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Distinct genetic architectures underlie divergent thorax, leg, and wing pigmentation between *Drosophila elegans* and *D. gunungcola*

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Pigmentation divergence between *Drosophila* species has emerged as a model trait for studying the genetic basis of phenotypic evolution, with genetic changes contributing to pigmentation differences often mapping to genes in the pigment synthesis pathway and their regulators. These studies of *Drosophila* pigmentation have tended to focus on pigmentation changes in one body part for a particular pair of species, but changes in pigmentation are often observed in multiple body parts between the same pair of species. The similarities and differences of genetic changes responsible for divergent pigmentation in different body parts of the same species thus remain largely unknown. Here we compare the genetic basis of pigmentation divergence between *Drosophila elegans* and *D. gunungcola* in the wing, legs, and thorax. Prior work has shown that regions of the genome containing the pigmentation genes *yellow* and *ebony* influence the size of divergent male-specific wing spots between these two species. We find that these same two regions of the genome underlie differences in leg and thorax pigmentation; however, divergent alleles in these regions show differences in allelic dominance and epistasis among the three body parts. These complex patterns of inheritance can be explained by a model of evolution involving tissue-specific changes in the expression of Yellow and Ebony between *D. elegans* and *D. gunungcola*.

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INTRODUCTION

Pigmentation differences within and between species of animals illustrate some of the most striking examples of phenotypic evolution in nature (Kronforst et al. 2012). In insects, pigmentation intensity in the body, legs, and wings varies widely, often distinguishing sexes, populations, and species (True 2003). The genetic and developmental processes determining pigment patterning are well understood, which has facilitated the use of insect pigmentation as a model system to investigate the genetic basis of phenotypic evolution (Kopp 2009; Wittkopp et al. 2003). Multiple studies have revealed that (1) the same genes have often evolved independently to cause pigmentation variation, (2) genetic variation within these genes often explains the majority of pigmentation differences, and (3) mutations affecting the expression of enzymes and transcription factors that regulate enzyme expression often underlie pigmentation evolution (reviewed in (Massey and Wittkopp 2016)).

The insect pigment synthesis pathway provides a roadmap for predicting which genes likely contribute to pigmentation differences within and between insect species. Differences in how dopamine is metabolized in this pathway (Fig. 1A) ultimately lead to combinations of black and brown melanins as well as yellow and colorless sclerotins that become part of the developing cuticle in sex-, population-, and species-specific ways. The genes *yellow*, *tan*, and *ebony* in this pathway have repeatedly evolved to contribute to differences in pigmentation within and between species (reviewed in (Massey and Wittkopp 2016)). The relative expression of these genes controls the relative amount of black, brown, and yellow pigments produced (Jeong et al. 2008; Wittkopp et al. 2002; Wittkopp et al. 2009). For example, Yellow expression is required to form black pigments, whereas Ebony expression is required to form yellow pigments (Fig. 1A). In *D. melanogaster*, increasing Yellow expression increased the amount of black pigments produced at the expense of yellow sclerotin, increasing Ebony expression did the opposite, and increasing both Yellow and Ebony expression simultaneously restored wildtype pigmentation (Wittkopp et al. 2002). Consistent with these observations, *cis*-regulatory changes affecting expression of *yellow* and *ebony* (and *tan*) have been identified that correlate with pigmentation divergence (Massey and Wittkopp 2016), emphasizing the importance of gene regulatory evolution in generating *Drosophila* pigmentation diversity.

To date, most studies of *Drosophila* pigmentation have focused on divergence of one particular element of pigmentation between each species pair (Lafuente et al. 2020), making it unclear how often the same genes and/or mutations underlie divergent pigmentation in different body parts of the same pair of species. For example, *D. elegans* and *D. gunungcola* differ in pigmentation of their wings, thorax, and legs, but prior work has focused only on divergence in wing pigmentation (Fig. 1B (Massey et al. 2020a; Yeh et al. 2006; Yeh and True 2014)). *D. elegans* from Hong Kong and Indonesia have a light brown thorax and legs and a male-specific dark black spot of

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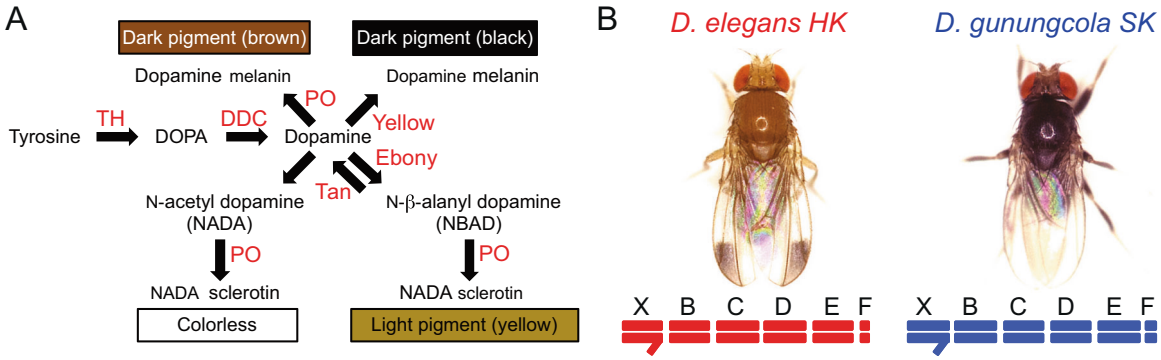

Fig. 1 Insect sclerotization and pigmentation synthesis pathway and pigmentation differences between *Drosophila elegans HK* and *D. gunungcola SK*. A Pigmentation enzymes (shown in red) convert substrates (shown in black) into pigmentation precursors that polymerize into darkly colored melanins or lightly colored sclerotins. Synthesis of black melanin depends on Yellow function, whereas Ebony converts dopamine into lightly colored sclerotins. Tan catalyzes the reverse reaction of Ebony, converting NBAD molecules back into dopamine. **B** *D. elegans HK (Hong Kong)* and *D. gunungcola SK (Sukarami)* show divergent thorax, leg, and wing pigmentation.

pigment at the tips of their wings (Hirai and Kimura 1997), whereas *D. gunungcola*, which diverged 2–2.8 million years ago (Prud'homme et al. 2006), has a dark black thorax and legs without any dark wing spots (Fig. 1B). Pigmentation is polymorphic within *D. elegans*, however, with Japanese and Taiwanese populations having dark black thoraxes and male-specific wing spots (Hirai and Kimura 1997), showing that divergence in wing and thorax pigmentation is likely genetically distinct.

