored in groups of 10 for one week on University of Michigan "M food," which is the standard cornmeal diet from the Bloomington Drosophila Stock Center (https://bdsc.indiana.edu/information/recipes/bloomfood.html) with 20% higher agar content. Virgin males from *D. elegans* were crossed to virgin females from *D. gunungcola*, and virgin males from *D. gunungcola* were crossed to virgin females from *D. elegans* in groups of 10 males and 10 females to generate fertile F_1 female and sterile F_1 male hybrids. These crosses took ~3–4 weeks to produce hybrid progeny. The switch from R food to M food for interspecific crosses was necessary, because R food tended to accumulate condensation

and bacterial growth much faster than M food when few flies occupied a vial. Because crossing *D. elegans* and *D. gunungcola* to generate F_1 hybrids tends to take several more weeks than within species crosses, the switch to M food diet allowed for maximum breeding time and the development of dozens of hybrid progeny. Once hybrid females eclosed from both interspecific cross directions, they were pooled into the same vial and aged for 10 days. We did not keep track of F_1 hybrid female maternity, because previous work (Yeh and True 2014) found no effect of F_1 hybrid maternity on trait means for wing spots and wing display in backcross populations. Multiple high-density groups of ∼60 F_1 hybrid females were then backcrossed to ∼60 virgin male *D. elegans* flies in individual vials on M food diet to create the *D. elegans* backcross recombinant population (724 individuals). To create the *D. gunungcola* backcross recombinant population (241 individuals), groups of ∼60 F_1 hybrid females were backcrossed to ∼60 virgin male *D. gunungcola* flies in individual vials on M food diet; this backcross was less successful at producing recombinant progeny than the *D. elegans* backcross direction.

BEHAVIORAL ASSAYS

Virgin *D. elegans* females were isolated upon eclosion, aged 10–20 days, and stored in groups of 30–40 for courtship assays. F_1 hybrid and recombinant backcross males were isolated individually in M food vials using CO_2 upon eclosion for at least 5 days before each courtship assay. For each assay, a single individual male was gently aspirated into a custom built 70-mm diameter bowl arena that matches the specifications in Simon and Dickinson (2010). Next, a single virgin *D. elegans* female was aspirated into the chamber and videotaped for the next 20 min, using a Canon VIXIA HF R500 camcorder mounted to Manfrotto (MKCOMPACTACN-BK) aluminum tripods. Videos were recorded between 0900h and 1600h at 23°C. *Drosophila elegans* virgin females were used in all courtship assays in case any *D. elegans* female cues were necessary to elicit male wing display behavior. After each assay, both the male and female were aspirated back into an M food vial and left for up to 5 days, after which each male was frozen in individual 1.5 mL Eppendorf tubes for wing spot quantification (see Methods—QUANTIFICATION OF WING SPOTS), genomic DNA (gDNA) extraction, and sequencing (see Methods—LIBRARY PREPARATION AND SEQUENCING). All courtship videos (∼900 total) are available here: https://deepblue.lib.umich.edu/data/concern/data_sets/j098zb17n?locale=en.

QUANTIFICATION OF WING DISPLAY BEHAVIOR

F_1 hybrid and recombinant males from both backcross directions performed variable wing display behaviors during courtship as described previously (Yeh et al. 2006; Yeh and True 2014). To generate quantitative measurements of wing display variation between individuals, each courtship video was played using QuickTime (version 10.4) (Apple Inc., Cupertino, CA) software in a MacOS environment, and digital screenshots were manually taken for each wing display bout, defined as a bilateral wing extension performed near the female (Fig. S1). Next, for each individual fly, wing display screenshots were compared to each other to identify the maximum wing display bout per fly, defined by comparing the distance between the tips of each wing relative to the center of the fly. These maximum wing display screenshots were then imported into ImageJ software (version 1.50i) (Wayne Rasband, National Institutes of Health, USA; http://rsbweb.nih.gov/ij/) to manually measure the "Maximum wing display angle" for F_1 hybrid and recombinant males. In ImageJ, each screenshot image was inverted using the "Find Edges" function to enhance the contrast between the arena background and the edges of the fly wings (Fig. S1). Next, the "Polygon Selections" tool was used to fit an ellipse around the fly body using the "Fit Ellipse" function (Fig. S1). A Macros function (File S1) was then used to generate major and minor axes inside the ellipse to identify the center of the fly body (Fig. S1). Finally, the "Angle Tool" was used to measure the "Maximum wing display angle" centering the vertex at the intersection of the major and minor axes and extended from wing tip to wing tip (Fig. S1). "Maximum wing display angle" varied between ∼50° and ∼220° between backcross recombinant individuals. Raw data for Figure 1E are available in File S2, and raw data for Figure 3 are deposited on Dryad (https://doi.org/10.5061/dryad.gb5mkkwm5).

QUANTIFICATION OF WING SPOTS

Because wing spots fully form ∼24 h after eclosion in *D. elegans*, all parental male *D. elegans*, *D. gunungcola*, F_1 hybrids, and backcross recombinants were aged at least 7 days before being frozen at –20°C in 1.5 mL Eppendorf tubes. Next, using a 20 Gauge stainless steel syringe tip (Techcon) (cat# TE720100PK), the right wing of each fly was cut away from the thorax and placed on a glass microscope slide (Fisherbrand) (cat# 12-550-15) to image using either a Leica MZFLIII stereoscope equipped with a Leica DC480 microscope camera or a Canon EOS Rebel T6 camera equipped with a Canon MP-E 65 mm macro lens. Each camera was calibrated using an OMAX 0.1-mm slide micrometer to define pixel density in ImageJ software. JPEG images of wings were imported into ImageJ to measure wing spot size relative to total wing area (wing spot size/total wing area). We quantified wing spot size, rather than wing spot intensity, because we aimed to map previously identified wing spot size QTL (Yeh et al. 2006; Yeh and True 2014) and their relationship with wing display behavior. Total wing area (wing length × wing width) was approximated using length and width proxies following methods described in Yeh and True (2014). Using the "Polygon Selections" tool, the margins of black pigmentation

