Drosophila pigmentation evolution: Divergent genotypes underlying convergent phenotypes

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Similar phenotypic changes have evolved independently in many animal taxa. It is unknown whether independent changes involve the same or different developmental and genetic mechanisms. Myriad pigment patterns in the genus *Drosophila* offer numerous opportunities to address this question. Previous studies identified regulatory and structural genes involved in the development and diversification of pigmentation in selected species. Here, we examine *Drosophila americana* and *Drosophila novamexicana*, interfertile species that have evolved dramatic pigmentation differences during the few million years since their divergence. Interspecific genetic analysis was used to investigate the contribution of five specific candidate genes and other genomic regions to phenotypic divergence by testing for associations between molecular markers and pigmentation. At least four distinct genomic regions contributed to pigmentation differences, one of which included the *ebony* gene. Ebony protein was expressed at higher levels in the more yellow *D. novamexicana* than the heavily melanized *D. americana*. Because Ebony promotes yellow pigment formation and suppresses melanization, the expression difference and genetic association suggest that evolution at the *ebony* locus contributed to pigmentation divergence between *D. americana* and *D. novamexicana*. Surprisingly, no genetic association with the *yellow* locus was detected in this study, and Yellow expression was identical in the two species. Evolution at the *yellow* locus underlies pigmentation divergence among other *Drosophila* species; thus, similar pigment patterns have evolved through regulatory changes in different genes in different lineages. These findings bear upon understanding classic models of melanism and mimicry.

Similar functional requirements or selective pressures have favored the independent evolution of similar structures or pattern elements in multiple lineages. The webbing of digits in aquatic or semiaquatic tetrapods, the wings of birds, bats, and pterosaurs, and wing color patterns in butterfly mimicry rings have evolved independently. One of the fundamental questions raised by these examples of convergent evolution is whether the genetic and developmental mechanisms underlying similar evolutionary changes are the same or different.

The evolution of pigmentation offers an attractive model for analyzing phenotypic convergence, because similar phenotypes have arisen frequently in a wide variety of organisms. For example, melanic forms have evolved in nearly all animal taxa (1). In some bird and mammal species, amino acid changes in the melanocortin receptor protein, which activates melanin synthesis, have caused melanism in natural populations (ref. 2 and refs. therein). Although this finding suggests that melanism in some vertebrates shares a common genetic and developmental basis, it is not known whether this is also the case in other taxa, such as insects. Pigmentation in vertebrates and insects forms through different cellular mechanisms (3, 4), which may affect the genetic constraints on pigmentation evolution in each lineage.

Pigmentation is one of the most variable traits in the genus *Drosophila*. The availability of genetic tools in *Drosophila melanogaster* has helped elucidate genetic and developmental mechanisms of pigment patterning and has suggested potential mechanisms of evolutionary change. Abdominal pigmentation has been studied most extensively, and a number of pleiotropic transcriptional regulators, including *bric-a-brac (bab)* and *optomotor-blind (omb)*, have been implicated in the development of specific pigment patterns within this tissue (5–12). Genes encoding enzymes required for the biochemical synthesis of pigments, including *yellow (y)*, *dopa-decarboxylase (ddc)*, and *ebony (e)*, have also been identified (4), and the spatial regulation of these genes delimits pigment patterns in *D. melanogaster* (13). Changing the expression of either regulatory or structural proteins can induce the formation of new pigmentation phenotypes (9, 10, 13–15), and species-specific expression patterns of *bab*, *y*, and *e* that correlate with pigmentation have all been identified (9, 13, 15). However, different species, pigment patterns, and candidate genes were examined in each case. It is unclear whether all of these genes contribute to pigmentation evolution in all lineages.

Here, we have examined two closely related species within the *Drosophila virilis* group to determine whether pigmentation differences between these species involve the same or different genes as pigmentation changes in other lineages. *Drosophila americana* and *Drosophila novamexicana* are interfertile sister species that shared a common ancestor with *D. virilis* ≈4 million years ago (16). Despite the relatively short divergence time between these species, dramatic differences in pigmentation have evolved: *D. americana* is heavily melanized, whereas *D. novamexicana* is mostly yellow with little melanization. Neither of these species has been developed as model organism; thus, very few mutant stocks or DNA sequences are available. However, *D. virilis*, their closest relative, is perhaps the most studied *Drosophila* species other than *D. melanogaster*, and genetic information available for *D. virilis* can be used to facilitate genetic analysis of *D. americana* and *D. novamexicana*.

