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Genetic Basis of Sex-Specific Color Pattern Variation in *Drosophila malerkotliana*

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ABSTRACT

Pigmentation is a rapidly evolving trait that can play important roles in mimicry, sexual selection, thermoregulation, and other adaptive processes in many groups of animals. In Drosophila, pigmentation can differ dramatically among closely related taxa, presenting a good opportunity to dissect the genetic changes underlying species divergence. In this report, we investigate the genetic basis of color pattern variation between two allopatric subspecies of *Drosophila malerkotliana*, a widespread member of the *ananassae* species subgroup. In *D. malerkotliana malerkotliana*, the last three abdominal segments are darkly pigmented in males but not in females, while in *D. malerkotliana pallens* both sexes lack dark pigmentation. Composite interval mapping in F_2 hybrid progeny shows that this difference is largely controlled by three quantitative trait loci (QTL) located on the 2L chromosome arm, which is homologous to the 3R of *D. melanogaster* (Muller element E). Using highly recombinant introgression strains produced by repeated backcrossing and phenotypic selection, we show that these QTL do not correspond to any of the candidate genes known to be involved in pigment patterning and synthesis in Drosophila. These results, in combination with similar analyses in other Drosophila species, indicate that different genetic and molecular changes are responsible for the evolution of similar phenotypic traits in different lineages. This feature makes Drosophila color patterns a powerful model for investigating how the genetic basis of trait evolution is influenced by the intrinsic organization of regulatory pathways controlling the development of these traits.

THE genetic basis of species differences is one of the most intriguing questions in the study of evolution. Although the particular genes responsible for variation in morphology, physiology, and behavior are necessarily different for different traits, some general questions go to the heart of evolutionary biology. One of these questions concerns the genetic basis of convergent evolution—Are the same genes responsible for similar phenotypic changes in different lineages, or can different genetic changes produce the same phenotypic outcome? To answer this question, we must seek out model traits that have undergone multiple independent transitions and whose genetic basis can be investigated in a reasonably large number of taxa. One of the most attractive models of this type is provided by the Drosophila cuticular pigmentation.

Pigmentation is a highly variable trait in many animals, including Drosophila. Differences between lightly and darkly pigmented species or morphs have evolved repeatedly in many Drosophila clades (Wittkopp *et al.* 2003a), providing a large number of independent phylogenetic contrasts in which the genetic basis of trait evolution can be studied. At the same time, the development of pigmentation in Drosophila is fairly well understood and offers important insights into the possible molecular mechanisms of evolutionary change. Cuticular pigments of Drosophila are based on catecholamine polymers (Wright 1987). Pigment precursors are secreted by epidermal cells and incorporated into the overlying cuticle, so that pigmentation is essentially cell autonomous. Many of the enzymes responsible for pigment synthesis have been identified, including the products of the *pale*, *Ddc*, *yellow*, *ebony*, and *tan* loci (Wright 1987; Han *et al.* 2002; Wittkopp *et al.* 2002a,b, 2003a; True *et al.* 2005). These genes are transcribed and translated in the epidermis during the pupal stage (Kraminsky *et al.* 1980; Walter *et al.* 1991; Wittkopp *et al.* 2003a), and the spatial color pattern is largely determined by the differential expression of these genes in different body regions (Wittkopp *et al.* 2002a,b; Futahashi and Fujiwara 2005; Ninomiya *et al.* 2006). This expression is controlled in turn by several transcription factors including *optomotor-blind* (*omb*), *Abdominal-B* (*Abd-B*), *doublesex* (*dsx*), and *bric a brac* (*bab*) (Kopp and Duncan 1997; Kopp *et al.* 2000). The regulatory connections between the transcription factors and the enzymes that ultimately mediate their functions are not yet clear. *Abd-B* is known to regulate *yellow* expression directly (Jeong *et al.* 2006), but other direct transcriptional targets of *Abd-B*, *bab*, and *omb* remain unknown.