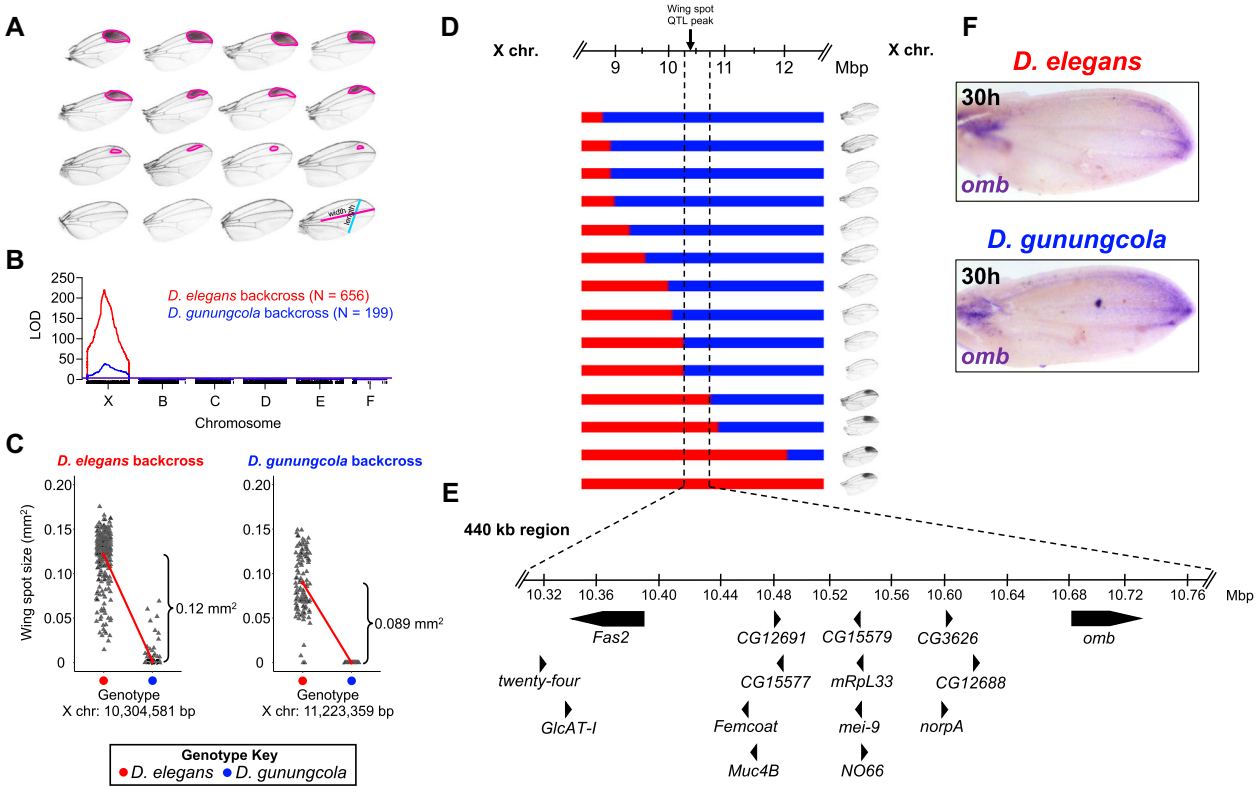

Figure 2. QTL analysis, effect plots, and *in situ* hybridization for wing pigmentation divergence. (A) Wing spots vary in size and shape in *D. elegans* and *D. gunungcola* backcross recombinants. Wing spots were traced (pink) and quantified relative to proxies for total wing area (length × width) using ImageJ software (see Methods). (B) Wing spot QTL map for the *D. elegans* (red) and *D. gunungcola* (blue) backcross. LOD (logarithm of the odds) is indicated on the *y*-axis. The *x*-axis represents the physical map of Muller Elements X, B, C, D, E, and F based on the *D. elegans* assembled genome (see Methods). *D. elegans* and *D. gunungcola* have six chromosomes (Yeh et al. 2006; Yeh and True 2014) that correspond to *D. melanogaster* chromosomes as follows: X = X, B = 2L, C = 2R, D = 3L, E = 3R, and F = 4. Individual SNP markers are indicated with black tick marks along the *x*-axis. Horizontal red and blue lines mark $P = 0.01$ for the *D. elegans* and *D. gunungcola* backcross, respectively. (C) Effect plots for the X chromosome QTL peak from the *D. elegans* backcross (left) and *D. gunungcola* backcross (right). Gray triangles represent individual replicates. (D) The chromosome region from X: 8–13 Mbp for backcross recombinants containing X chromosome breakpoints immediately flanking the wing spot QTL peak were aligned to compare the effects of each on wing pigmentation. Regions in red represent *D. elegans* linked loci, and regions in blue represent *D. gunungcola* linked loci. Recombinants possessing *D. elegans* loci to the left of ~10.32 Mbp are spotless, whereas recombinants possessing *D. elegans* loci to the right of ~10.74 Mbp possess dark wing spots. (E) Two recombinants define the wing spot locus to a ~440 Kbp region containing 15 candidate genes. *omb* is a wing pigmentation candidate gene given evidence from prior work (see Results and Discussion). (F) In situ hybridization of *D. elegans* and *D. gunungcola* pupal wings probed for *omb* mRNA (purple) at 30 h after pupal formation (APF) (see Fig. S8 for additional replicates).

defining each "Wing spot size" were traced and the polygon area quantified in mm^2 using the "Measure" function. "Wing spot size" varied between 0 (spotless) and 0.15 mm^2 between recombinant individuals. Raw data for Figure 1D are available in File S3, and raw data for Figure 2 are deposited on Dryad (https://doi.org/10.5061/dryad.gb5mkkwm5).

LIBRARY PREPARATION AND SEQUENCING

We estimated chromosome ancestry "genotypes" for 724 *D. elegans* backcross progeny and 241 *D. gunungcola* backcross progeny with a single MSG (Andolfatto et al. 2011) library using 965 barcoded adaptors following methods described in Cande et al. (2012). In brief, to extract gDNA from all male backcross individuals, single flies were placed into individual wells of 96-well (Corning, cat# 3879) plates containing a single steel grinding bead in each well (Qiagen, cat# 69989). Eleven plates in total were prepared for 965 individual gDNA extractions. gDNA was isolated and purified using the solid tissue extraction procedure from a Quick-DNA 96 Kit (Zymo, cat# D3012) and a paint shaker to homogenize tissue. gDNA was tagmented using a hyperactive version of Tn5 transposase charged with annealed adaptor oligos following the methods described in Picelli et al. (2014). Unique

barcoded adaptor sequences were ligated to each sample of tagmented gDNA with 14 cycles of PCR using OneTaq 2x Master Mix (NEB, cat# M0482S), and all samples were pooled into a single multiplexed sequencing library. Agencourt AMPure XP beads (Beckman Coulter, cat# A63881) were used to size select ∼150–800 bp fragments and eluted in 35 μL of molecular grade water (Corning, cat# MT46000CI). The library was quantified by qPCR and sequenced in a single lane of Illumina HiSeq by the Janelia Quantitative Genomics Team.