Interspecific genetic analysis was used to test for associations between specific candidate genes and pigmentation, as well as to examine the genetic architecture underlying pigmentation divergence. We used 23 species-specific molecular markers, including 5 in previously identified pigmentation genes, to test for associations with pigmentation, and found at least 4 predicted quantitative trait loci (QTL). Surprisingly, only one of the markers in a candidate gene (*ebony*) showed an association with pigmentation. We found that abdominal expression of the Ebony protein is higher in *D. novamexicana* than in the darker *D. americana*, consistent with its ability to inhibit melanization (13). The absence of an association between pigmentation and other candidate genes that have been implicated in pigmentation divergence among other *Drosophila* species (9, 13–15) shows that similar pigmentation patterns have evolved through different genetic mechanisms in different lineages.

Materials and Methods

***Drosophila* Strains, Rearing, and Crosses.** Wild-type strains of *D. novamexicana* and *D. americana* (stock numbers 15010-1031.0, isolated in Colorado, and 15010-0951.0, isolated in Indiana,

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Abbreviations: QTL, quantitative trait locus; LOD, log odds ratio.

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Table 1. Molecular markers

Marker name	Cytological location			GenBank accession no.		
	vir	*amer*	*nova*	*vir*	*amer*	*nova*
yellow (y)	1D	1C	1C	AY128944	AY165536	AY165561
period (per)	3A2	3A	3A	L81321	AY165530	AY165555
optomotor blind (omb)	8B	8B	8B	Unpubl.	AY165529	AY165560
yolk protein 1 (yp1)	15A	18B	18B	U52124	AY165537	AY165562
ebony (e)	20B	20	21	Unpubl.	AY165522	AY165547
hunchback (hb)	21C	21	20	X15359	AY165524	AY165549
Actin E2 (ActE2)	22D	22	22	AF358263	AY165516	AY165541
pros28.1b	27	24	27	AF017650	AY165532	AY165557
Kunitz inhibitor like protein 1 (Kil1)	31C	31C	31C	AJ249250	AY165526	AY165551
Cdc37	NA	NA	NA	L37055	AY165519	AY165544
heat shock protein 83 (hsp83)	33E	33E	33E	XD3813	AY165523	AY165548
knirps (kni)	NA	NA	NA	L36177	AY165525	AY165550
bric-a-brac (bab)	NA	NA	NA	Unpubl.	AY165518	AY165543
Rhodopsin 4 (Rh4)	39C	39C	36B	M77281	AY165533	AY165558
maverick (mav)	43B	43	43	U93213	AY165528	AY165553
Osvaldo-like element (OLE)	NA	NA	NA	AJ133521*	AY165535	AY165555
dopa decarboxylase (ddc)	NA	NA	NA	AF293749	AY165520	AY165545
RpL31	47	47	45	AF006573	AY165534	AY165559
Alcohol dehydrogenase (Adh)	49	49	49	AB033640	AY165517	AY165542
Actin C2 (ActC2)	50	50	51	AF358264	AY165514	AY165539
Early gland protein 1 (Egp1)	55B	57	55	M92914	AY165521	AY165546
mastermind (mam)	59	59	59	Z49942	AY165527	AY165552
Aats-tyr (aats)	72	72	72	AF096709	AY165513	AY165538

Estimated cytological locations in *D. americana* and *D. novamexicana* were determined using the location of the marker in *D. virilis* (26) and the inversion maps in refs. 16 and 21. NA, Not available. *D. virilis* sequences for the *e*, *omb*, and *bab* genes were provided by J. True, J. Brisson, and N. Gompel, respectively.

*The *OLE* sequence (amplified using primers designed based on the *D. virilis sina* sequence)is most closely related to an Osvaldo retrotransposon from *D. buzzatii*.

respectively) were obtained from the National *Drosophila* Species Stock Center (Bowling Green, OH). Flies were raised and crossed on standard cornmeal/molasses media at 25°C. Throughout this work, the female is always designated first in crosses, and the mother's genotype is listed first in hybrids. To generate the 3,117 backcross progeny scored, 150 *D. americana*/*D. novamexicana* hybrid females, and 250 *D. novamexicana*/*D. americana* hybrid females were mated to *D. novamexicana* males, with 5 virgin females and 5 males per vial.

Imaging Adult Flies. Flies were killed by exposure to ethyl acetate vapor, mounted, and imaged immediately. Abdominal cuticles were dissected, mounted, and imaged as described in (13).

Scoring Abdominal Pigmentation Phenotypes. Melanization gradually increases during the first few days after eclosion (data not shown); thus, flies were aged 5–10 days after eclosion before scoring. Independent observations by P.J.W. and B.L.W. identified five distinct phenotypic classes in the backcross population. All flies were scored over 2 consecutive days by using the same microscope and light conditions.