In many species of the *melanogaster* group, abdominal pigmentation is sexually dimorphic, with the last two or

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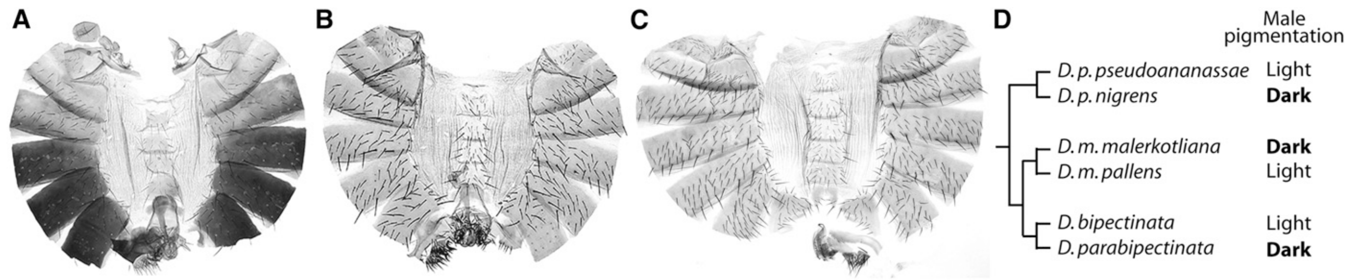

FIGURE 1.—Abdominal pigmentation in *D. malerkotliana*. Adult abdomens were cut along the dorsal midline and mounted flat so that ventral cuticle is in the middle, dorsal cuticle is on both sides, and posterior is down. (A) *D. m. malerkotliana* male. Note the dark pigmentation of segments 4, 5, and 6, and intermediate pigmentation of segment 3. (B) *D. m. pallens* male. Note complete absence of dark pigmentation. (C) Females of both subspecies lack dark abdominal pigmentation. (D) Phylogeny of the *bipectinata* species complex (KOPP and BARMINA 2005b), showing the distribution of male pigmentation phenotypes. Females of all species are pigmented identically.

three segments completely covered by dark melanin in males but not in females. This pattern is controlled by a regulatory circuit involving the *Abd-B*, *dsx*, and *bab* genes (KOPP *et al.* 2000). Male-specific abdominal pigmentation is a derived character state in the *melanogaster* species group, and the origin of this novel spatial pattern was caused, at least in part, by evolutionary changes in the transcriptional regulation of *bab* by *Abd-B* and *dsx* (KOPP *et al.* 2000; WILLIAMS *et al.* 2008). Although sex-specific pigmentation is fixed in the closest relatives of *D. melanogaster*, it is highly variable in the more basal *ananassae* species subgroup, where some species and subspecies have dark male abdominal pigmentation while others lack it entirely (KOPP and BARMINA 2005a) (M. MATSUDA, C. S. NG, M. DOI, Y. TOBARI and A. KOPP, unpublished results). Genetic analysis of these differences may offer key insights into the origin of sex-specific pigmentation.

Of particular interest is the *bipectinata* species complex, which includes four closely related species—*D. bipectinata*, *D. parabipectinata*, *D. malerkotliana*, and *D. pseudoananassae*—distributed throughout Southeast Asia (BOCK 1971; KOPP and BARMINA 2005a). In each of the latter two species, two allopatric subspecies are recognized: *D. malerkotliana malerkotliana* and *D. malerkotliana pallens* and *D. pseudoananassae pseudoananassae* and *D. pseudoananassae nigrens*. *D. malerkotliana malerkotliana*, *D. parabipectinata*, and *D. p. nigrens* show very dark male-specific abdominal pigmentation, while in *D. m. pallens*, *D. bipectinata*, and *D. p. pseudoananassae* males as well as females lack such pigmentation (Figure 1). Species of the *bipectinata* complex are very closely related, with noncoding sequence divergence of ~2% and F_{ST} of only 0.18–0.31 between species (KOPP and BARMINA 2005a; KOPP and FRANK 2005). Phylogenetic analysis indicates that the latest common ancestors of the *bipectinata* complex and of the *ananassae* subgroup as a whole were equally likely to have had sexually dimorphic or monomorphic pigmentation (M. MATSUDA, C. S. NG, M. DOI, Y. TOBARI and A. KOPP, unpublished results). Male-specific pigmentation may have been gained or lost repeatedly in this group; alternatively, its current taxonomic distribution may reflect a sorting of ancient polymorphism that was present in the common ancestor of the *bipectinata* complex. Importantly, all four species can be hybridized in the lab, making the *bipectinata* complex an excellent model for investigating the genetic basis of color pattern evolution.

We have carried out a quantitative genetic analysis of the difference in male-specific abdominal pigmentation between *D. m. malerkotliana* and *D. m. pallens*. We show that this difference is controlled predominantly by three quantitative trait loci (QTL) of large effect. Surprisingly, these QTL do not correspond to any of the candidate genes known to be involved in the development of Drosophila pigmentation. An important conclusion from this and other analyses is that different loci are responsible for the evolution of similar color patterns in different Drosophila species.