In addition to generating the backcross sequencing library, both *D. elegans HK* and *D. gunungcola SK* parental species were sequenced at 20× coverage using an Illumina MiSeq Reagent Kit (version 3, 600 cycle PE). In brief, gDNA was extracted using a Quick-DNA Microprep Kit (Zymo, cat# D4074) from 10 pooled females for each species and quantified on a Qubit 2.0 (Invitrogen). These samples were sent to the University of Michigan DNA Sequencing Core to prepare 300 bp paired-end libraries, which were quantified by qPCR and sequenced in a single lane of Illumina MiSeq.

GENOME ASSEMBLY

In brief, Illumina reads from all 965 backcross recombinants were used to perform MSG on the Baylor College of Medicine *D. elegans* genome assembly (accession number: GCA_000224195.2). Using custom scripts in R and Python (https://github.com/masseyj/elegans), the recombination fraction between the Baylor and MSG contigs was calculated and plotted to manually tabulate joins and splits between newly assembled contigs. These new contigs were then used to assemble approximately chromosome length scaffolds in *D. elegans* (accession number: WVIB00000000) and partially assembled scaffolds in *D. gunungcola* (accession number: WTSR00000000).

MARKER GENERATION WITH MSG

Following methods described previously (Andolfatto et al. 2011; Cande et al. 2012), we used the MSG software pipeline (https://github.com/JaneliaSciComp/msg/tree/master/instructions) to perform data parsing and chromosome ancestry estimation to generate markers for QTL analysis. In brief, using data from the Illumina backcross sequencing library (see File S4 for the number of reads per individual), we mapped reads to the assembled *D. elegans* and *D. gunungcola* parental genomes to estimate chromosome ancestry for each backcross individual. We generated 3425 and 3121 markers for the *D. elegans* and *D. gunungcola* backcrosses, respectively, for QTL analysis (markers, phenotypes, and procedures for QTL mapping are deposited on Dryad [https://doi.org/10.5061/dryad.gb5mkkwm5]). PDFs of chromosomal breakpoints for each recombinant are available here: https://deepblue.lib.umich.edu/data/concern/data_sets/j098zb17n?locale=en.

QTL ANALYSIS

QTL analysis was performed using R/qtl (Broman et al. 2003; Broman and Sen 2009) in R for Mac version 3.3.3 (R Core Team 2018) in a MacOS environment. Ancestry data for both backcross directions were imported into R/qtl using a custom script (https://github.com/dstern/read_cross_msg), which directly imports the conditional probability estimates produced by the Hidden Markov Model (HMM) of MSG (Andolfatto et al. 2011). We performed genome scans with a single QTL model using the "scanone" function of R/qtl and Haley-Knott regression (Haley and Knott 1992) for "Wing spot size" and "Maximum wing display angle." Note, for "Wing spot size," 68 and 42 recombinants from the *D. elegans* and *D. gunungcola* backcross populations, respectively, were excluded from the QTL mapping because their wings were too damaged to quantify spot variation. Similarly, for "Maximum wing display angle," 314 and 94 recombinants from the *D. elegans* and *D. gunungcola* backcross populations, respectively, were excluded from the QTL mapping because these males did not perform any courtship behavior during the assay. Significance of QTL peaks at $\alpha = 0.01$ was determined by performing 1000 permutations of the data. Effect sizes for each QTL peak were individually estimated by comparing the mean "Wing spot size" or "Maximum wing display angle" between individuals that inherited either *D. elegans* or *D. gunungcola* alleles at each QTL peak position (markers, phenotypes, and procedures for QTL mapping are deposited on Dryad [https://doi.org/10.5061/dryad.gb5mkkwm5]).

Because we detected multiple QTL peaks on separate chromosomes for "Maximum wing display angle," we tested for the presence of epistatic interactions using two methods: First, we performed two- and three-way ANOVAs comparing the effect of each QTL peak in multiple QTL peak genetic backgrounds and found no evidence of an interaction. For two-way ANOVAs, we tested for any statistically significant interactions for max wing display angles between two different QTL peaks in the *D. elegans* backcross. For three-way ANOVAs, we tested for any statistically significant interactions for max wing display angles between three different QTL peaks in the *D. gunungcola* backcross. Second, we performed genome-wide pairwise tests using the "scantwo" function of R/qtl and Haley-Knott regression to test for non-additive interactions across all markers; LOD significance thresholds at $\alpha = 0.05$, 0.01, and 0.001 were determined by performing 1000 permutations of the data for each model (Fig. S2; Tables S1 and S2).

ANNOTATING THE WING SPOT QTL INTERVAL

To annotate genes within the ∼440 Kbp fine-mapped wing spot locus, we performed nucleotide BLAST (BLASTn) (Johnson et al. 2008) searches against the *D. melanogaster* genome (taxid: 7227) using ∼10-Kbp windows of assembled *D. elegans*

chromosomal regions spanning the wing spot QTL interval. Using the "GBrowse" tool on Flybase (Thurmond et al. 2018), we mapped regions of microsynteny to identify the orientation of each gene and exported the respective *D. melanogaster* coding region (CDS) FASTA sequences to align with the *D. elegans* X chromosome.

IN SITU HYBRIDIZATION

Fly genomic DNA (gDNA) was extracted from 10 homogenized *D. elegans* and *D. gunungcola* females using a Quick-DNA Microprep Kit (Zymo, cat# D3021). The following forward and reverse primers were designed and synthesized by Integrated DNA Technologies (IDT) to PCR amplify 321 bp DNA templates targeting exon 5 of the *omb* locus in *D. elegans*: 5′-GCTGAGGATCCATTCGCTAGATTTG-3′ and 5′-GTTGTTGGAACTAGAGTTGTTGGTG-3′, and *D. gunungcola*: 5′- GCTGAGGATCCATTCGCTAGATTTG-3′ and 5′-GTTGTTGGAACTGGAGTTGTTGGTG-3′. Reverse primers were designed beginning with a T7 RNA polymerase binding sequence (TAATACGACTCACTATAG) to facilitate in vitro transcription. Raw PCR products were then used to generate digoxigenin-labeled RNA probes using a T7 RNA in vitro transcription kit (Promega/Life Technologies). RNA was ethanol precipitated and resuspended in water to analyze on a Nanodrop. Each probe was stored at –20°C in 50% formamide before in situ hybridization.