Developing Molecular Markers. Primers were designed based on available *D. virilis* sequences to amplify orthologous sequences from *D. americana* and *D. novamexicana* genomic DNA in a PCR. PCR fragments were purified from an agarose gel and sequenced by using Big Dye sequencing kit (V.3.0, Perkin–Elmer). Sequences were aligned by using Sequencher, and *D. americana* allele-specific PCR primers were designed with the 3′ end at polymorphic sites. PCR conditions were optimized such that a product was observed in a reaction with *D. americana* but not *D. novamexicana* genomic DNA. For the *omb* marker, a 36-bp size difference of the PCR product was used for genotyping. GenBank accession numbers are shown in Table 1. Primer sequences and PCR annealing temperatures are provided in Table 2, which is published as supporting information on the PNAS web site, www.pnas.org.

Selective Genotyping of Backcross Progeny. Genomic DNA was extracted from 48 (24 each from A/N and N/A F_1 mothers) of the lightest and 47 (23 from A/N and 24 from N/A F_1 mothers) of the darkest males from the backcross population of 3,117 flies, following the method of (17). Allele-specific PCR using 50 ng of genomic DNA as template was used to detect the presence of the *D. americana* allele. Primers that amplify a region of *ddc* in both species were used as positive control. In the few cases where these primers interfered with allele-specific amplifications, the allele-specific reactions were replicated at least twice without a positive control. Complete genotypes of each individual are provided in Table 3, which is published as supporting information on the PNAS web site.

Statistical Analyses. A sample size of 48 affords 90% power to distinguish between allele frequencies that differ by at least 30% at the 5% significance level ($\alpha = 0.05$). Given the neutral expectation that an allele is inherited with a probability of 0.5, alleles present at <20% or >80% in a phenotypic class were considered significant. Thus, markers within 20 cM of a QTL should produce a significant association.

χ^2 goodness of fit tests and tests for independence were used to test for interactions between pigmentation class, F_1 mother, sex, and marker genotype. To test for segregation distortion, genotypes from both phenotypic classes were pooled and compared with the expected 1:1 *D. americana*:*D. novamexicana* ratio by using a χ^2 goodness of fit test. Only *Rh4* deviated significantly from neutral expectations ($n = 97$, $\chi^2 = 25$, df = 1, $P < 0.05$).

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This marker was underrepresented in the light class but segregated freely in the dark class, suggesting that the indication of segregation distortion is a product of the selective genotyping. The χ^2 test of independence was used to identify interactions among pairs of markers. The genotypes from both the light and dark populations were combined for this analysis, and the significance of the 253 pairwise tests is provided in Table 4, which is published as supporting information on the PNAS web site.

To test for linkage between a marker and a QTL, we calculated the log odds ratio (LOD), which compares the probability of observing the data given that the marker is linked to a QTL to the probability of observing the data if it is not. The formula $\text{LOD} = \log[\{[(1 - r)/2]^x(r/2)^y\}/(0.25)^{(x+y)}]$, where r = recombination distance, x = number of nonrecombinants, and y = number of recombinants was used. One allele at each marker was always derived from *D. novamexicana*; thus, only the second allele was considered. Light and dark classes were analyzed separately, assuming that the QTL allele was from either *D. americana* or *D. novamexicana*. Thus, four sets of LOD scores were calculated. For example, to test for an association between a *D. americana* marker allele and a *D. americana* QTL allele that contributes to dark pigmentation, *D. americana* alleles present in the dark population were counted as nonrecombinants (x), and *D. novamexicana* alleles were assumed to be recombinants (y). To test for a *D. novamexicana* QTL allele that contributed to dark pigmentation, these values were reversed with the *D. novamexicana* alleles now counted as nonrecombinants. Similar calculations were used to investigate linkage to "light" pigmentation QTLs. The value of r that makes the observed data most likely (i.e., results in the largest LOD score) was determined by differentiating the LOD equation (maximum likelihood estimate for recombination distance $= y/(x + y)$. Only recombination distances between 0 and 0.5 with LOD > 2.7 ($\alpha = 0.05$, after a Bonferroni correction for 23 tests) were considered significant.

Immunohistochemistry. Detection of the Ebony and Yellow proteins in pharate adult flies with specific polyclonal antibodies was performed as described in ref. 13 by using a preabsorbed, biotin-conjugated secondary antibody followed by streptavidin conjugated to FITC (The Jackson Laboratory). Primary antibody was omitted to determine background florescence caused by the secondary and tertiary antibodies. Z-series images were collected on a Bio-Rad MRC 1024 confocal microscope and merged into single images by using the associated software.