MATERIALS AND METHODS

Drosophila strains and crosses: Flies were maintained on standard cornmeal-agar-yeast media at room temperature. Parental strains used for genetic crosses were inbred by at least 12 generations of single-pair, full-sib matings. *D. m. malerkotliana* strain *mal0-isoC* was derived in this manner from the strain 14024-391.0 collected in Mysore, India, and obtained from the Tucson Drosophila species stock center. *D. m. pallens* strains *palQ120-isoG* and *palQ121-isoA* were derived from different isofemale strains collected on Palawan, Philippines, and kindly provided by Y. Fuyama and M. Matsuda. Polytene chromosome squashes showed that all three inbred strains were free of segregating chromosome inversions. A similar analysis of F_1 hybrids showed *mal0-isoC* and *palQ121-isoA* to be homosequential, while *mal0-isoC* and *palQ120-isoG* differ by a single inversion that covers ~20% of the 2L chromosome arm (Muller element E) (data not shown). F_2 hybrid progeny for QTL mapping were produced by crossing *mal0-isoC* females to either *palQ120-isoG* or *palQ121-isoA* males in mass cultures, and then mass mating F_1 females and males.

To construct introgression strains, 10–20 of the darkest F_2 hybrid males, at least 1 week old, were backcrossed to females of the appropriate *D. m. pallens* parental strain (*palQ120-isoG* or *palQ121-isoA*) in mass cultures. In the next generation, virgin females, which show no color variation, were crossed to *palQ120-isoG* or *palQ121-isoA* males. These crosses were re-

peated for at least 20 generations, alternating recombining (random hybrid females × *D. m. pallens* males) and phenotyping (darkest hybrid males × *D. m. pallens* females) generations (Figure 2). This procedure is expected to introduce increasingly small genomic regions containing the genes responsible for the dark male-specific pigmentation of *D. m. malerkotliana* into an otherwise *D. m. pallens* genetic background.

Pigmentation scores: Pigmentation of the three most posterior abdominal tergites (segments 5, 6, and 7) was recorded in at least 1000 F_2 hybrid males from each cross. Scoring was performed under a dissecting microscope in live 14-day-old flies in the *mal0-isoC* × *palQ120-isoG* cross, and in 4-day-old flies in the *mal0-isoC* × *palQ121-isoA* cross. A score ranging from 0 (unpigmented, identical to the *D. m. pallens* parent) to 10 (shiny black, identical to the *D. m. malerkotliana* parent) was assigned to each male on the basis of both the pigmented area and the intensity of pigmentation. Each fly was scored independently by two people. The two sets of scores were in strong agreement, and the average scores were used for QTL mapping.

In the *mal0-isoC* × *palQ120-isoG* cross, we followed a selective genotyping strategy by choosing 94 males with a "0" pigmentation score and 84 males with a "10" score. Compared to random genotyping, selective use of phenotypic extremes increases the power to detect QTL, although it is less useful for estimating their effects (Lynch and Walsh 1998). In the *mal0-isoC* × *palQ121-isoA* cross, 188 males were selected at random. The 94 lightest and 94 darkest remaining males were then added to the sample, for a total of 376 hybrid progeny. This approach allowed us to assemble a linkage map of *D. malerkotliana* and perform unbiased QTL mapping, while at the same time increasing our power to detect weak or moderate QTL.

Molecular markers and genotyping: To identify molecular polymorphisms distinguishing the parental strains, >50 nuclear gene regions were amplified from *mal0-isoC*, *palQ120-isoG*, and *palQ121-isoA* using primers based on the *D. ananassae* and *D. melanogaster* genome sequences. PCR fragments were gel extracted and sequenced directly using amplification primers. Sequence chromatograms were assembled and edited as needed using the FinchTV (Geospiza, Seattle) and SeqMan (DNAStar, Madison WI) software, and aligned using ClustalX (Thompson *et al.* 1997). The MapDraw program (DNAStar) was then used to predict allele-specific restriction sites that could be used to discriminate parental alleles in the hybrid progeny. Marker locations, primer sequences, genotyping conditions, and GenBank sequence accession numbers for the *mal0-isoC* × *palQ120-isoG* and *mal0-isoC* × *palQ121-isoA* crosses are shown in supplemental Tables 1 and 2, respectively.