Prior work has shown that the presence or absence of a wing-spot in *D. elegans* and *D. gunungcola* is controlled by a region of the X chromosome containing the transcription factor *optomotor-blind* but not the nearby pigmentation gene *yellow*, whereas the size of the wing spot when it develops is controlled by an overlapping region of the X chromosome including the *yellow* gene. A region of the genome containing the *ebony* gene on Muller element E and a locus on Muller element C were also found to influence wing spot size (Massey et al. 2020a). Here, we map loci contributing to differences in thorax and leg pigmentation between the light *D. elegans* morph from Hong Kong (HK) and *D. gunungcola* from Sukarami, Indonesia (SK) using the same backcross populations and introgression lines used to map differences in wing pigmentation. We find that regions of the X chromosome and Muller element E containing the *yellow* and *ebony* genes, respectively, also contribute to divergence in thorax and leg pigmentation, but that epistatic interactions between the *D. elegans HK* and *D. gunungcola SK* loci in these regions as well as dominance relationships between Muller element E alleles differ among the three traits. To explain these observations, we propose a model of divergence including tissue-specific changes in expression of both *yellow* and *ebony* that is consistent with the inheritance patterns observed in this study as well as prior studies of *Drosophila* pigmentation development and evolution.

MATERIALS AND METHODS

Fly stocks

Strains of *D. elegans HK (Hong Kong)* and *D. gunungcola SK (Sukarami)* were gifts from John True (Stony Brook University). Stock maintenance and food recipes are described in (Massey et al. 2020a). In brief, stocks were maintained at 23 °C on a 12 h light-dark cycle. At the third instar larval stage of development, adults were transferred onto new food, and Fisherbrand filter paper (cat# 09-790-2A) was added to the larval vials to facilitate pupariation. A previously described introgression line containing 1.5 Mb of the end of Muller element E including the *ebony* gene from *D. gunungcola* introgressed into *D. elegans* was also examined in this study. Construction and genetic analysis of this introgression line is described in (Massey et al. 2020a).

Generating interspecific F_1 hybrids and backcross progeny

Male and female *D. elegans HK* flies will reproduce with male and female *D. gunungcola SK* in the laboratory to produce fertile F_1 hybrid female and sterile F_1 hybrid male offspring (Yeh et al. 2006). Creating these F_1 hybrids in populations large enough for genetic analysis, however, is difficult. Detailed methods are described in (Massey et al. 2020a). In brief, both *D. elegans HK* and *D. gunungcola SK* species stocks were expanded to establish populations of more than 10,000 flies. Next, virgin males and females from each species were placed in heterospecific crosses (*D. elegans HK* males with *D. gunungcola SK* females and vice versa) in groups of ten males and females to generate fertile F_1 hybrid female offspring. Dozens of these crosses were set to create ~120 F_1 hybrid female offspring. Since F_1 hybrid males are sterile (Yeh et al. 2006; Yeh and True 2014), the F_1 hybrid females were crossed to males from the parental strains to generate two backcross populations for quantitative trait loci (QTL) mapping. For the *D. elegans HK* backcross population, ~60 F_1 hybrid females were crossed in the same vial with ~60 *D. elegans HK* males and transferred onto new food every 2 weeks for ~2.5 months, resulting in 724 recombinant individuals. For the *D. gunungcola SK* backcross population, ~60 F_1 hybrid females were crossed in the same vial with ~60 *D. gunungcola SK* males and transferred onto new food every 2 weeks for ~2.5 months, resulting in 241 recombinant individuals.

Pigmentation phenotyping

To map the genetic basis of thorax pigmentation, male recombinants from each backcross population were organized into three thorax pigmentation classes. The lightest recombinants showed thorax pigmentation intensities similar to *D. elegans HK* and were given a score of 0; recombinants with intermediate thorax pigmentation intensities were given a score of 1; and recombinants with dark thorax pigmentation intensities similar to *D. gunungcola SK* were given a score of 2. To quantify the effects of the Muller element E introgression region on thorax pigmentation, individuals were placed thorax-side up on Scotch double sided sticky tape on glass microscope slides (Fisherbrand) (cat# 12-550-15) and imaged at the same exposure using a Canon EOS Rebel T6 camera mounted to a Canon MP-E 65 mm macro lens equipped with a ring light. The images were then imported into ImageJ software (version 1.50i) (Wayne Rasband, National Institutes of Health, USA; http://rsbweb.nih.gov/ij/) and converted to 32 bit grayscale. Using the "straight line segment" tool, a line was drawn between the anterior scutellar bristles to measure the mean grayscale value of the cuticle.

To map the genetic basis of leg pigmentation, methods were identical to the thorax procedures above except recombinants were organized into light (0), intermediate (1), and dark (2) classes based on the pigmentation intensity of the medial side of their right hindleg femur. This region of the leg was chosen because it contains few cuticular bristles that could obscure pigmentation. To quantify the effects of the Muller element E introgression region on leg pigmentation, the same methods as above were used except right medial hindleg images were captured. In ImageJ, the "polygon selections" tool was used to draw a polygon selection around the medial side of the right hindleg femur to measure the mean grayscale value of the cuticle.

The genetic basis of wing spot size was mapped previously using the same mapping populations described above (Massey et al. 2020a). Briefly, right wings from male recombinants from each backcross population were imaged, and spots were quantified in ImageJ using the "polygon selections" tool to quantify wing spot size relative to wing size. The same procedure was used to quantify the effects of the Muller element E introgression region.

Genetic mapping

QTL affecting thorax and leg pigmentation were mapped using the same backcross populations and methods used to map loci affecting wing spots in *D. elegans* and *D. gunungcola* (Massey et al. 2020a). Regions of the genome inherited from the *D. elegans* and *D. gunungcola* parents in each recombinant fly were determined by MSG-seq (Andolfatto et al. 2011). These data included 3425 and 3121 genetic markers for the *D. elegans HK* and *D. gunungcola SK* backcrosses, respectively. The marker genotypes; thorax, leg, and wing spot phenotypes; and code used for QTL mapping are available on Dryad (https://doi.org/10.5061/dryad.gb5mkkwm5). Schematics showing chromosomal breakpoints for each recombinant are available here: https://deepblue.lib.umich.edu/data/concern/data_sets/j098zb17n?locale=en.