Results and Discussion

Interspecific Differences in Pigmentation Are Polygenic. *D. americana* and *D. novamexicana* are closely related species with significant differences in pigmentation. The dorsal thorax and abdomen of *D. americana* is heavily melanized, whereas the same structures in *D. novamexicana* have little melanization (Fig. 1 *a* and *b*). *D. novamexicana* also lacks the melanization along the dorsal midline of the abdomen that is present in *D. americana*. Furthermore, the general cuticle color of *D. novamexicana* has more yellow pigment than *D. americana.* Cuticular pigmentation is identical in males and females of both of these species (data not shown).

Hybrids between *D. americana* and *D. novamexicana* displayed pigmentation characteristics of both species (Fig. 1 *c–e*): dark melanization of the thorax and abdomen, characteristic of *D. americana*, as well as a yellowish overall cuticle color and lack of melanization along the dorsal midline of the abdomen, seen in *D. novamexicana*. Pigmentation of female hybrids from both directions of cross was indistinguishable (Fig. 1*d*, data not shown), indicating that there is no apparent maternal effect. Abdominal pigmentation of males from reciprocal crosses differed however, with pigmentation resembling the female parent (in Fig. 1, compare *a* with *c*, and *b* with *e*). These males carry different X chromosomes,

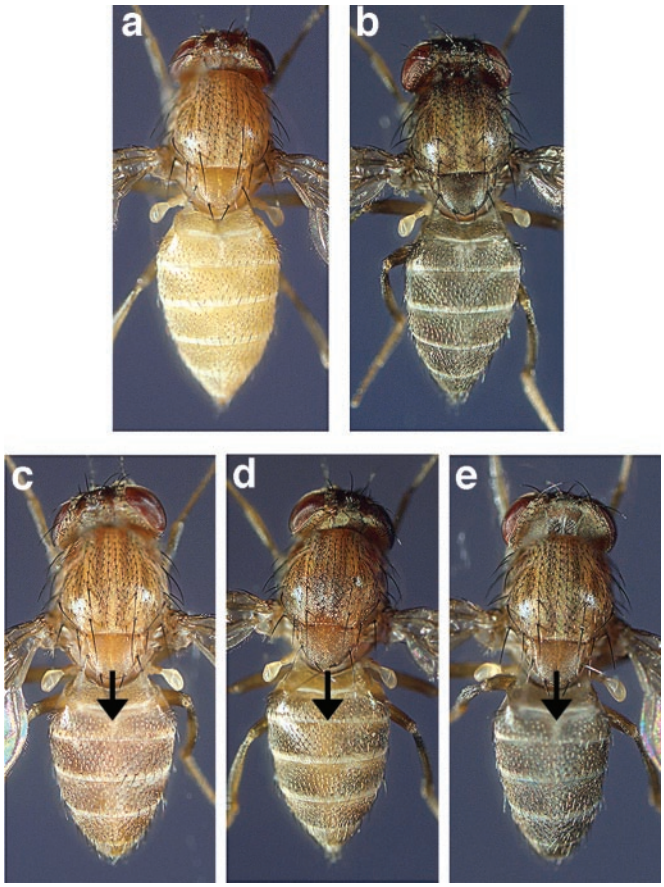

Fig. 1. Pigmentation of *D. novamexicana*, *D. americana*, and hybrid flies. (*a*) Males and females of *D. novamexicana* have a yellowish cuticle color with little melanization (female shown). (*b*) In contrast, both sexes of *D. americana* are heavily melanized and produce little yellow pigment (female shown). (*c*) Hybrid males from a *D. novamexicana* mother have an increase in body melanization characteristic of the *D. americana* parent but lack melanization along the abdominal dorsal midline (arrow), similar to *D. novamexicana*. (*d*) Hybrid females from either direction of cross have identical phenotypes (hybrid from a *D. novamexicana* mother shown), with more body melanization and slightly less yellow pigment than males from *D. novamexicana* mothers. (*e*) Hybrid males from *D. americana* mothers have nearly as much body melanization as *D. americana*, with only slightly more yellow pigment. A reduction of dorsal midline melanization is still observed in these males, but the decrease in yellow pigment reduces the contrast between melanized and nonmelanized cuticle, making it hard to discern in photographs (*e*, arrow). Flies in *c–e* each carry a different complement of X chromosomes: the fly in *c* has a *D. novamexicana* X chromosome; the fly in *d* has both a *D. novamexicana* and a *D. americana* X chromosome; and the fly in *e* has a *D. americana* X chromosome. All flies are the same magnification (×13.5).

suggesting that at least one gene on this chromosome affects abdominal pigmentation. One or more autosomal genes also contribute to pigmentation differences, because hybrid males carrying the *D. novamexicana* X chromosome are darker than *D. novamexicana* (in Fig. 1, compare *c* with *a*).