Genomic DNA was extracted from each F_2 hybrid male after pigmentation scoring was complete. For genotyping, each marker region was amplified from each individual male, and the PCR fragments were digested with the appropriate restriction enzymes and separated on an agarose gel. The resulting restriction fragment length polymorphisms (RFLPs) allowed most hybrid individuals to be identified as *mal/mal* homozygotes, *pal/pal* homozygotes, or *mal/pal* heterozygotes at each of the marker loci.

QTL mapping: A linkage map was constructed using MapMaker software (Lander *et al.* 1987) on the basis of the genotypes of 188 random F_2 males at 41 loci in the cross between *mal0-isoC* and *palQ121-isoA*. Recombination rates and map distances between consecutive markers are shown in supplemental Table 3. QTL responsible for the differences in pigmentation between *D. m. malerkotliana* and *D. m. pallens* were mapped in the F_2 population comprising the 188 random progeny as well as the 94 lightest and 94 darkest males using composite interval mapping (CIM) implemented in the QTL Cartographer program (Wang *et al.* 2005). CIM tests whether an interval between two markers contains a QTL while simultaneously controlling for the effects of QTL located outside this interval (Zeng 1994). The likelihood ratio (LR) test statistic is $-2\ \ln(L_0/L_1)$, where L_0/L_1 is the ratio of likelihoods under the null hypothesis (there is no QTL in the test interval) and the alternative hypothesis (there is a QTL in the test interval). Model 6 of QTL Cartographer with five background markers was chosen by forward regression, with a window size of 10 cM. Significance thresholds that account for multiple testing and correlations among markers were determined by permutation tests (Churchill and Doerge 1994; Doerge and Churchill 1996). We permuted trait and marker data 1000 times and recorded the maximum LR statistic across all intervals for each permutation. LR statistics calculated from the original data were considered significant at the experimentwise 5% level if they exceeded the 50th highest LR statistic from the permuted data.

Epistatic interaction analysis: Epistatic interactions were examined using the method implemented in the QTLNetwork-2.0 software (Yang *et al.* 2007, 2008). This method begins by selecting candidate marker intervals that may be linked to a QTL. These intervals are used as cofactors in a 1D genome scan to identify putative QTL and detect significant marker–interval interactions. Subsequently, a 2D genome scan is performed to detect epistasis conditioned on the previously detected QTL and marker–interval interactions. These analyses are implemented in a mixed linear model framework (Searle *et al.* 1992) to construct an *F*-statistic whose significance can then be determined by permutation tests (Doerge and Churchill 1996). Finally, all detected QTL and their interactions are fitted by a full QTL model to estimate both main and epistatic effects.

RESULTS

Selective genotyping: Genotypes at 33 RFLP markers were determined for the 94 lightest and 84 darkest 14-day-old F_2 males from the cross between *D. m. malerkotliana* strain *mal0-isoC* and *D. m. pallens* strain *palQ120-isoG*. Using single-marker regression performed in the QTL Cartographer (Wang *et al.* 2005), we tested each marker for association with color differences. For several markers on Muller element B and all markers on Muller elements D and E, the probability of having dark (light) pigmentation was significantly associated with the presence of the *D. m. malerkotliana* (*D. m. pallens*) parental allele (Table 1). The strongest association was observed for markers located on Muller element E.

This analysis allows us to exclude a number of candidate genes known to be involved in the development of Drosophila pigmentation (Wittkopp *et al.* 2003a). In particular, the *yellow*, *tan*, and *omb* loci on Muller A, and the *black* and *Dat* loci on Muller C, do not make major contributions to color pattern differences (Table 1), although we cannot rule out that some of these genes have weak phenotypic effects that are below our detection threshold. The number and locations of QTL on Muller elements B, D, and E cannot be determined from these data. Selective genotyping strongly distorts linkage relationships in the vicinity of major QTL, leaving it unclear whether several linked markers are all associated with a single QTL or multiple QTL. In addition, *mal0-isoC* and *palQ120-isoG* differ by a chromosomal inversion on Muller E, where