QTL mapping was performed using R/qtl (Broman and Sen 2009) in R for Mac version 3.3.3 (Pinheiro et al., (2016)). We imported ancestry data for both backcross populations into R/qtl using a custom script (https://github.com/dstern/read_cross_msg). This script directly imports the conditional probability estimates produced by the Hidden Markov Model of MSG (described in detail in 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 thorax, leg, and wing spot pigmentation. For QTL mapping using the *D. elegans HK* backcross population, we excluded 18 and 20 individuals for thorax and leg pigmentation, respectively, because fly samples were either too poor to image or sequencing reads were too shallow to map. For the *D. gunungcola SK* backcross population, we excluded 12 and 9 individuals for thorax and leg pigmentation, respectively, for the same reasons. Significance of QTL peaks at $\alpha = 0.01$ was determined by performing 1000 permutations of the data. For wing pigmentation, we previously identified a 440 kb region on the X chromosome explaining the presence or absence of wing spots (Massey et al. 2020a) and mapped QTL on the X chromosome and Muller elements C and E that affected wing spot size using only backcross progeny with a visible wing spot (Supplementary Fig. 4 in (Massey et al. 2020a)).

Statistics

Statistical tests were performed in R for Mac version 3.3.3 (Pinheiro et al., (2016)). ANOVAs were performed with post-hoc Tukey HSD for pairwise comparisons adjusted for multiple comparisons. See "QTL analysis" methods for statistical tests used for QTL mapping and "*Assessing epistasis between QTL-X and QTL-E*" for statistical tests used to measure epistasis.

Creating an ebony null allele in *Drosophila elegans HK*

Using methods described in (Bassett et al. 2014), we in vitro transcribed (MEGAscript T7 Transcription Kit, Invitrogen) two single guide RNAs (sgRNAs) (Supplementary File S1) with target sequences designed based on conserved sites between *D. elegans HK* and *D. gunungcola SK* in exon 2 of the *ebony* gene. After transcription, sgRNAs were purified using an RNA Clean and Concentrator 5 kit (Zymo Research), eluted with nuclease-free water, and quantified using a Qubit RNA BR Assay Kit (Thermo Fisher Scientific). Embryos for injection were collected as described in (Massey et al. 2020b). Briefly, mature (≥2 weeks old) *D. elegans HK* males and females were transferred to 60 mm embryo lay cages (GENESEE Part Number: 59–100) on top of 60 mm grape plates (3% agar + 25% grape juice + 0.3% sucrose) at high densities (>300 flies) after brief CO_2 anesthesia. After CO_2 anesthesia, mature, mated *D. elegans HK* females will often eject an embryo from their abdomen where it sticks briefly to the anus. Tapping the grape plate + embryo cage down on a hard surface 10 times caused the embryos to stick to the grape agar. Flies were then transferred back into food vials and embryos were lined up on glass cover slips taped to a glass microscope slide. For CRISPR/Cas9 injections into *D. elegans HK* embryos, Cas9 protein (PNA Bio #CP01), phenol red, and both sgRNAs were mixed together at 400 ng/µl, 0.05%, and 100 ng/µl final injection concentrations, respectively. All CRISPR injections were performed in-house, using previously described methods (Miller et al. 2002). In all, we injected 992 *D. elegans HK* embryos, 455 (46%) of the injected embryos developed into larvae and ~200 emerged as adults. We screened for germline mutants based on changes in body and pupal case pigmentation and confirmed loss of Ebony protein by western blot. To the best of our knowledge, these are the first successful gene editing experiments in *D. elegans HK*. We attempted the same experiments in *D. gunungcola SK* (injecting ~500 embryos) but failed to recover any *ebony* mutants.

Western blotting

To determine whether the *ebony* mutants recovered carried a null allele, we performed western blotting to check for expression of the Ebony protein. Western blots were performed as described (Wittkopp et al. 2002). In brief, for each replicate per genotype, four newly eclosed (within 60 min) male flies were homogenized in 100 µl of 125 mM Tris pH 6.8, 6% SDS and centrifuged for 15 min. The supernatant was then transferred to a new protein low-bind Eppendorf tube with an equal amount of 2X Laemmli sample buffer [4% SDS, 20% glycerol, 120 mM Tris-Cl (pH 6.8)], boiled for 10 min, and stored at −80 °C. Before gel electrophoresis, samples were thawed at room temperature for 30 min, and 20 µl of each sample was loaded into individual wells of an Invitrogen NuPAGE 4–12% Bis-Tris Gel. The gel was run at 175 V for 60 min, washed, and transferred to an Invitrogen iBlot 2 PVDF Mini Stack Kit. The mini stack was run on an iBlot 2 (ThermoFisher, Catalog Number: IB21001) to transfer proteins to the membrane, which was then blocked using an Invitrogen Western Breeze anti-rabbit kit (Catalog Number: WB7106). After two washes with deionized H_2O, samples were incubated in 1:400 rabbit anti-Ebony using the blocking buffer from the Invitrogen Western Breeze anti-rabbit kit to dilute (Wittkopp et al. 2002) overnight at 4 °C. Finally, samples were washed using the Western Breeze wash solution, incubated in a secondary antibody solution containing alkaline-phosphatase conjugated anti-Rabbit antibodies (Catalog Number: WP20007) for 30 min, washed for 2 min with deionized H_2O, and prepared for chromogenic detection.

We analyzed *D. elegans HK*, flies homozygous for the introgressed region of *D. gunungcola SK* Muller element E containing *ebony* into *D. elegans HK*, and the *D. elegans HK ebony* mutant. The Ebony antibody used for this work has previously been shown to also detect a shorter protein unrelated to Ebony in *D. melanogaster* (Wittkopp et al. 2002). We observed this secondary band and used it to correct for differences in total protein loaded in each lane when comparing Ebony expression among genotypes. For quantification, the western blot image was imported into ImageJ software (version 1.50i) (Wayne Rasband, National Institutes of Health, USA; http://rsbweb.nih.gov/ij/) and converted to 32 bit grayscale. Using the "straight line segment" tool, the mean grayscale value of each band was quantified and inverted so that higher values indicated darker band intensity. A Student's *t*-test was used to compare expression between *D. elegans HK* and the introgression line, with three biological replicates for each genotype.