To estimate the number of genes underlying pigmentation differences, we first examined the phenotypic distribution of backcross progeny from female hybrids and *D. novamexicana* males. Pigmentation of backcross progeny was not continuously distributed, but rather five discrete phenotypes were recognized. Each of these phenotypes was assigned a score from "1" (lightest) to "5" (darkest), reflecting the intensity of abdominal pigmentation (Fig. 2). We scored 1,288 progeny (670 females, 619 males) from hybrid females with a *D. americana* mother, and 1,829 progeny (952 females, 877 males) from hybrid females with a *D. novamexicana* mother. The frequency of each phenotype is shown in Fig. 2*f*. The distribution of phenotypes is inconsistent

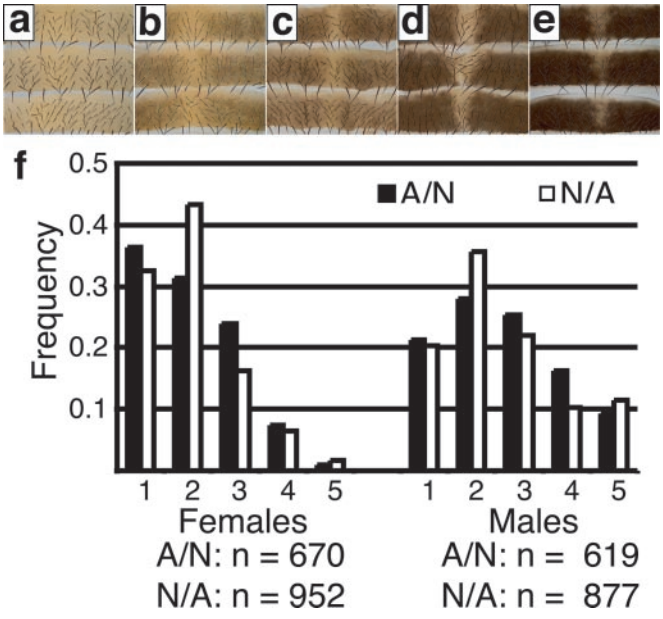

Fig. 2. The five classes of backcross progeny. Abdominal pigmentation phenotypes among progeny from a backcross between hybrid females and *D. novamexicana* males fell into five phenotypic classes, scored 1–5 (*a*–*e*), respectively. An inverse correlation between yellow and black pigments was observed among these flies. (*f*) The phenotypic distribution of backcross progeny is shown separated by both sex (male and female) and F_1 mother (A/N and N/A). Progeny from *D. americana* mothers (A/N) are shown with black bars; those from *D. novamexicana* mothers (N/A) are shown with white bars. The number of flies (*n*) scored from each backcross are also shown. χ^2 tests of independence (df = 4) indicated that both sex and F_1 mother affected the distribution of abdominal pigmentation intensity ($\chi^2 = 209.5$, $P < 10^{-43}$; $\chi^2 = 44.3$, $P < 10^{-8}$, respectively). The significance of the interaction between F_1 mother and abdominal pigmentation, however, may be caused by scoring error rather than biology, because no significant effect of F_1 mother was detected in the genotyped sample set.