TABLE 1

Single marker analysis for the *mal0-isoC* × *palQ120-isoG* cross

Muller element	Marker	Maximum likelihood				
		b_0	b_1	$-2\ln(L_0/L_1)$[a]	F[b]	P[c]
A	*Sev*	4.721	−1.784	2.467	2.456	0.119
	y	4.531	−2.264	3.038	3.029	0.083
	LD22118	4.607	−1.447	1.375	1.365	0.244
	omb	4.663	−0.821	0.442	0.438	0.509
	trr	4.706	−0.306	0.062	0.061	0.805
	Bx	4.762	1.095	0.843	0.836	0.362
	t	5.035	3.554	7.923	8.009	0.005
B	*cbt*	4.594	1.389	6.796	6.848	0.01
	ed	4.805	0.943	3.764	3.762	0.054
	Wnt4	4.644	1.152	4.337	4.341	0.039
	yC	4.392	2.638	23.868	25.224	<0.001
	Adh	4.73	2.133	19.314	20.152	<0.001
	yB	4.514	2.201	14.441	14.864	<0.001
	grk	4.709	1.065	3.848	3.846	0.051
	b	4.832	3.648	9.912	10.075	0.002
C	*ptc*	4.578	0.871	2.625	2.615	0.108
	bw	4.723	0.627	1.146	1.137	0.288
	Hr46	4.883	1.753	11.166	11.39	0.001
	Dat	4.667	−0.405	0.177	0.175	0.676
D	*baldspot*	5.939	3.913	57.493	66.868	<0.001
	klu	4.626	4.993	125.476	178.664	<0.001
	Sod	3.51	3.774	47.134	53.209	<0.001
	bab2	5.166	4.948	196.003	348.422	<0.001
	pcdr	1.569	4.253	41.323	45.88	<0.001
	pale	3.582	3.156	52.247	59.853	<0.001
E	*tsl*	9.867	9.17	340.564	989.331	<0.001
	burs	5.229	5.098	273.133	627.293	<0.001
	yellow-f2	5.273	4.937	395.832	1404.239	<0.001
	Lsdp1	5.163	5.061	577.339	4114.763	<0.001
	bcd	4.471	5.068	431.892	1751.381	<0.001
	Scr	3.317	5.545	258.356	564.337	<0.001
	yellow-e	5.062	4.805	325.684	897.535	<0.001
	e	3.656	3.167	61.221	71.976	<0.001

Data were fit to a linear regression model, $y_i = b_0 + b_1 x_i + e$, where y_i is the phenotype of the ith individual and x_i is an indicator variable for the marker genotype, b_0 is the intercept, and b_1 is the slope of the least squares regression line fit to the data.

[a] A likelihood ratio test statistic for the model.

[b] The *F*-statistic.

[c] Probability of the *F*-statistic assuming 1 and $n - 1$ degrees of freedom for the numerator and denominator, respectively (Basten *et al.* 2005). Linkage between a marker and a QTL was examined by testing whether b_1 is significantly different from zero. The *F*-statistic compares the hypothesis H_0: $b_1 = 0$ to an alternative H_1: $b_1 \neq 0$. The *P*-value is a measure of support for H_0. Genetic association is considered significant if the *P*-value is lower than the Bonferroni-corrected value of 0.0015 (0.05/33).

one or more QTL are detected, further complicating the mapping of these QTL.

Composite interval mapping: Composite interval mapping was performed using 41 RFLP markers in the F_2 population including 188 randomly selected, 94 darkest, and 94 lightest 4-day-old males from the cross between *D. m. malerkotliana* strain *mal0-isoC* and *D. m. pallens* strain *palQ121-isoA*. Three large-effect QTL were detected on the Muller elements D and E in the *CG7145-AbdB*, *Tbp1-kkv*, and *e-CG6342* intervals (Figure 3). The first of these regions spans the proximal portions of Muller elements D and E, with a bias toward Muller E, while the last two are located entirely within Muller E. An additional, much weaker QTL may also be present on the XL chromosome arm (Muller A). QTL located on Muller E account for 0.26, 0.28, and 0.30 of the phenotypic variation in the F_2 progeny, and together explain 84% of this variation (Table 2). The additive effects of these QTL were 1.14, 1.69, and 0.84 phenotypic units, respectively (Table 2), so that their combined effects including both homozygotes and heterozygotes amount to 7.34 units. Since phenotypic scores in the F_2 progeny range from 0 to 8 (supplemental Figure 1), it is likely that we have detected all major QTL affecting the

TABLE 2

QTL intervals associated with differences in pigmentation between *D. m. malerkotliana* and *D. m. pallens*

QTL	LOD	Effect (SE)[a]	Effect/σ_p	R^2
CG7145-AbdB	30.93	1.14 (0.12)	0.42	0.28
Tbp-1-kkv	24.94	1.69 (0.09)	0.61	0.30
e-CG6342	10.43	0.84 (0.10)	0.30	0.26