Assessing epistasis between QTL-X and QTL-E

To test for epistatic interactions between QTL on the X chromosome and on Muller element E, we compared pigmentation values for each tissue (thorax, leg, and wing) among the following genotypic combinations:

$$QTL - X^{ele}/\mathrm{Y}, QTL - E^{ele}/QTL - E^{ele} \quad (1)$$

$$QTL - X^{ele}/\mathrm{Y}, QTL - E^{ele}/QTL - E^{gun} \quad (2)$$

$$QTL - X^{ele}/\mathrm{Y}, QTL - E^{gun}/QTL - E^{gun} \quad (3)$$

$$QTL - X^{gun}/\mathrm{Y}, QTL - E^{ele}/QTL - E^{ele} \quad (4)$$

$$QTL - X^{gun}/\mathrm{Y}, QTL - E^{ele}/QTL - E^{gun} \quad (5)$$

$$QTL - X^{gun}/Y, QTL - E^{gun}/QTL - E^{gun} \quad (6)$$

where QTL-X and QTL-E represent genotype positions with the maximum estimated LOD scores from QTL mapping in each backcross (Table 1; Massey et al. 2020a). For thorax pigmentation, these positions were: *QTL-X*^{ele}: 9,309,667 bp, *QTL-X*^{gun}: 7,704,294 bp, *QTL-E*^{ele}: 409,133 bp, and *QTL-E*^{gun}: 3,766,760 bp; for leg pigmentation, *QTL-X*^{ele}: 9,456,223 bp, *QTL-X*^{gun}: 10,117,133 bp, *QTL-E*^{ele}: 606,610 bp, and *QTL-E*^{gun}: 606,610 bp (note, we used the same QTL-E peak position for *QTL-E*^{gun} as *QTL-E*^{ele}, because we detected a much larger effect in the *D. elegans HK* backcross than the *D. gunungcola SK* backcross); for wing spot size, *QTL-X*^{ele}: 10,303,766 bp, *QTL-X*^{gun}: 10,303,766 bp (note, we used the same QTL-X peak for *QTL-X*^{gun} as *QTL-X*^{ele}, because we detected a significant QTL only in the *D. elegans HK* backcross due to the removal of spotless recombinants in this analysis), *QTL-E*^{gun}: 12,536 bp, and *QTL-E*^{ele}: 12,536 bp (note, we used the same QTL-E peak for *QTL-X*^{ele} as *QTL-X*^{gun}, because we detected a significant QTL only in the *D. gunungcola SK* backcross). We performed two-way ANOVAs in R (version 3.3.3) to assess genotype (QTL-X) X genotype (QTL-E) interactions for each pigmentation tissue. Epistasis was indicated by a significant ($P < 0.01$) interaction effect. The same analysis was also performed using genotype markers in the *yellow* gene on the X chromosome and the *ebony* gene on Muller element E with similar results (data not shown).

Table 1. QTLs detected for thorax and leg pigmentation divergence.

Trait	Backcross	Chr.	QTL interval (bp)[a]	QTL peak (bp)	LOD	Candidate (bp)
Thorax pigmentation	*D. elegans HK*	X	8,996,888–10,120,567	9,309,667	34.2	*yellow:* 11,412,900
Thorax pigmentation	*D. elegans HK*	E	244,349–6,527,255	409,133	27.8	*ebony:* 1,580,200
Leg pigmentation	*D. elegans HK*	X	8,727,567–14,805,348	9,456,223	34.0	*yellow:* 11,412,900
Leg pigmentation	*D. elegans HK*	C	14,797–7,500,000	27,017	5.27	none
Leg pigmentation	*D. elegans HK*	E	7600–6,588,062	606,610	27.9	*ebony:* 1,580,200
Thorax pigmentation	*D. gunungcola SK*	X	6,969,794–9,711,406	7,704,294	20.0	*yellow:* 11,412,900
Thorax pigmentation	*D. gunungcola SK*	E	2,083,292–3,843,775	3,766,760	30.3	*ebony:* 1,580,200
Leg pigmentation	*D. gunungcola SK*	X	10,029,176–11,595,407	10,117,133	39.5	*yellow:* 11,412,900
Leg pigmentation	*D. gunungcola SK*	E	517,241–27,451,006	11,251,902	6.41	*ebony:* 1,580,200

[a]LOD drop 1.5 support interval (the region where the LOD score is within 1.5 of its maximum; Broman and Sen 2009).

