of the *cis*-regulatory mutation may only be noticed in environmentally- or developmentally-specific environments, when a sufficient amount of the relevant transcription factor is active.

SUPPLEMENTAL TABLES

Table S1. Traits used to mark developmental stages P8 - A2. Based on Bainbridge and Bownes (1981). To avoid circularity, the color of the abdominal cuticle was not considered as a developmental marker. Eye, wing and bristle pigmentation - which develop separately from cuticle pigmentation - were used.

Stage	Developmental marker(s)
P8	Eyes become bright yellow.
P9	Eyes darken to deep amber.
P10	Eyes become bright red.
P11	Head bristles, followed by thoracic bristles, darken.
P12	Wings become grey.
P13	Wings blacken; tarsal bristles darken.
P14	Abdominal bristles reach mature length and darkness; green patch of meconium appears in the abdomen.
P15	Meconium has migrated to the posterior tip of the

	abdomen; legs twitch when pupal cover is removed.
A1	Flies newly eclosed from pupae; wings are still compressed.
A2	Wings fully expanded.

Table S2. Significance tests for differential expression of *D. americana* versus *D. novamexicana* alleles of *tan* and *ebony*. Results were obtained from PROC MIXED models in SAS, as described in the Methods. Parental data were obtained using the "indirect" method (Fig. S1A) for stages P12 and P13, and the "direct" method (Fig. S1B) for stages P14 - A1. $P<0.0025$ (Bonferroni-corrected alpha = 0.05 significance level for 20 comparisons) indicates that the *D. americana* and *D. novamexicana* alleles are expressed at different levels; that is, that the $\log_2$ of their ratio is not equal to zero.

	Stage	Sample	LSM	*t*	*P*
tan	P12	Parents	-0.188	-0.97	0.3388
		F1	0.093	1.26	0.2294
	P13	Parents	0.447	2.32	0.0273
		F1	0.187	2.52	0.0243
	P14	Parents	0.835	5.303	<0.0001
		F1	0.367	4.290	0.0007
	P15	Parents	0.949	6.029	<0.0001
		F1	0.214	2.883	0.0120
	A1	Parents	0.018	0.158	0.8766
		F1	0.042	0.560	0.5845
ebony	P12	Parents	-1.079	-4.26	<0.001
		F1	-0.830	-8.38	<0.0001

P13	Parents	-0.488	-1.81	0.0743
	F1	-0.781	-7.88	<0.0001
P14	Parents	-1.126	-4.71	<0.0001
	F1	-1.142	-9.99	<0.0001
P15	Parents	-1.684	-7.05	<0.0001
	F1	-1.047	-10.57	<0.0001
A1	Parents	-1.585	-9.38	<0.0001
	F1	-0.901	-8.78	0.0004

Table S3. Primers and sequences used for pyrosequencing estimates of mRNA expression. All sequences are listed 5′ to 3′. Abbreviations: F, Forward; R, Reverse; *Da*, *Drosophila americana*; *Dn*, *Drosophila novamexicana*. *, biotinylated marker. Polymorphic sites used to differentiate between alleles are underlined. Dispensation order for each sequence includes bases (indicated with lowercase letters) before and after those corresponding to the polymorphic site (underlined) that are expected to yield no signal, as a negative control.

		tan	***ebony***
PCR primers	**F** **R**	*GAAATGGCACCGAATCCG GCGGTACCTCCTTGATGCTC	AGCCCGAGGTGGACATCA *GTATGGGTCCCTCGCAGAA
Pyrosequencing primers		TGACTCGTTCTTCCG (R)	CGAGGTGGACATCAAGT (F)
Analyzed sequence	***Da*** ***Dn***	G**T**CCGGATTC G**G**CCGGATTC	C**C**AAGCTGCT C**G**AAGCTTCT
Dispensation order		a**GT**aCGATC	t**CG**cAGCTGCT

SUPPLEMENTAL FIGURE

Figure S1. Direct measurements confirm indirect estimates of species expression differences, but provide more precise estimates. For both *tan* (A) and *ebony* (B), direct estimates (dark blue) indicated a slightly greater expression difference between species than the data obtained from indirect estimation (light blue).

SUPPLEMENTAL METHODS

Pyrosequencing measures the relative abundance of two alleles, differentiated by a SNP. Each pyrosequencing data point is an estimate of how many of each of the two alternate nucleotides can be incorporated at the end of a growing DNA sequence. Homopolymers (repeated nucleotides) in the analyzed sequence lead to the addition of multiple identical nucleotides onto a single strand, yielding a pyrosequencing output that is summed over multiple nucleotide positions rather than just the single polymorphic site.

Both *tan* and *ebony* contained a homopolymer at the polymorphic site used for expression analysis. At *ebony*, the *D. americana* allele is a C**C** homopolymer, while the *D. novamexicana* allele is C**G** (the analyzed site is the second position, consisting of a **C/G** polymorphism). At *tan*, the *D. americana* allele is G**T** while the *D. novamexicana* allele is a G**G** homopolymer (the analyzed site is **T/G**). We were therefore unable to calculate a simple ratio of *D. americana* to *D. novamexicana* alleles within each sample, but instead used the following formulas to determine the correct ratio of alleles at the polymorphic site, excluding information from the neighboring position:

$$\frac{a}{n} = \frac{C - G}{2 \bullet G}$$ allele abundance ratios for *ebony*

$$\frac{a}{n} = \frac{2 \bullet T}{G - T}$$ allele abundance ratios for *tan*

The intuitive reasoning behind the equations may be best understood by imagining an F_1 hybrid, with one copy of the *D. americana tan* allele (G_A T…) and one copy of the *D. novamexicana* allele (G_N G_N …). The abundance of the *D. americana* G, denoted G_A, at site 1 should be equal to the abundance of the *D. americana* T at site 2. Total *D. americana* abundance for sites 1-2 is thus G_A+T or 2*T. The total abundance of the *D. novamexicana* allele for sites 1-2 is the total quantity of G detected (G), minus the amount of G that pertains to *D. americana* (G_A, which is the same quantity as T). This value of G – G_A can be re-written as G – T. The ratio of *D. americana* / *D. novamexicana* is therefore 2*T / (G – T). Similar logic applies to equation 1, but with the homopolymer occurring in *D. americana*.

Using these formulas, we determined the ratio of *D. americana* / *D. novamexicana* allele abundance ratios for both cDNA and genomic DNA prepared from each biological replicate. The mean of three cDNA technical replicates (per biological replicate) was divided by the mean of the two corresponding genomic DNA technical replicates, in order to correct for species differences in body size, extraction efficiency, and/or PCR amplification efficiency (Landry et al. 2005). This value represents the corrected allelic ratio for a single parental or F_1 biological replicate, and reflects mRNA expression of the two alleles present in each sample. In order to more clearly depict the range of values, we $\log_2$-transformed each biological replicate. Figures 4 and 5 show the mean of four biological replicates per developmental time point.