All tissues underwent primary dissection in PBS (1X phosphate–buffered saline), fixed for 30 min in 4% PFA, washed three times in PBT (PBS + 1% Triton X–100), and underwent secondary dissection in PBT, and then washed two times in MeOH and two times in EtOH before being stored at –20°C . Male *D. elegans* and *D. gunungcola* L3 wing discs were dissected first to validate that our *omb* probes detected an mRNA expression pattern similar to *D. melanogaster* (Grimm and Pflugfelder 1996; Fig. S3). Next, pupal wings were dissected at 30 and 48 h after pupal formation (APF) to probe for *omb* mRNA. To prepare pupal wings, appropriately staged pupae underwent a primary dissection: they were cut in half along the anterior-posterior axis using Astra Platinum Double Edge Razor Blades, and fat body was washed out of the pupal casing using a pipette and PBS prior to fixation. After fixation, pupal wings underwent a secondary dissection to pull off the cuticle surrounding each wing and then washed using the procedure described above. Finally, in situ hybridization was carried out as previously described (Vincent et al. 2019). Briefly, we used an InsituPro VSi robot to rehydrate in PBT, fix in PBT with 4% PFA, and prehybridize in hybridization buffer for 1 h at 65°C. Samples were then incubated with probe for 16 h at 65°C before washing with hybridization buffer and PBT. Samples were blocked in PBT with 1% bovine serum albumin (PBT + BSA) for 2 h. Samples were then incubated with anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Roche) diluted 1:6000 in PBT + BSA. After additional washes, color reactions were performed by incubating samples with NBT and BCIP (Promega) until purple stain could be detected under a dissecting microscope. Samples were mounted in glycerol on microscope slides coated with poly-L-lysine and imaged at 10× magnification on a Leica DFC450C camera.

GENERATING ADVANCED RECOMBINANT INTROGRESSIONS ON THE X CHROMOSOME

To try to isolate the QTL effects for "Wing spot size" and "Maximum wing display angle" localized to the X chromosome according to the *D. elegans* backcross experiment, F_1 hybrid females were generated using the procedures described above. F_1 hybrid females were then backcrossed to *D. elegans* males in ∼12 replicate vials, and backcross males lacking wing spots were isolated to measure "Maximum wing display angles" during courtship as described above. This procedure was repeated for seven generations to generate BC3-BC9 backcross individuals: backcross females were backcrossed en masse (across ∼12 vials) to *D. elegans* males, and BC3 backcross males lacking wing spots were isolated to measure "Maximum wing display angles" during courtship with *D. elegans* virgins (and so on to BC9). At each generation, an attempt was made to create stable introgression lines of advanced recombinant males lacking wing spots, but all failed to produce offspring, suggesting that *D. gunungcola* X-linked loci might also contain hybrid sterility factors. After seven generations of backcrossing, gDNA from all backcross males lacking wing spots was extracted and sequenced for MSG as described above. Backcross males lacking wing spots from BC4-BC9 were homozygous for *D. elegans* genomic regions across all autosomes but varied for the amount of *D. gunungcola* genome regions on the X chromosome.

INTROGRESSION OF BLACK BODY COLOR ALLELES FROM D. GUNUNGCOLA INTO D. ELEGANS

In the *D. gunungcola* backcross, QTL mapping for wing spot size revealed QTL peaks linked to Muller Element C and E when spotless recombinants were excluded from the analysis (Fig. S4; Table S3). The Muller Element E QTL peak is located near the *ebony* gene, which appears to contribute to variation in body color between *D. elegans* and *D. gunungcola* (unpubl. data). We therefore reasoned that introgressing dark body color from *D. gunungcola* into *D. elegans* would introgress the Muller Element E QTL peak underlying wing spot size differences. After six generations of backcrossing dark brown female recombinants with *D. elegans* males, we crossed dark brown male and female recombinants together to create black offspring homozygous for

the introgressed region. We then performed MSG on a single, dark black introgression line and found that it was homozygous for ∼1.5 Mb of *D. gunungcola* alleles linked near the Muller Element E QTL peak (Fig. S4A,C).

OBSERVING AND COLLECTING WILD D. GUNUNGCOLA AND D. ELEGANS IN INDONESIA

Throughout early July 2018, *D. elegans* and *D. gunungcola* were recorded performing courtship in East Java, Indonesia on *Ipomoea* sp. and *Brugmansia* sp. flowers using Canon VIXIA HF R500 camcorders mounted to Manfrotto (MKCOMPACTACN-BK) aluminum tripods. Both species were observed in sympatry on flowers near Coban Rondo Waterfall in Batu, Batu City, East Java, Indonesia (–7.884985, 112.477311). After video recording courtship, males and females were captured using a mouth pipette and gently aspirated into glass vials containing standard fly media (glucose, corn meal, yeast extract, and agar). Isofemale lines of *D. gunungcola* from Bumiaji District (Batu City, East Java Province, Indonesia) were established in the laboratory on standard fly media at 24°C temperature. We quantified (see Methods—QUANTIFICATION OF WING DISPLAY BEHAVIOR), to the best of our knowledge, the first recorded observations of *D. gunungcola* wing displays on flowers in the field and in the laboratory (Fig. S11; Videos 8, 9, and 11). Across ∼10 independent observations of courting *D. gunungcola* pairs at Bumiaji District and Coban Rondo, all males performed a type of wing display despite completely lacking wing spots. To confirm species identification of *D. gunungcola* and *D. elegans* from the field sites mentioned above, we dissected and imaged male genitalia and compared with the laboratory strains (*D. gunungcola SK* and *D. elegans HK*) used in this study and described previously (Sultana et al. 1999; Kopp and True 2002) (Fig. S9). The distal paramere (also called the pregonite; Rice et al. 2019) was especially diagnostic of species identity (Fig. S9). We also performed low-coverage sequencing of the new *D. gunungcola* strains' genomes from Coban Rondo (see Methods—LIBRARY PREPARATION AND SEQUENCING) and aligned coding sequences from the *omb* locus with the *D. gunungcola SK* lab strain (Fig. S10). A nonsynonymous coding change that distinguished the laboratory *D. gunungcola SK* strain from *D. elegans HK* also distinguished the new *D. gunungcola* Coban Rondo strain from *D. elegans* HK, matching the *D. gunungcola SK* sequence (Fig. S10).