[a] Additive effects (per one allele) were estimated by MCMC algorithm implemented in QTLNetwork 2.0 (Yang *et al.* 2007, 2008) and are given in phenotypic units reflecting pigmentation scores on a scale from 0 to 10. σ_p is the phenotypic standard deviation of the progeny. R^2 is the proportion of the variance accounted by the QTL and is estimated as $R^2 = (s_0^2 - s_1^2)/s^2$, where s^2 is the variance of the trait, s_0^2 is the sample variance of the residuals, and s_1^2 is the variance of the residuals (Basten *et al.* 2005).

difference in pigmentation between *D. m. malerkotliana* and *D. m. pallens* in young males. It is also possible, however, that QTL effects were overestimated due to the selective inclusion of progeny with extreme phenotypes. All QTL from each species, including the weak QTL on Muller A, affect pigmentation in the same direction, consistent with adaptive evolution driven by positive selection.

The phenotypes of 4-day-old hybrid progeny were strongly skewed toward the lightest values (supplemental Figure 1). To test whether the excess of light individuals affected our ability to detect and map major QTL, we repeated CIM in two subsamples of the F_2 progeny: in a sample that excluded all individuals with the 0 phenotypic score, and in a sample that included only the 188 random progeny. In both analyses, three major QTL were detected at the same locations as in the total sample, although their relative significance and effect estimates were different in each analysis (supplemental Figure 2).

Epistasis tests revealed only one significant interaction: between the QTL in the *e-CG6342* interval on Muller E and the *omb-LD22118* interval on Muller A, with a weak effect of −0.0312 phenotypic units. Thus, QTL responsible for color pattern differences between *D. m. malerkotliana* and *D. m. pallens* appear to act in a largely additive fashion. No additional epistatic interactions were detected in the analysis of subsamples described above.

Analysis of candidate genes: Previous research has identified a substantial number of genes responsible for pigment patterning and synthesis in Drosophila (Wittkopp *et al.* 2003a). Several of these candidate genes are located on the same chromosome arms as the QTL responsible for the difference in male abdominal pigmentation between *D. m. malerkotliana* and *D. m. pallens* (Figure 3). We tested whether any of these genes corresponded to the detected QTL using introgression strains generated by repeated backcrossing of dark hybrid males to the *D. m. pallens* parent (Figure 2), followed by several generations of full-sib inbreeding. These strains have male abdominal pigmentation as dark as that of the *D. m. malerkotliana* parent, indicating that they carry the "dark" parental alleles at all major QTL. The majority of dark hybrid males were homozygous for the "light" (*D. m. pallens*) alleles at the *Abdominal-B*, *bric a brac*, *doublesex*, *ebony*, *yellow*, *tan*, and *pale* loci (Table 3). Thus, none of these candidate genes contribute significantly to color pattern variation in *D. malerkotliana*.

DISCUSSION

Genetic basis of pigmentation in *D. malerkotliana*: The dramatic difference in male abdominal pigmentation between *D. m. malerkotliana* and *D. m. pallens* (Figure 1) appears to have an oligogenic genetic basis. Composite interval mapping reveals three major QTL responsible for this difference on Muller element E (chromosome arm 2L of *D. malerkotliana*), with an additional minor QTL on Muller A (chromosome XL) (Figure 3). In contrast to some other studies (Carbone *et al.* 2005; Steiner *et al.* 2007), epistatic interactions among QTL do not play an important role in shaping color pattern variation in this species. Although it is possible that weak epistasis would be detected in a larger sample of progeny, the three major QTL appear to act in an additive manner.

There is some disagreement between the results of two independent crosses. Whereas the presence of QTL on Muller E is detected in both crosses, markers on Muller B (chromosome 3R of *D. malerkotliana*) show a significant association with phenotype in the *mal0-isoC* × *palQ120-isoG* cross, but not in the *mal0-isoC* × *palQ121-isoA* cross. Conversely, the weak QTL on Muller A is seen in the latter cross, but not in the former. There are two, not mutually exclusive explanations for these results. First, the selective genotyping strategy used in the former cross is expected to have higher power to detect weak QTL (Lynch and Walsh 1998). Muller element B may carry such weak QTL that were missed by composite interval mapping the *mal0-isoC* × *palQ121-isoA* cross. Second, phenotypic analysis was performed at different ages in the two crosses: 4 days for CIM *vs.* 14 days for selective genotyping. Male abdominal pigmentation becomes progressively darker with age in *D. m. malerkotliana* and in *D. m. malerkotliana* × *D. m. pallens* hybrids, reaching full intensity after 1 week. Thus, the discrepancy between the two crosses may reflect the existence of age-specific QTL, with a gene or genes on Muller B having a visible effect only in older males, and the gene on Muller A only in younger individuals. In fact, the search for such age-specific QTL was the main motivation for phenotyping younger males in the *mal0-isoC* × *palQ121-isoA* cross. A third possible explanation for the difference between the two crosses is that different loci are in fact responsible for phenotypic variation in different strains. This, however, appears