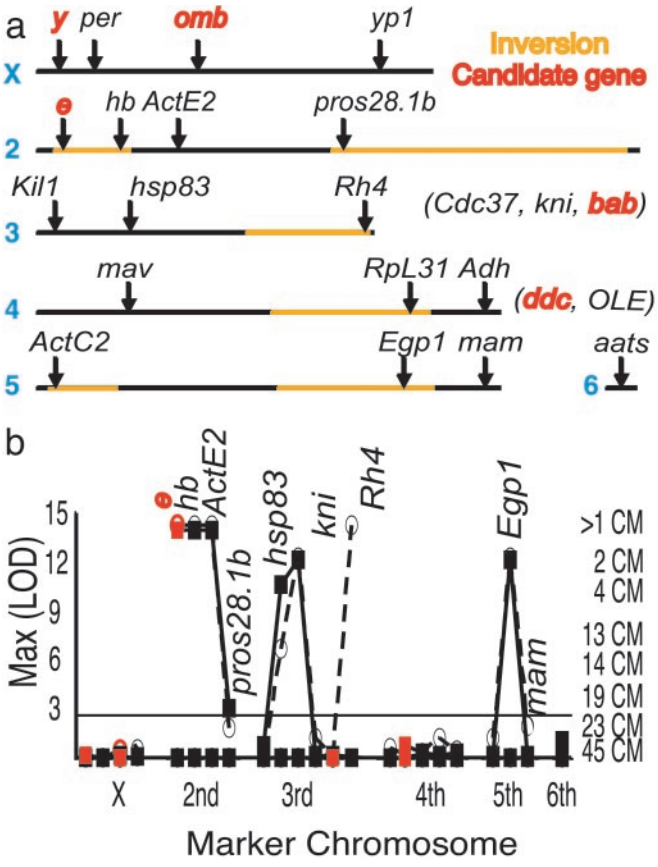

Fig. 3. Genomic distribution of candidate genes and molecular markers. (*a*) Approximate cytological locations of molecular markers in *D. americana* (indicated by arrows) are shown relative to each cytological inversion (shown in orange). Markers in parentheses are located on that chromosome, but the precise cytological location is unknown. Candidate genes are shown in red; chromosome numbers are shown in blue. Note that the *e* marker is located within an inversion. (*b*) Dotted lines show tests for linkage between each marker and a *D. novamexicana* allele that contributes to either light (circle) or dark (square) pigmentation. Similarly, solid lines show tests for linkage between markers and *D. americana* alleles that serve as light (circle) or dark (square) QTLs. LOD scores for candidate genes are shown in red, and the markers with names shown had a significant association in the χ^2 analysis. Maximum LOD scores are shown on the left and the corresponding recombination distances are shown on the right in centimorgans (CM). LOD scores >2.7 (indicated with line) are significant ($\alpha = 0.05$ after Bonferroni correction for 23 tests). Note that all ''dark'' QTL alleles are linked to *D. americana* marker alleles, and all ''light'' QTL are linked to *D. novamexicana* alleles. The seven markers that deviated significantly from neutral expectations in both populations (*e, hb, ActE2, pros28.1b, hsp83, kni,* and *Egp1*) show evidence of linkage to both a ''dark'' *D. americana* QTL allele and a ''light'' *D. novamexicana* QTL allele. *Rh4* and *mam* alleles were associated with light, but not dark, pigmentation. *Rh4* shows linkage to a ''light'' *D. novamexicana* QTL allele but not to any ''dark'' QTL. Also note that *e* is the only candidate gene with significant linkage to a QTL.

with either an extremely polygenic trait or a one- or two-gene model, suggesting that a moderate number of genes contribute to this trait.

At Least Four Genomic Regions Contribute to Pigmentation Differences. To identify the genomic regions contributing to pigmentation differences, we developed molecular markers that distinguish between *D. americana* and *D. novamexicana* alleles at 23 loci, and genotyped the lightest 1.5% and darkest 1.5% of males from the backcross population (18). Five of the markers were located within genes previously implicated in the development and evolution of abdominal pigmentation [*y* (13, 15, 19); *e* (13, 20); *ddc* (4, 14); *omb* (10); and *bab* (9)], and the remaining 18 molecular markers were spaced throughout the genome (Fig. 3*a*). A list of these markers and their estimated cytological locations is shown in Table 1. Backcross progeny were either homozygous for *D. novamexicana* alleles or heterozygous for *D. americana* and *D. novamexicana* alleles at each locus. We scored for the *D. americana* allele and inferred a homozygous *D. novamexicana* genotype in its absence. Complete genotypes of each individual are provided in Table 3. The direction of the cross from which the hybrid mother was derived did not affect the inheritance at any of the markers (data not shown); thus, data from both F_1 mother genotypes was combined for all subsequent analyses.

Intrachromosomal rearrangements exist between *D. americana* and *D. novamexicana* that affect recombination and compromise the mapping power of this study. An inversion is present on every chromosome except the X and the very small sixth chromosome, with two nonoverlapping inversions on the second and fifth chromosomes (Fig. 3*a*; refs. 16 and 21). In all, ≈30% of the genome is inverted. Recombination is suppressed within inverted regions, but the presence of an inversion on one chromosome can increase the recombination rate on other chromosomes (22). As a result, the frequency of recombination varied unpredictably across the hybrid genome (see *Materials and Methods*).

Assuming no segregation distortion, the *D. americana* allele of a marker locus should be observed at a frequency of 50% in both the light and dark classes if the marker is not linked to a QTL.