STATISTICS

Statistical tests were performed in R for Mac version 3.3.3 (R Core Team 2018) using Student's *t*-test (two-tailed) to test for statistically significant effects of pairwise comparisons of continuous data with normally distributed error terms. For tests comparing more than two groups, ANOVAs were performed with post hoc Tukey HSD for pairwise comparisons adjusted for multiple comparisons. See "QTL analysis" methods for statistical tests used during QTL mapping.

Results and Discussion

X-LINKED SEQUENCE DIVERGENCE CONTRIBUTED TO WING SPOT AND WING DISPLAY DIVERGENCE

Drosophila elegans males perform elaborate wing display dances (Video 1) in front of females during courtship, displaying the presence of darkly pigmented wing spots (Fig. 1B), whereas its sibling species, *D. gunungcola*, lost wing spots (Yeh et al. 2006; Prud'homme et al. 2006) and wing displays (Fig. 1B; Video 2). Despite these differences in sexual traits, *D. elegans* and *D. gunungcola* can mate and form viable F_1 hybrids in the lab (Yeh et al. 2006; Yeh and True 2014). Sequence divergence on the X chromosome has previously been implicated in the divergence of wing spots and wing display behavior (Yeh et al. 2006; Yeh and True 2014). To confirm this effect of the X-chromosome, we quantified variation in wing spot size and wing display behavior in F_1 hybrid males from reciprocal crosses between *D. elegans* and *D. gunungcola*. These F_1 hybrids inherited their X chromosome from either *D. elegans* or *D. gunungcola* (whichever species was their mother) and autosomes from both species. Consistent with prior work, F_1 hybrid males inheriting the X chromosome from *D. elegans* mothers (F_1E) possessed wing spots, whereas F_1 hybrid males inheriting the X chromosome from *D. gunungcola* mothers (F_1G) did not (Fig. 1C,D). These wing spots of F_1E males were smaller, however, than the wing spots seen in *D. elegans* (Fig. 1D; Student's *t*-test, $P = 0.02$). Differences in wing display behavior were also apparent between F_1E (Video 3) and F_1G hybrids (Video 4), which is also consistent with prior work (Yeh et al. 2006; Yeh and True 2014). More specifically, we found that although both F_1 hybrids performed wing displays during courtship, F_1E hybrids tended to open their wings more widely than F_1G hybrids during display performance (Fig. 1C). We quantified variation in this wing display trait between F_1 hybrids by measuring the maximum bilateral wing display angles (Fig. 1C) during courtship (see Methods). We found that F_1E hybrids performed wing displays comparable to *D. elegans* males (Fig. 1E; post hoc Tukey HSD, $P = 0.6$), whereas F_1G males showed, on average, lower display angles (Fig. 1E; post hoc Tukey HSD, $P = 7.1 \times 10^{-5}$). Together these data confirm that divergence of one or more loci on the X chromosome contributes to divergence in wing spot size and wing display behavior between *D. elegans* and *D. gunungcola*.

Table 1. QTLs detected for wing spot size and maximum wing display angle divergence.

Trait	Backcross	Chromosome	QTL interval (bp)[a]	QTL peak (bp)	LOD
Wing spot size	*D. elegans*	X	10,297,836–10,744,020	10,304,581	220
Max wing display angle	*D. elegans*	X	8,729,737–15,691,924	9,006,035	18.9
Max wing display angle	*D. elegans*	B	5,773,911–13,325,000	9,001,485	4.66
Wing spot size	*D. gunungcola*	X	10,474,499–11,584,862	11,223,359	38.9
Max wing display angle	*D. gunungcola*	X	16,885,658–25,539,528	24,196,217	4.23
Max wing display angle	*D. gunungcola*	B	7,078,659–12,180,268	10,093,006	6.28
Max wing display angle	*D. gunungcola*	E	3,813,413–11,535,144	9,604,970	7.59

[a]LOD drop 1.5 support interval.

EVOLUTION OF AT LEAST THREE LOCI CONTRIBUTE TO WING SPOT DIVERGENCE

To identify the location of X-linked (as well as autosomal) loci contributing to divergence in wing spot size, we quantified wing spot size variation in 656 recombinant males produced by backcrossing F_1 hybrid females to *D. elegans* males and 199 recombinant males produced by backcrossing F_1 hybrid females to *D. gunungcola* males. These backcross males showed a range of wing spot sizes (Fig. 2A). Using MSG (Andolfatto et al. 2011), we inferred the allele most likely inherited from the F_1 mother (*D. elegans* or *D. gunungcola*) for each genomic position in each recombinant. We then performed QTL mapping for wing spot size and identified a single, highly significant QTL peak on the X chromosome (Fig. 2B and Table 1). In both backcross directions, variation linked to this wing spot QTL peak explained almost all of the difference in wing spot size between *D. elegans* and *D. gunungcola* (Fig. 2C). Repeating the QTL mapping after excluding recombinant individuals lacking wing spots, however, allowed us to identify additional QTLs of smaller effect on Muller Elements C (chromosome 2R in *D. melanogaster*) and E (chromosome 3R in *D. melanogaster*) in the *D. gunungcola* (but not *D. elegans*) backcross population (Fig. S4A; Table S3). Observing these QTL only in the *D. gunungcola* backcross populations suggests that they are caused by recessive *D. gunungcola* alleles, which were never homozygous in the *D. elegans* backcross population. Introgressing the QTL region on Muller Element E from *D. gunungcola* into *D. elegans* through five generations of backcrossing (Fig. S4C) reduced the size of wing spots (Fig. S4D,E). This region includes the *ebony* gene, which has previously been shown to be able to inhibit the development of dark pigments in *D. melanogaster* (Wittkopp et al. 2002b). Crossing this introgression line to *D. elegans* masked most of the reduction in spot size (Fig. S4D,E), consistent with the *D. gunungcola* QTL allele being recessive to the *D. elegans* allele. Taken together, these data indicate that the majority of wing spot divergence between *D. elegans* and *D. gunungcola* maps to a single, large-effect QTL on the X chromosome, but that wing spot size is also influenced by loci on Muller Elements C and E.