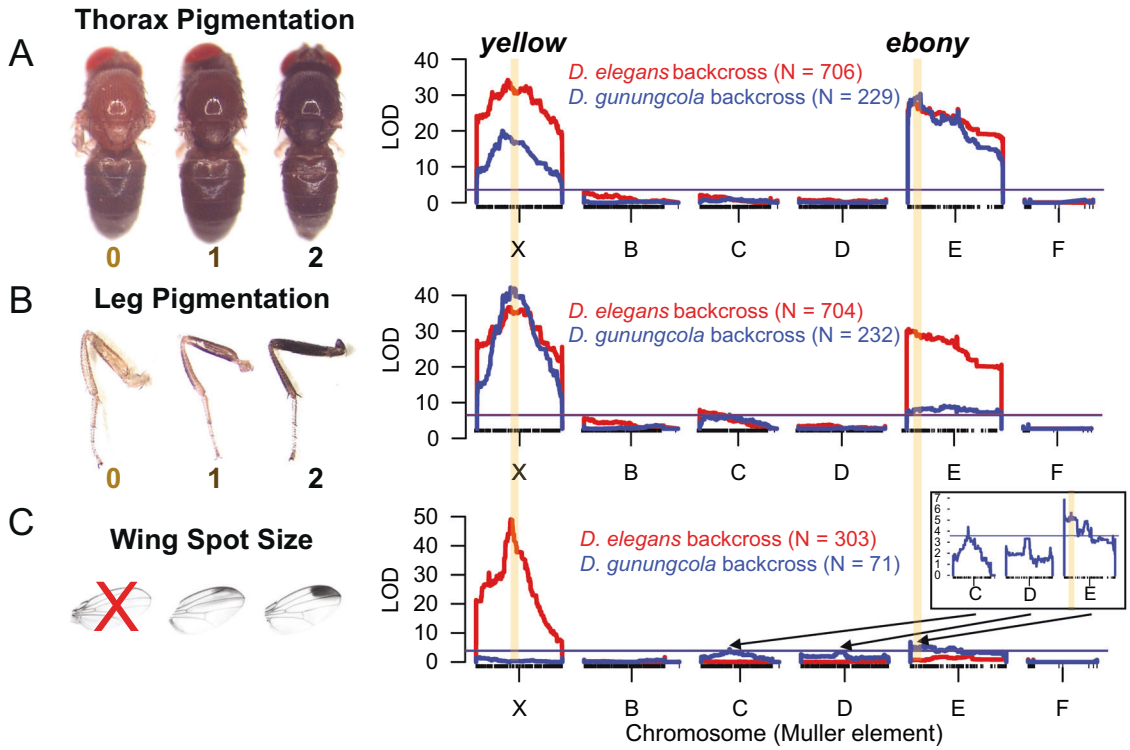

Fig. 2 Quantitative trait locus (QTL) mapping of thorax, leg, and wing pigmentation divergence. A Thorax pigmentation was organized into three classes (light, intermediate, and dark). QTL map for thorax pigmentation is shown for *D. elegans HK* backcross (red) and *D. gunungcola SK* backcross (blue). **B** Leg pigmentation was organized into three classes (light, intermediate, and dark). QTL map for leg pigmentation is shown for *D. elegans HK* backcross (red) and *D. gunungcola SK* backcross (blue). **C** Wing spot pigmentation was quantified relative to wing size; recombinants completely lacking wing spots were excluded (red "X") from the QTL analysis to detect effects of other QTL on size differences. QTL map for spot size is shown for *D. elegans HK* backcross (red) and *D. gunungcola SK* backcross (blue) (Reprinted from Massey et al. (2020a), Copyright (2020), with permission from John Wiley and Sons). Transparent yellow bars indicate the chromosomal location of pigmentation candidate genes. 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 HK* assembled genome (see Methods). *D. elegans HK* and *D. gunungcola SK* have six chromosomes (Yeh et al. 2006; Yeh and True 2014) that correspond to *D. melanogaster* chromosomes as follows: X = X, B = 2 L, C = 2 R, D = 3 L, E = 3 R, 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 HK* and *D. gunungcola SK* backcross, respectively.

RESULTS

QTLs on the X chromosome and Muller element E are primarily responsible for divergent thorax and leg pigmentation between *D. elegans HK* and *D. gunungcola SK*

To identify loci contributing to divergent thorax and leg pigmentation, we performed QTL mapping using backcross populations produced by crossing F_1 hybrid females to males from each parental species. Thorax and leg pigmentation were scored by eye in male recombinants, with light (*D. elegans*-like) pigmentation scored as 0, dark (*D. gunungcola*-like) pigmentation scored as 2, and intermediate pigmentation scored as 1 (Fig. 2A, B). For thorax pigmentation, we identified QTLs on

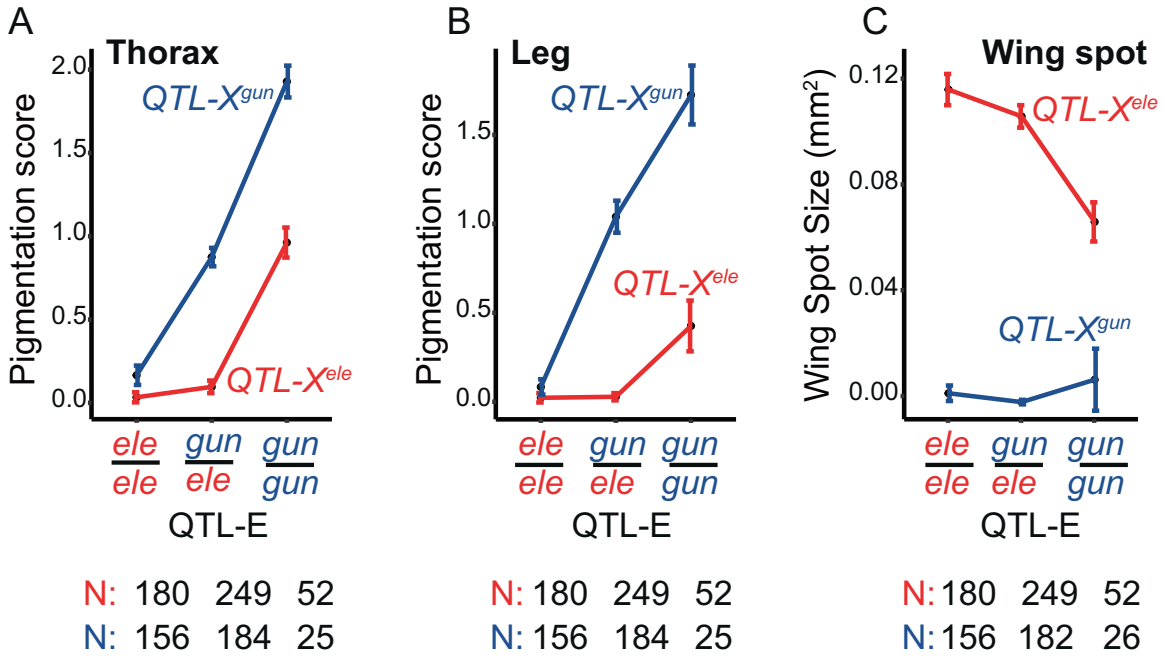

Fig. 3 Genetic interactions among QTL on the X chromosome and Muller element E differ among body parts. Mean pigmentation scores for the thorax, leg, and wing spot are shown based on genotype positions linked to the estimated max LOD score from QTL mapping in each backcross (see Table 1 and Methods). The superscripts "*ele*" (red) and "*gun*" (blue) indicate alleles at peak QTL positions from *D. elegans HK* and *D. gunungcola SK*, respectively. Error bars indicate 95% confidence intervals for the phenotype of each genotypic class. The number of flies (N) with each genotype (red for *D. elegans HK* X allele; blue for *D. gunungcola SK* X allele) included for each trait is shown below each plot, excluding backcross progeny with missing or ambiguous genotypes or missing phenotype data.

the X chromosome and Muller element E in both backcrosses (Fig. 2A; Table 1). For leg pigmentation, we also identified QTLs on the X chromosome in both backcrosses, but the statistical support for a QTL on Muller element E was much lower in the *D. gunungcola SK* backcross than in the *D. elegans HK* backcross (Fig. 2B; Table 1).