If, however, the marker is linked to a QTL, its frequency should deviate from 50% in at least one of the two phenotypic classes. Fourteen markers showed no significant deviation from 50%, with *D. americana* and *D. novamexicana* alleles inherited equally in both phenotypic classes. These markers, which include four candidate loci (*yellow*, *ddc*, *omb*, and *bab*), are therefore not linked to a pigmentation QTL.

Of the remaining nine markers, the *D. americana* allele of eight (*e*, *hb*, *ActE2*, *pros28.1b*, *hsp83*, *kni*, and *Egp1*) was significantly over-represented in the dark class and under-represented in the light class (see Fig. 5, which is published as supporting information on the PNAS web site). However, the cosegregation of four of these markers (*e*, *hb*, *ActE2,* and *pros 28.1b*) can be explained by interactions among loci that are independent of pigmentation (e.g., physical linkage, see Table 4); thus, all may be linked to the same QTL. Two other markers (*hsp83* and *kni*) are believed to be adjacent and may also be linked to a single QTL. Finally, the segregation of the *Egp1* marker suggested linkage to a QTL but not to any other marker. Therefore, these seven markers indicate as few as three pigmentation QTLs. The remaining two markers, *Rh4* and *mam*, deviated significantly from the expected allele frequency only in the light class (Fig. 5), suggesting linkage to QTLs with nonadditive effects on abdominal pigmentation. The significance of the association with *mam* ($P = 0.0015$) was slightly lower than the significance threshold ($P = 0.002$), but neither the interaction between *mam* and other pigmentation associated markers (Table 4), nor the likelihood of linkage to a QTL was significant (see Fig. 3*b*). Thus, we did not consider the association with *mam* significant, and any QTL that may be linked to it was not included in our estimate of gene number. Because linkage was not always observed between adjacent loci, the markers used in this study do not provide complete genome coverage, and our estimate of four QTLs is a minimum. In addition, neither the relative contribution of each QTL nor the proportion of the phenotypic differences explained by the four QTLs can be estimated from these data because of the selective genotyping approach used.

To estimate the most likely distance between each marker and a putative pigmentation QTL, the recombination distance (r) that maximizes the LOD score was determined. QTLs may be either *D. americana* or *D. novamexicana* alleles that either increase or decrease melanization. Maximum LOD scores and the corresponding r values for each potential type of QTL allele are summarized in Fig. 3*b*. All markers, except *mam*, that showed a significant association with pigmentation by χ^2 analysis also showed significant linkage to a QTL. All "dark" QTL alleles detected came from *D. americana*, and all "light" alleles came from *D. novamexicana*. Significant linkage was detected if the QTL was within ≈20 cM of a marker, consistent with our *a priori* estimates of statistical power.

Differences in the Expression of Ebony, but Not Yellow, Correlate with Pigmentation. The only candidate gene marker with significant linkage to a QTL was *ebony* (Fig. 3*b*). This marker was estimated to be 0 cM from a QTL; however, it lies within an inversion, and nearly 15% of the chromosome is completely linked to *ebony*. This inversion prevents the use of fine-scale mapping to distinguish the effects of *ebony* from surrounding loci. To investigate further this association, we compared the expression pattern of the Ebony protein between *D. americana* and *D. novamexicana*. Ebony was clearly expressed in the developing pupal abdomen at a higher relative level in *D. novamexicana* than in *D. americana* and at an intermediate level in hybrids between the two (Fig. 4 *a–c*). Differences in protein expression may reflect differences in transcription or posttranscriptional processing (e.g., mRNA processing, translation, protein stability). These differences may be caused by DNA changes in *ebony* or other genes. The genetic association between *ebony* and pigmentation suggests that the protein expression difference is due, at least in part, to evolution at the *ebony* locus.