A 440-kb LOCUS BEHAVES LIKE A GENETIC SWITCH FOR WING SPOTS

To further refine the X-linked QTL, we more closely examined the genotypes and phenotypes of recombinants with inferred crossover positions immediately flanking the wing spot QTL peak (Figs. 2D and S5). Doing so allowed us to identify a ~440-kb region containing a QTL that acts like a genetic switch controlling the presence or absence of the wing spot (Figs. 2D and S5). This region includes 15 genes (Fig. 2E) and notably excludes the X-linked pigmentation gene, *yellow*, which has previously been suggested to contribute to wing spot development and evolution through changes in a spot-specific *cis*-regulatory element (Wittkopp et al. 2002a; Gompel et al. 2005; Prud'homme et al. 2006; Yeh et al. 2006; Arnoult et al. 2013; Yeh and True 2014; Fig. S6). One of these 15 genes is *optomotor-blind* (*omb*) (Fig. 2E), which encodes a T-box-containing transcription factor (Pflugfelder et al. 1992a; Pflugfelder et al. 1992b) that has previously been implicated in pigmentation patterning (Thompson 1959; Kopp and Duncan 1997), pigmentation evolution (Brisson et al. 2004), and distal wing patterning (Grim and Pflugfelder 1996). In *D. melanogaster*, gain- and loss-of-function *omb* alleles cause expansion and contraction of abdominal pigmentation bands, respectively (Kopp and Duncan 1997), and variation in abdominal pigmentation patterning in *Drosophila polymorpha* is strongly associated with polymorphisms at the *omb* locus (Brisson et al. 2004).

Although we identified two nonsynonymous protein coding changes between *D. elegans* and *D. gunungcola* (File S5), *omb* is required for the development of many structures throughout the body (Pflugfelder 2009); we, therefore, reasoned that genetic divergence in *omb* would be more likely to affect its expression than its protein function (Stern and Orgogozo 2008). To look for differences in *omb* expression between *D. elegans* and *D. gunungcola* that might affect wing spot development, we used in situ hybridization to detect *omb* mRNA in the developing wing of both species (Fig. 2F). In *D. melanogaster*, *omb* is expressed in a broad stripe that overlaps the wing pouch region in larval L3 wing discs (Grimm and Pflugfelder 1996). *omb* expression in the

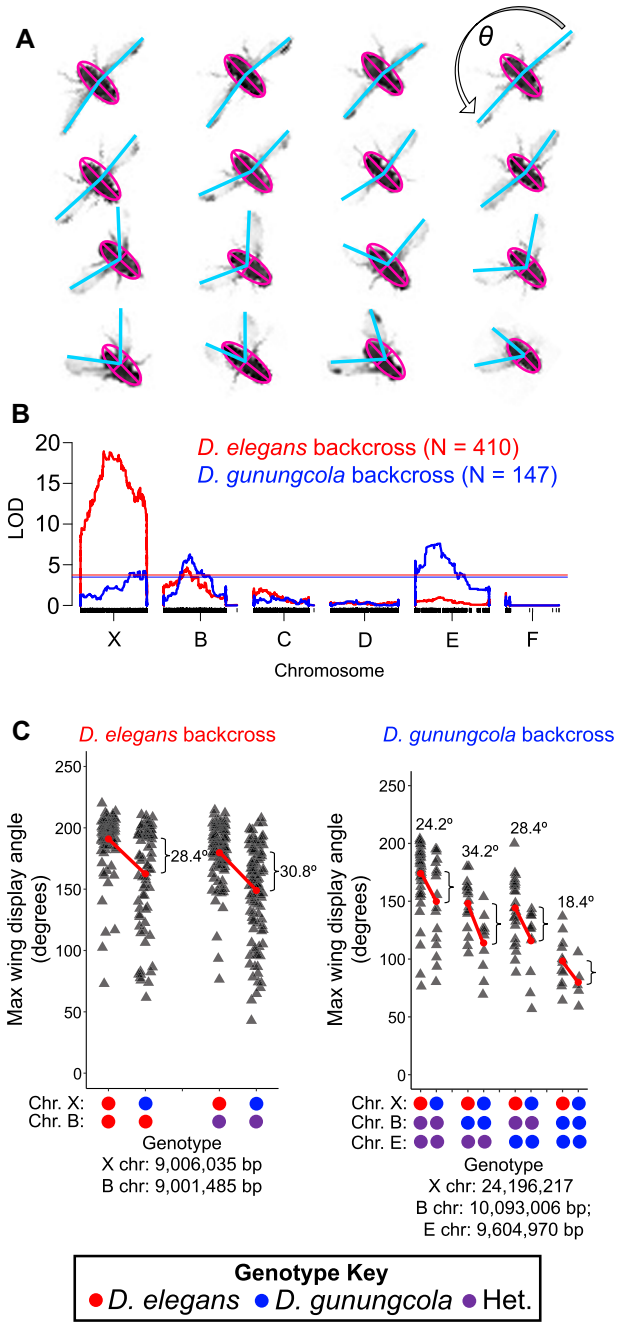

Figure 3. QTL analysis and effect plots for wing display divergence. (A) Maximum wing display angles varied in *D. elegans* and *D. gunungcola* backcross recombinants. Maximum wing display angles were quantified by measuring the angle between each wing tip using ImageJ software (see Methods). (B) Maximum wing display QTL map for the *D. elegans* (red) and *D. gunungcola* (blue) backcross. LOD is indicated on the *y*-axis. Individual SNP markers are indicated with black tick marks along the *x*-axis. Horizontal red and blue lines mark $P = 0.01$ for the *D. elegans* and *D. gunungcola* backcross, respectively. (C) Effect plots for the X chromosome and Muller Element B QTL peaks from the *D. elegans* backcross (left) and for the X, Muller Element B, and E QTL peaks from the *D. gunungcola* backcross (right). No epistatic interactions were detected between QTLs (see Methods) (two-way ANOVA: $F_{1,402} = 0.146$; $P = 0.70$ for the *D. elegans* backcross; three-way ANOVA: $F_{1,137} = 0.050$ (X:B), 0.034 (X:E), 1.75 (B:E), and 0.799 (X:B:E); $P = 0.82$ (X:B), 0.86 (X:E), 0.19 (B:E), and 0.37 (X:B:E) for the *D. gunungcola* backcross). Gray triangles represent individual replicates.

wing pouch is required for distal wing development, as demonstrated by *D. melanogaster omb* hypomorphs that show disrupted distal wing tip development in adults (Grimm and Pflugfelder 1996). We hypothesized, therefore, that differences in *D. elegans* and *D. gunungcola omb* expression patterning during pupal wing development might prefigure changes in wing spot pigmentation observed in adult males, similar to the changes in *wingless* expression shown to prefigure wing spots in *Drosophila guttifera* (Werner et al. 2010). Consistent with the expression of *omb-lacZ* in pupal wings of *D. melanogaster* (Álamo Rodrıǵuez et al. 2004), we detected *omb* mRNA in the wing hinge and distal wing tip 30 h APF in *D. elegans* and *D. gunungcola* (Fig. 2F). We were unable to identify any consistent differences in the *omb* expression patterns between *D. elegans* and *D. gunungcola*, although it is possible that we may not have detected subtle differences in expression patterns. In addition, it is possible that the changes in *omb* protein sequence contribute to differences in wing spot patterning, or that other genes in the minimal mapped interval are the true cause of the difference in wing spot patterning.