Although the entire chromosomes often showed statically significant associations with pigmentation, the strongest associations (peaks) were seen near the location of *yellow* on the X-chromosome and *ebony* on Muller element E (Fig. 2A, B; Table 1). Significant associations throughout the chromosome are consistent with the low recombination rate in the mapping population: among 951 backcross flies, an average of 0.68 and 0.25 crossovers were observed per fly on the X chromosome and Muller element E, respectively (Supplementary Fig. 1). QTL mapping for variation in wing spot size using only flies in these recombinant backcross populations with visible wing spots also showed different genetic architectures between the two backcrosses (Fig. 2C). As reported previously, a QTL peak on the X chromosome for wing spot size (rather than wing spot presence) near the *yellow* gene is present for the *D. elegans HK* but not the *D. gunungcola SK* backcross, whereas a QTL on Muller element E (as well as on Muller element C) is detected in the *D. gunungcola SK* but not the *D. elegans HK* backcross (Fig. 2C, reproduced from Supplementary Fig. 4A in (Massey et al. 2020a), Copyright (2020), with permission from John Wiley and Sons)). The absence of an X-linked QTL for wing spot size in the *D. gunungcola SK* backcross is consistent with recombinants possessing the X-linked *D. gunungcola SK* allele (71 out of 199) that suppresses wing spot development being excluded from this analysis (Massey et al. 2020a). Excluding these recombinants revealed the spot-size QTL on Muller elements C and E (Fig. 2C). Together, these data suggest that divergence of the same genes might underlie divergent pigmentation in the thorax, leg, and wing, but that their interactions with genetic background differ for the three pigmentation traits.

X chromosome and Muller element E QTL show tissue-specific genetic interactions

To test directly for epistatic interactions between the QTL identified on the X chromosome and Muller element E, we used our mapping data to more closely examine the genotype-phenotype relationships in each tissue. That is, we assessed whether the effect of species-specific alleles at one QTL depended on the presence of species-specific alleles at another QTL, as suggested by the observed differences in QTL maps between backcrosses. We calculated and plotted mean thorax, leg, and wing spot pigmentation for male backcross genotypes with either *D. elegans HK* or *D. gunungcola SK* alleles at the respective QTL peak region (see Table 1 and Methods) on the X chromosome (*QTL-X*) and Muller element E (*QTL-E*) (Fig. 3). We detected evidence of genetic interactions between *QTL-X* and *QTL-E* in all three tissues, but the nature of the interactions differed among tissues (Fig. 3; Two-way ANOVA, Thorax: $F_{2,826} = 106.6$, $P < 2 \times 10^{-16}$; Leg: $F_{2,827} = 178.9$, $P < 2 \times 10^{-16}$; Wing: $F_{2,455} = 20.14$, $P = 4.17 \times 10^{-9}$). In both the thorax and leg, we found that the *D. elegans HK QTL-E* was completely dominant to *D. gunungcola SK QTL-E* when in combination with *D. elegans HK QTL-X* but the two *QTL-E* alleles interacted additively when in combination with *D. gunungcola SK QTL-X* (Fig. 3A, B). Flies homozygous for *D. gunungcola SK QTL-E* showed darker pigmentation than flies heterozygous for this QTL in both the thorax and leg regardless of which species' *QTL-X* allele was present (Fig. 3A, B). For the wing spot, we observed a different pattern of inheritance. When in combination with *D. elegans HK QTL-X*, effects of *QTL-E* on wing spot size were nearly additive with *D. gunungcola SK* alleles (Fig. 3C). When in combination with *D. gunungcola SK QTL-X*, however, these effects were masked, because the *D. gunungcola SK QTL-X* suppresses wing spots (Fig. 3C, Massey et al. 2020a). These data show that the effects of *QTL-X* and *QTL-E* on all three body parts differ depending on whether *D. elegans HK* or *D. gunungcola SK* alleles are present at each locus.