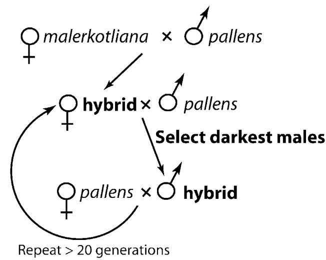

FIGURE 2.—Crossing scheme used to generate introgression lines between *D. m. malerkotliana* and *D. m. pallens.*

unlikely, since there is no visible variation in pigmentation in either subspecies, and the two strains of *D. m. pallens* were collected from the same local population.

Different genes underlie convergent phenotypic changes: A key conclusion from this and other recent work is that genetic changes at different loci are responsible for color pattern differences that evolved convergently in different Drosophila species. Several genes involved in pigment synthesis [including *yellow* (*y*), *tan* (*t*), and *ebony* (*e*)] or in the spatial patterning of pigmentation [*optomotor-blind* (*omb*) and *bric a brac* (*bab*)] show an association with intra- or interspecific pigmentation differences in various distantly related lineages. For example, dark abdominal pigmentation has been lost in *D. santomea*, so that males of this species and its closest relative *D. yakuba* show the same difference in abdominal color pattern as *D. m. pallens* and *D. m. malerkotliana* (LLOPART *et al.* 2002; CARBONE *et al.* 2005). Genetic mapping and transgenic analysis have implicated *t* as one of the loci responsible for the loss of abdominal pigmentation in *D. santomea* (JEONG *et al.* 2008). We find, however, that this gene makes no significant contribution to phenotypic differences in *D. malerkotliana*. Similarly, *omb* and *bab* are associated

TABLE 3

Genotypes of individuals in the introgression strains

	palQ120-isoG[a]:	*palQ121-isoA*[a]	
	Darkest[b]	Darkest[b]	Lightest[b]
y	0, 0, 6	0, 0, 11	0, 0, 14
tan	0, 0, 24	0, 0, 4	0, 0, 8
bab2	0, 0, 24	0, 3, 8	0, 0, 11
ple	—	0, 0, 10	0, 0, 14
AbdB	—	1, 4, 1	0, 0, 8
e	0, 0, 24	0, 8, 1	0, 1, 14
dsx	0, 0, 24	3, 5, 1	0, 0, 15

[a] The *D. m. pallens* parental strain that was used for constructing the introgression strains. *mal0-isoC* was used as the *D. m. malerkotliana* parent for both strains. The *palQ120-isoG* × *mal0-isoC* cross was in the 22nd generation and the *palQ121-isoA* × *mal0-isoC* cross in the 16th when the progeny were genotyped.

[b] Phenotypic extremes used for genotyping. The number of individuals with each genotype is shown in the following order: *mal/mal* homozygote, *mal/pal* heterozygote, *pal/pal* homozygote.

with intraspecific variation in abdominal color patterns in *D. polymorpha* and *D. melanogaster*, respectively (KOPP *et al.* 2003; BRISSON *et al.* 2004), but not in *D. malerkotliana*. More global differences in the intensity of pigmentation appear to be controlled by *ebony* and *tan* in *D. americana* and *D. novamexicana* (P. J. WITTKOPP, personal communication), by *ebony* and *yellow* in *D. elegans* and *D. gunungcola* (S. YEH and J. TRUE, personal communication), and by *ebony* in some populations of *D. melanogaster* (POOL and AQUADRO 2007; TAKAHASHI *et al.* 2007). Again, none of these loci make a detectable contribution to the difference in pigmentation between *D. m. malerkotliana* and *D. m. pallens*. In general, the genetic architecture of color pattern differences ranges