EVOLUTION AT MULTIPLE LOCI CONTRIBUTED TO WING DISPLAY DIVERGENCE

To identify loci contributing to divergence in wing display behavior, we quantified variation in maximum wing display angles (see Methods) in 410 *D. elegans* and 147 *D. gunungcola* backcross recombinant males, again observing a range of phenotypes (Fig. 3A). We identified multiple significant QTL contributing to variation in wing display (Fig. 3B; Table 1). In the *D. elegans* backcross, we mapped a QTL on the X chromosome that overlaps with the wing spot QTL (Fig. 3B; Table 1). We also mapped a QTL on Muller Element B (chromosome 2L in *D. melanogaster*) (Fig. 3B; Table 1). In the *D. gunungcola* backcross, we mapped QTLs on the X chromosome as well as Muller Elements B and E (Fig. 3B; Table 1). The observation of a peak on Muller Element E only in the *D. gunungcola* backcross suggests the presence of alleles affecting wing display behavior that are recessive and/or interact epistatically with divergent sites elsewhere in the genome.

To test for epistatic interactions contributing to wing display divergence, we performed a two-dimensional genome scan to search for non-additive interactions across all markers in both backcross directions and found no significant interactions

(Fig. S2; Tables S1 and S2). We also tested for evidence of non-additive interactions among the wing display QTL peaks themselves by performing two- and three-way ANOVAs in the *D. elegans* and *D. gunungcola* backcrosses, respectively, and found no evidence of significant interactions between loci (Fig. 3C). Instead, each wing display QTL peak appears to behave approximately additively, with *D. gunungcola* alleles contributing to lower maximum wing display angles (Fig. 3C). Surprisingly, the effect of the X-linked QTL on wing display angle in the *D. gunungcola* backcross in multiple genetic backgrounds was similar to the estimated effect size of the X-linked QTL in the *D. elegans* backcross (compare panels in Fig. 3C) despite the much lower LOD score of the X-linked QTL in the *D. gunungcola* backcross population (Fig. 3B; Table 1). We suggest that although the detected QTL in the *D. gunungcola* backcross appear to interact additively with each other, undetected QTL elsewhere in the genome are likely masking the X-effect in the *D. gunungcola* backcross map. Although the purpose of the two-dimensional genome scan (Fig. S2; Tables S1 and S2) was to detect these effects, our sample size is likely too small to identify small-effect epistatic interactions.

MALES LACKING WING SPOTS PERFORM NORMAL WING DISPLAYS

Although it remains unclear which gene evolved to cause the majority of wing spot divergence, fine-mapping the locus controlling the presence or absence of the wing spot allowed us to test whether the locus that turns off wing spots in *D. gunungcola* also affects wing display behavior. To perform this test, we introgressed *D. gunungcola* alleles causing a loss of the wing spot into *D. elegans* by repeated backcrossing (see Methods). We recovered three introgression lines lacking wing spots and found that all three lines had inherited the ~440-kb region observed in mapping experiments to act like a genetic switch controlling wing spot development (Fig. 4A,B), independently confirming the causal role of the switch region in wing spot divergence. We noticed, however, that several advanced recombinants developed a wing spot "shadow" (Fig. 4B), possibly due to the effects of other *D. elegans* alleles affecting wing spot development. We next asked whether the spotless advanced recombinants performed wing displays with lower wing display angles than *D. elegans* males. Surprisingly, we found that all advanced recombinants inheriting the *D. gunungcola* allele eliminating the wing spot performed wing displays indistinguishable from *D. elegans* males during courtship (Fig. 4B,C; Videos 5–7). Thus, the loci controlling the wing spot and courtship behavior are genetically separable.

The repeated co-evolution of male-specific wing spots and wing display behavior in multiple species (Kopp and True 2002) combined with the presence of overlapping QTL for these traits

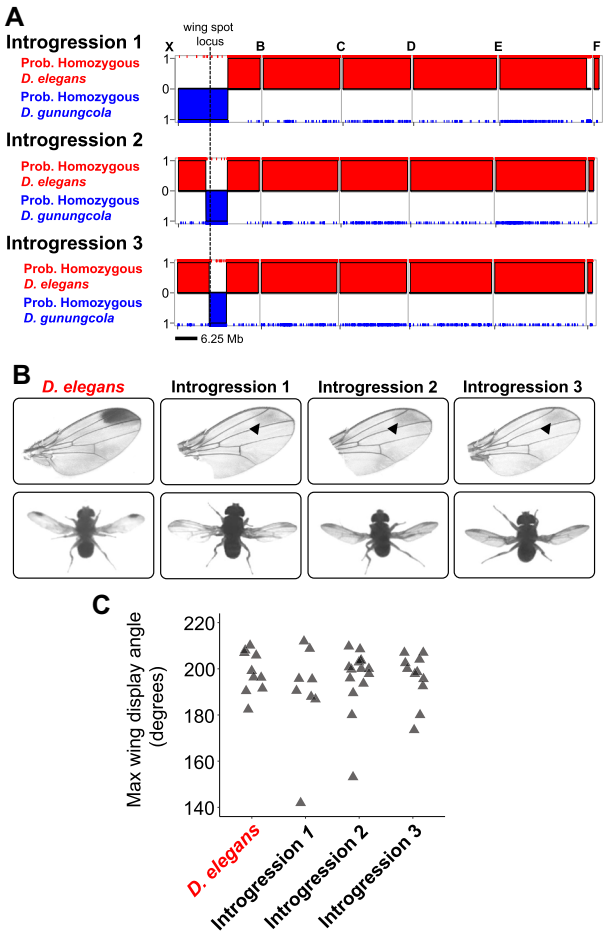

Figure 4. ***Drosophila elegans*** **males possessing the** ***D. gunungcola*** **wing spot locus perform normal wing displays. (A) Multiplexed Shotgun Genotyping (MSG) (Andolfatto et al. 2011) was used to estimate genome-wide ancestry assignments for three introgression lines generated by repeatedly backcrossing the** ***D. gunungcola*** **wing spot QTL region into a** ***D. elegans*** **genetic background (see Methods). The posterior probability that a region is homozygous for** ***D. elegans*** **(red) or** ***D. gunungcola*** **(blue) ancestry is plotted along the** ***y*****-axis. The dotted line marks the location of the fine-mapped wing spot region (Fig. 2D,E; Table 1). (B) None of the introgressions possessed dark wing spots (although a light wing spot "shadow" is visible). (B and C) Flies from all introgression lines performed max wing display angles indistinguishable from** ***D. elegans*** **males (one-way ANOVA:** $F_{3,42} = 0.449$; $P = 0.72$**). Gray triangles represent individual replicates.**