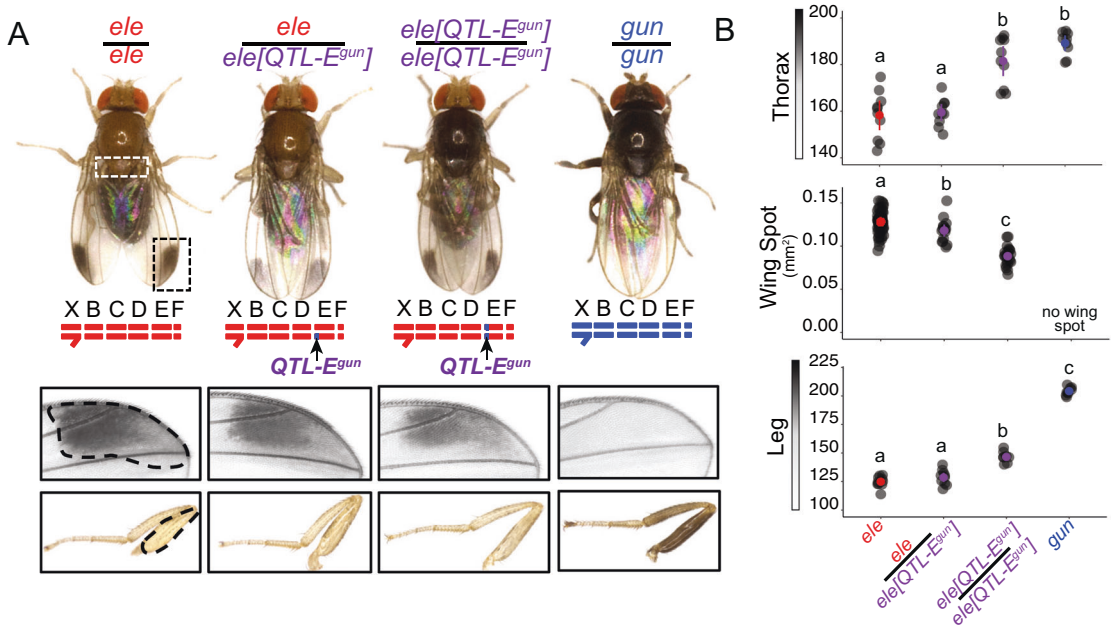

Fig. 4 *D. gunungcola SK* alleles linked to *ebony* on Muller element E have varied effects on thorax, leg, and wing pigmentation divergence. A Images highlighting pigmentation and chromosome differences between species and the Muller Element E introgression line. The white dashed box highlights the scutellar region of the thorax used to quantify thorax pigmentation in (**B**) (see Methods). Dashed black lines highlight regions of the wing and leg used to quantify wing spot and leg pigmentation in (**B**) (see Methods). **B** Quantification of thorax pigmentation differences between species and the Muller element E introgression line (One-way ANOVA $F_{3,35} = 33.9$; $P = 1.84 \times 10^{-10}$; groups not sharing the same letter are significantly different based on post-hoc Tukey HSD at $P < 0.00001$). Quantification of wing spot pigmentation differences between species and the Muller element E introgression line (One-way ANOVA $F_{2,88} = 78.6$; $P < 2.0 \times 10^{-16}$; groups not sharing the same letter are significantly different based on post-hoc Tukey HSD at $P = 0.02$) (Reprinted from Massey et al. (2020a), Copyright (2020), with permission from John Wiley and Sons). Quantification of leg pigmentation differences between species and the Muller element E introgression line. (One-way ANOVA $F_{3,33} = 481$; $P < 2.0 \times 10^{-16}$; groups not sharing the same letter are significantly different based on post-hoc Tukey HSD at $P < 5 \times 10^{-7}$). Transparent circles represent individual replicates for (**B**).

A Muller element E introgression line confirms tissue-specific pigmentation effects

A region of 1.5 Mb (~7%) of Muller element E containing *ebony* was introgressed from *D. gunungcola SK* into *D. elegans HK* to assess its contribution to pigmentation divergence (Massey et al. 2020a). *D. elegans HK* flies heterozygous for this introgression showed a significantly smaller wing spot than *D. elegans HK*, and flies homozygous for this introgression showed an even smaller wing spot (Fig. 4A, B, wing spot data also shown in Supplementary Fig. 4D, E in Massey et al. (2020a)). These phenotypes are consistent with the additive effects of the *D. gunungcola SK* QTL allele on Muller element E in the presence of *D. elegans HK* X chromosome QTL (Fig. 3). In the thorax and legs, flies heterozygous for this introgression showed light pigmentation statistically indistinguishable from that of *D. elegans HK* (Fig. 4), consistent with the dominance of the *D. elegans HK* Muller element E QTL allele in the presence of the *D. elegans HK* X chromosome seen in the recombinant population used for QTL mapping (Fig. 3). Flies homozygous for this introgression showed significantly darker pigmentation than *D. elegans HK*, with pigmentation statistically equivalent to *D. gunungcola SK* in the thorax and intermediate between *D. elegans HK* and *D. gunungcola SK* in the legs (Fig. 4). These observations indicate that the 1.5 Mb introgressed region of Muller element E contains evolved sites with different effects on pigmentation of the wing, thorax, and leg.

The *ebony* gene is a strong candidate for these effects within the 1.5 Mb region. Using CRISPR/Cas9 genome editing (Bassett et al. 2014), we attempted to generate *ebony* null mutant alleles in *D. elegans HK* and *D. gunungcola SK* to test for pigmentation differences in F1 hybrids possessing a single copy of either *D. elegans HK* or *D. gunungcola SK* with a reciprocal hemizygosity test (Stern 2014); however, we could generate an *ebony* null mutant allele only in *D. elegans HK* (see Methods). In this mutant line, we observed pigmentation changes in the thorax, legs, and wings. Specifically, flies homozygous for this *ebony* mutant allele had darker thorax pigmentation than *D. elegans HK*, legs that appeared less yellow than *D. elegans HK*, and wings that showed additional gray pigmentation surrounding wing veins similar to that seen in *D. melanogaster ebony* mutants (Wittkopp et al. 2002) but no apparent changes in wing spot size (Supplementary Fig. 2).

DISCUSSION

D. elegans HK and *D. gunungcola SK* differ in thorax, leg, and wing pigmentation. We found that QTL on the X chromosome and Muller element E underlie divergence in all three pigmentation traits; however, the effect of each QTL allele differs among traits and depends on the alleles present at the other QTL. For example, dominance of the *D. elegans HK* QTL allele on Muller element E was observed in the thorax and leg only in the presence of the *D. elegans HK* QTL allele on the X, resulting in epistatic interactions between these two QTL. Distinct epistatic interactions were observed for the wing spot, with the X-chromosome from *D. gunungcola SK* masking the effects of the QTL on Muller element E (Massey et al. 2020a). Introgression of the Muller element E QTL from *D. gunungcola SK* into *D. elegans HK* caused different pigmentation changes in each tissue, as did the elimination of *ebony* activity in *D. elegans HK*, suggesting that distinct pigmentation changes in different body parts can be caused by genetic variation in the same genes.

Combining the results from this study with prior work suggests a model of pigmentation divergence between *D. elegans HK* and *D. gunungcola SK* in which tissue-specific changes in expression of two interacting pigmentation genes can explain the dominance,