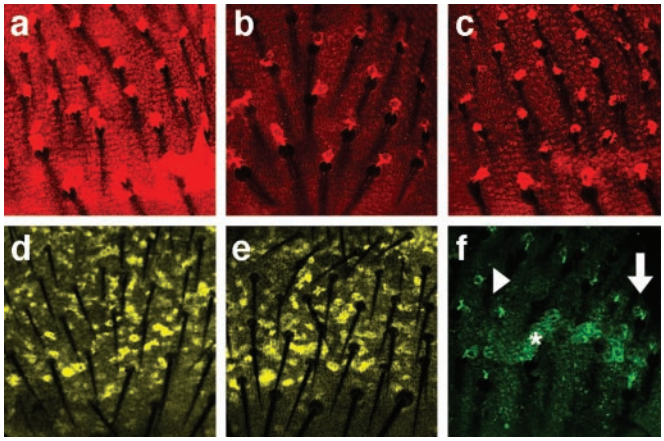

Fig. 4. Changes in Ebony expression correlate with pigmentation differences. Ebony protein (shown in red) is expressed at a higher relative level in the abdominal tergite of *D. novamexicana* (*a*) than *D. americana* (*b*), with an intermediate level observed in hybrids (*c*). This expression positively correlates with yellow pigment and inversely correlates with melanization (compare with Fig. 1). In contrast, expression of the Yellow protein (shown in yellow) is similar between *D. novamexicana* (*d*) and *D. americana* (*e*), despite the differences in melanization. (*f*) No fluorescent signal was observed in epidermal cells (arrowhead) in the absence of the Ebony or Yellow antibodies. There was, however, some background fluorescence (shown in green) within a bristle-associated cell (arrow) and in cells located underneath the epidermis (asterisk). Because the signal from immunolocalizations is nonlinear and difficult to standardize between species, we can make only a qualitative statement about relative levels of antigen and not an absolute quantitative measurement of Ebony or Yellow protein levels. In all panels, a section of pharate adult dorsal abdominal cuticle from either the A3 or A4 segment is shown with anterior at the top.

ebony encodes the enzyme *N*-β-alanyldopamine (NBAD) synthetase that converts dopamine to NBAD, which is subsequently oxidized to produce a yellowish pigment (4, 23). In *D. melanogaster*, ectopic expression of the Ebony protein increases production of this yellow pigment while reducing the amount of black melanin formed (13). Therefore, if interspecific differences in Ebony expression affect melanization, differences in the amount of yellow pigment should also be observed. Consistent with this hypothesis, there is an inverse correlation between the yellowness of the overall cuticle color and intensity of abdominal melanization among backcross progeny (Fig. 2 *a–e*).

Ectopic expression of the Yellow protein can counteract the effects of ectopic Ebony expression (i.e., inducing black melanin at the expense of yellow pigment; ref. 13). Furthermore, evolutionary changes in Yellow expression that correlate with divergent melanin patterns have been identified in other *Drosophila* species (13, 15). However, no association between the *yellow* locus and pigmentation was observed in this study. It is formally possible that other QTLs could affect pigmentation by altering Yellow protein expression. To test this possibility, we examined *yellow* expression in both species and found, to our surprise, that Yellow expression was comparable between *D. americana* and *D. novamexicana* (Fig. 4 *d* and *e*), revealing that contrary to the situation in other species (15), differences in melanization have evolved without changes in Yellow expression.

Different Genetic Mechanisms Underlying Convergent Phenotypes. Studying the evolution of pigmentation provides the opportunity to shed light upon long-standing evolutionary puzzles such as mimicry and industrial melanism. Pigmentation, melanism in particular, plays diverse roles in crypsis, predator avoidance,

thermoregulation, and mate choice. The rapid increase in the frequency of melanic forms of moth species in industrialized regions of the United Kingdom remains one of the most clear examples of adaptive evolution. Nearly half of all moth species in this region have evolved melanic forms; each is believed to have arisen independently (1). Genetic analysis of these species suggests that melanism is often due to a few loci, including single genes of large effect (1), but none of these genes has been identified, and it remains unknown whether the same loci are involved in all lineages. Because many of the basic mechanisms of pigmentation are believed to be conserved among all insects (4, 24), identifying the genetic changes underlying pigmentation divergence within the *Drosophila* lineage may provide critical insights to the evolution pigment patterns in other species.

In a previous study, we showed that divergent *yellow* expression patterns, due in part to changes in a *yellow cis*-regulatory region, correlate with differences in abdominal pigmentation among three long-diverged species. Here, we found that neither *yellow* nor three other candidate genes contribute to differences in abdominal pigmentation between the more closely related *D. novamexicana* and *D. americana*. Instead, we found that the *ebony* gene and at least three other unidentified QTLs were associated with pigmentation differences between these species. These results suggest that abdominal melanization can evolve by means of at least two distinct mechanisms, one involving changes in Yellow expression and the other affecting the distribution of the Ebony protein. In addition, the presence of at least three additional QTLs suggests that there are additional mechanisms to be uncovered.

Our findings indicate that there is no *a priori* prediction one can make about which candidate genes contribute to melanic pattern evolution in a specific *Drosophila* lineage. It follows, then, that diversity of wing pigment patterns among Hawaiian *Drosophila* (14) or in mimicry rings of various butterfly species (25) may have evolved through a variety of genetic mechanisms. Similarly, the convergent melanic phenotypes of moth species may also be caused by different genetic changes. The approach used here to test for associations between specific candidate genes and pigmentation could also be used to investigate these classic models of phenotypic evolution.

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