on the X chromosome (Yeh et al. 2006; Yeh and True 2014; and this study) suggested that a single pleiotropic gene might be contributing to the evolution of both traits. The finding that *D. elegans* introgression lines lacking a wing spot performed a normal wing display argues against this hypothesis and indicates instead that these two traits arose independently between this species pair. To further investigate how these divergent traits might have evolved, we recorded courtship behavior in a wild

population of *D. gunungcola* in Indonesia; to the best of our knowledge, all prior studies of *D. gunungcola* pigmentation and courtship used the one previously available lab strain (Sultana et al. 1999). Surprisingly, we found that all *D. gunungcola* males observed in the wild population lacked wing spots (Fig. S7) but performed wing displays (Fig. S11; Videos 8 and 9), confirming that these are genetically distinct traits. The wing displays performed by these flies appeared to show a lower maximum wing extension angle than *D. elegans* (Fig. S11), similar to the wing display behavior seen in F_1 hybrids between *D. elegans* and *D. gunungcola* with *D. gunungcola* mothers (Fig. 1C; Video 4). Analysis of new lab strains founded by flies captured from this *D. gunungcola* population showed similar male courtship behavior in the lab as observed on flowers (Fig. S11; Video 11).

It remains unknown whether the absence of wing display behavior in the *D. gunungcola SK* lab strain seen since Yeh et al. (2006) (collected originally from Sumatra in 1999) is either (1) segregating within and/or among wild populations of *D. gunungcola* or (2) limited to the SK strain and might have been lost by chance or adaptation to the lab environment. Observing that flies from the newly isolated strain of *D. gunungcola* displayed a similar wing display behavior in the field and after being reared in the lab (Fig. S11) does, however, argue that the absence of wing display behavior in the SK line is unlikely due to phenotypic plasticity caused by the lab setting. Additional sampling of natural *D. gunungcola* populations from throughout its species range (or at least resampling of the population from which the SK lab line was derived) is needed to distinguish between these possibilities. What we can say at this time, however, is that the new strain of *D. gunungcola* performed a similar wing display in the field and in the lab, with the angle of the wing display appearing to be consistently less than the wing display angle measured for *D. elegans* (Fig. S11). Because we mapped QTL explaining variation in the wing display angle (Fig. 3) rather than the presence or absence of wing display, some QTL identified here might also contribute to variation in wing display angle segregating within wild populations. We therefore conclude that although the absence of wing spots appears fixed in *D. gunungcola*, the absence of wing display behavior does not. These observations suggest that the loss of male-specific wing spots predates the loss of male wing display behavior in this species.

Conclusions

Male-specific wing spots and wing display behavior have co-evolved in *Drosophila* multiple times (Kopp and True 2002). By studying the genetic basis of these divergent traits between *D. elegans* and *D. gunungcola*, we showed that the changes in wing spot and wing display were not caused by changes in a single, pleiotropic gene despite overlapping QTL (Yeh et al. 2006; Yeh and True 2014). Rather, we found that distinct loci contribute to divergence in each of these traits, with the genetic architecture of divergent wing behavior being more complex than that of the divergent wing spot pigmentation. Both traits were affected by divergent gene(s) located on the X chromosome that are in physical linkage, however, causing alleles of these distinct loci to be co-inherited. This linkage might have facilitated the coordinated evolution of these traits.

The specific genes contributing to divergence in wing spot and wing display remain unknown, but *optomotor-blind* is a strong candidate for the X-linked gene contributing to the loss of the wing spot. Introgression lines and additional sampling of *D. gunungcola* from a wild population also showed that the loss of wing spots and wing display are not inexorably linked: in both cases, males lacking wing spots still performed a wing display behavior. Coordinated evolution of morphological and behavioral traits such as these is often observed in animal species, but it is often unclear which change evolved first. In this case at least, it seems that the divergence of morphology preceded the divergence of behavior.

AUTHOR CONTRIBUTIONS

JHM was associated with conceptualization, data curation, formal analysis, funding acquisition, validation, investigation, visualization, methodology, and writing (original draft, and review and editing). GRR was associated with formal analysis, validation, investigation, methodology, and writing (review and editing). ASF was associated with investigation, methodology, writing (review and editing). Chi-Yang Chen was associated with investigation and methodology. SDY was associated with funding acquisition, investigation, and methodology. DLS was associated with supervision, funding acquisition, conceptualization, data curation, formal analysis, investigation, visualization, writing (original draft and review and editing), and project administration PJW was associated supervision, funding acquisition, conceptualization, data curation, formal analysis, investigation, visualization, writing (original draft and review and editing), and project administration.

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DATA ARCHIVING

All supporting data can be accessed at University of Michigan Deep Blue (https://deepblue.lib.umich.edu/data/concern/data_sets/j098zb17n?locale=en) and Dryad (https://doi.org/10.5061/dryad.gb5mkkwm5).

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplementary Table S2. Results of two-QTL scan for max wing display angle in *D. elegans* backcross
Supplementary Table S3. Results of two-QTL scan for max wing display angle in *D. gunungcola* backcross
Supplementary Table S1. QTLs detected for wing spot size, excluding spotless individuals
Video 1 D. elegans HK wing display behavior
Video 2 D. gunungcola SK courtship and copulation
Video 3 F_1E wing display behavior
Video 4 F_1G wing display behavior
Video 5 Introgression 1 wing display behavior
Video 6 Introgression 2 wing display behavior
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Video 8 *D. gunungcola* wing display behavior at Coban Rondo Waterfall in East Java, Indonesia (Version 1)
Video 9 *D. gunungcola* wing display behavior at Coban Rondo Waterfall in East Java, Indonesia (Version 2)
Video 10 *D. elegans* wing display behavior in Tumpang, Indonesia
Video 11 *D. gunungcola* (Batu City, Indonesia) wing display behavior in the laboratory