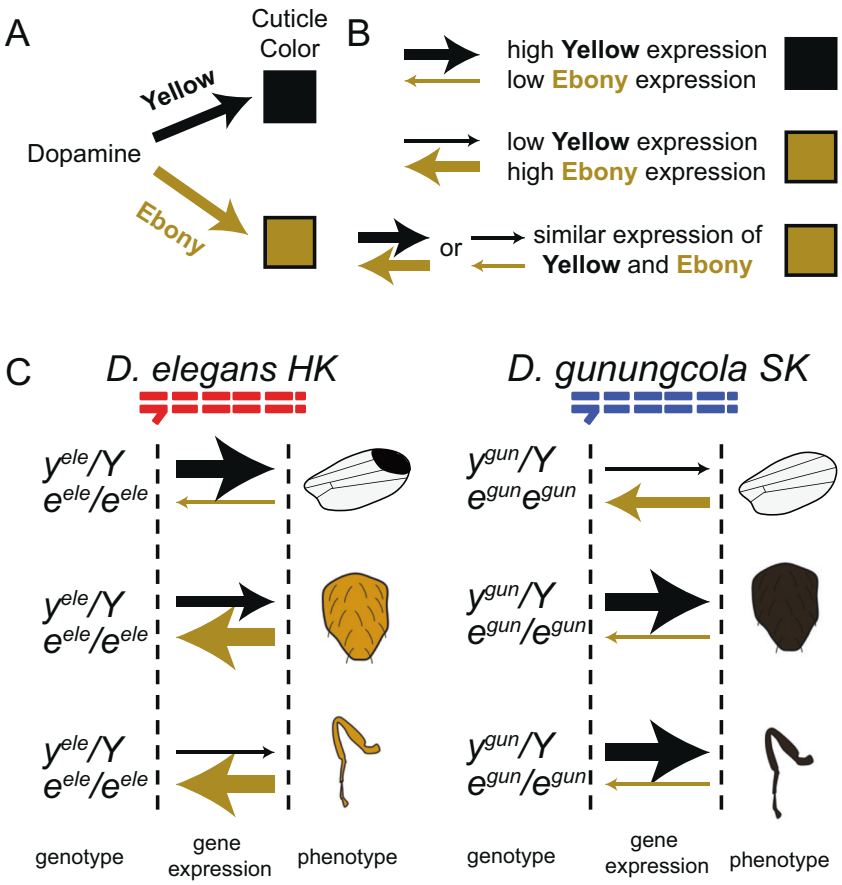

Fig. 5 Model of pigmentation divergence connecting genotypes to gene expression to phenotypes. A The Yellow protein promotes the synthesis of black melanins derived from dopamine that give the adult cuticle a black color. The Ebony protein promotes the synthesis of yellow sclerotins from dopamine that give the adult cuticle a yellow color. Note that the gene names are opposite to their functions because these genes were named for their loss-of-function mutant phenotypes. **B** Expression of Yellow and Ebony in the same tissue has opposing effects because both proteins draw from the same pool of dopamine to synthesize black and yellow pigments, as shown in **A**, with the relative expression of Yellow and Ebony within a tissue determining the cuticle color. The relationships between gene expression and pigmentation shown are supported by work in *D. melanogaster* showing that increasing Yellow expression increases dark pigmentation when Ebony expression is low, increasing Ebony expression lightens pigmentation by replacing dark pigments with yellow pigments, and increasing expression of both proteins results in a predominantly yellow cuticle (Wittkopp et al. 2002). In this schematic, the thickness of the black and yellow arrows represents the expression levels of Yellow and Ebony, respectively, and the boxes show the resulting cuticle color. **C** Schematics show the relationships between *yellow* (y^{ele}/Y or y^{ele}/Y) and *ebony* (e^{ele}/e^{ele}, e^{ele}/e^{gun}, or e^{gun}/e^{gun}) genotypes, hypothesized expression of Yellow and Ebony in the wing spot, thorax, and leg, and pigmentation phenotypes in the wing spot, thorax, and leg in males for *D. elegans HK* and *D. gunungcola SK*. The *yellow* gene is on the X chromosome, so males carry only one allele and *yellow* genotypes are shown with a Y chromosome. *Ebony* is located on Muller element E, so two alleles are shown for each genotype. The superscripts "*ele*" and "*gun*" indicate alleles from *D. elegans HK* and *D. gunungcola SK*, respectively. As in (**B**), the thickness of the black and yellow arrows indicate the hypothesized expression levels of Yellow and Ebony, respectively.

epistasis, and pigmentation effects observed. In this model, the pigmentation genes *yellow* and *ebony*, which are located close to the pigmentation QTL we mapped on the X and Muller element E, respectively (Table 1), are assumed to be responsible for the QTL effects observed. Yellow and Ebony have opposing effects on pigmentation (Figs. 1A and 5A), and their relative expression within a tissue determines its pigmentation (Wittkopp et al. 2002, Fig. 5B). Among *Drosophila* species, expression of Yellow and Ebony often correlates with pigmentation (reviewed in Massey and Wittkopp 2016), and these expression patterns are controlled by multiple, tissue specific, *cis*-regulatory elements for each gene (Liu et al. 2019; Rebeiz et al. 2009; Wittkopp et al. 2002), providing a mechanism for *yellow* and/or *ebony* expression to vary and diverge independently among tissues (Stern and Orgogozo 2008; Wittkopp 2006; Wittkopp and Kalay 2011; Wray 2007).

Pigmentation phenotypes observed in *D. elegans HK*, *D. gunungcola SK*, and *D. elegans HK ebony* null mutants suggest that differences in Yellow and Ebony vary among tissues and between species (Fig. 5C). The higher expression of Yellow in *D. elegans HK* than in *D. gunungcola SK* shown for the wing spot in this model is supported by empirical data from Prud'homme et al. (2006). The pattern of Ebony expression has not been reported for *D. elegans* and *D. gunungcola* wings, but the expression patterns shown in our model are consistent with those seen between two other species that do (*D. biarmipes)* and do not (*D. melanogaster*) develop a wing spot similar to *D. elegans* (Wittkopp et al. 2002, Arnoult et al. 2013). The patterns of Yellow and Ebony expression have also not been reported for the thorax or legs of *D. elegans* or *D. gunungcola*, but correlations between expression of these genes and pigmentation in other *Drosophila* species suggest that the darker pigmentation of *D. gunungcola SK* relative to *D. elegans HK* results from higher Yellow and lower Ebony expression in these tissues (Fig. 5C). Within *D. elegans HK*, the higher expression of Yellow in the thorax than the legs shown in Fig. 5C is supported by the darker thorax than leg pigmentation seen in *D. elegans HK ebony* null mutants (Supplementary Fig. 2B). Additive inheritance of these inferred gene expression patterns can explain the pigmentation phenotypes seen in this study (Supplementary Fig. 3), but

important work remains to test this model, including confirming whether variation in *yellow* and *ebony* are responsible for the observed QTL and whether the hypothesized expression patterns of Yellow and Ebony shown in Fig. 5C are real and caused by *cis*-regulatory divergence.

This study adds to the rich literature on *Drosophila* pigmentation evolution by revealing similarities and differences in the genetic architecture of pigmentation divergence in different body regions between the same pair of species. The same pigmentation genes have recurrently been found to harbor genetic variation responsible for pigmentation divergence in different species (reviewed in Massey and Wittkopp 2016), and our data suggest that the same is true for distinct changes in pigmentation evolving in different body parts between the same pair of species. Epistatic interactions between pigmentation QTL have previously been reported for differences in abdominal pigmentation between *D. yakuba* and *D. santomea* (Carbone et al. 2005; Liu et al. 2019), and our study of pigmentation divergence in multiple body parts between *D. elegans* and *D. gunungcola* also shows evidence of epistatic interactions among QTL and that these interactions differ among tissues. By combining knowledge of genetic and developmental mechanisms underlying pigmentation divergence from multiple species and contexts, this work illustrates the power of using metamodels such as *Drosophila* pigmentation (Kopp 2009) to advance our understanding of how phenotypes evolve.

DATA AVAILABILITY

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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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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