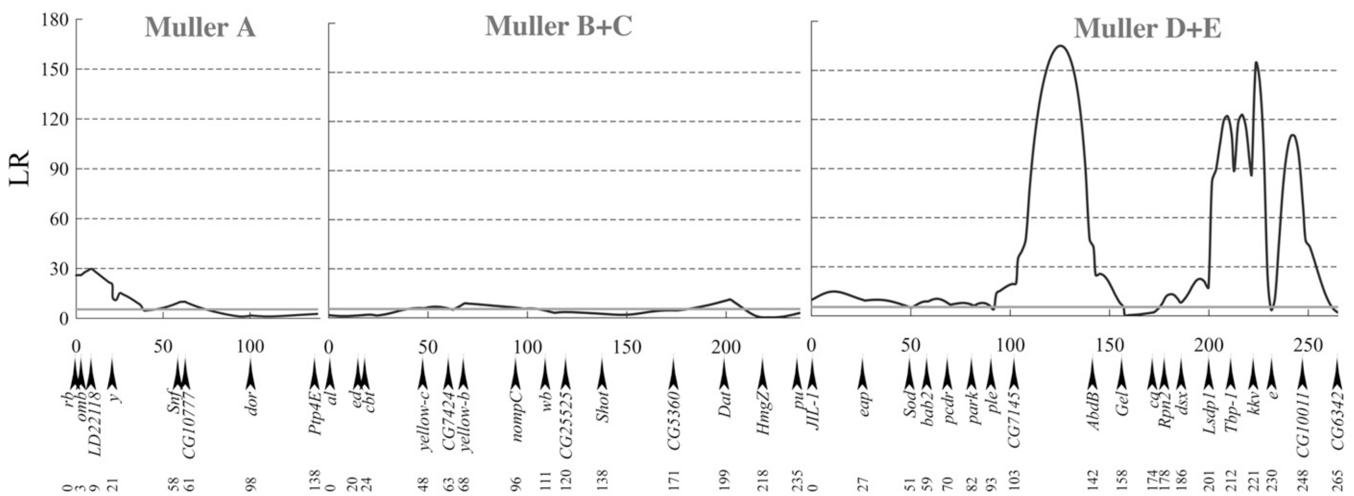

FIGURE 3.—QTL responsible for the difference in pigmentation between *D. m. malerkotliana* and *D. m. pallens*. Plots are LR test statistics for pigmentation differences as determined by composite interval mapping. Significance threshold was determined by permutation and is LR = 5.37, denoted by the horizontal line.

from a single Mendelian factor in *D. kikkawai* and *D. jambulina* (Ohnishi and Watanabe 1985) to polygenic systems involving complex gene interactions in *D. arawakana* and *D. nigrodunni* (Hollocher *et al.* 2000a,b). *D. malerkotliana*, and indeed most studied species, fall somewhere between these extremes, and a moderately oligogenic basis of variation appears to be typical for this trait (Martinez and Cordeiro 1970; Spicer 1991; Wittkopp *et al.* 2003b; Carbone *et al.* 2005).

Although it is possible that some of the known candidate genes have minor effects on color variation in *D. malerkotliana*, this is unlikely since the selective genotyping and introgression line approaches used in some of our experiments had high power to detect weak QTL. In any event, most of the difference between the two subspecies is due to other, unknown loci. An important corollary to this conclusion is that our knowledge of the Drosophila pigmentation pathway is still incomplete. The QTL responsible for color pattern differences in *D. malerkotliana* could represent an intermediate tier of regulatory genes between the spatial patterning pathway that controls color patterns (*omb*, *bab*, *Abdominal-B*, and *doublesex*), and the metabolic pathway responsible for pigment synthesis (*e*, *y*, *t*, *pale*, *Ddc*, and other enzymes) (True 2003; Wittkopp *et al.* 2003a). Alternatively, these QTL may represent unknown enzymes essential for melanin synthesis (Wright 1987), or components of the hormonal cascade responsible for their activation (Davis *et al.* 2007). Further genetic analysis in *D. malerkotliana* is necessary to identify these genes and determine their biological functions.

A different genetic basis of similar color patterns in different species may be explained by the branched organization of the Drosophila pigmentation pathway. In this pathway, different metabolic reactions draw on a shared pool of soluble precursors to produce several distinct light and dark pigments (Wright 1987; True 2003; Wittkopp *et al.* 2003a). At least one of these reactions is reversible, with the opposing reactions catalyzed by the products of the *ebony* and *tan* loci (True *et al.* 2005). This nonlinear structure of the pigment synthesis pathway means that changes in the output of different enzymatic reactions can produce similar phenotypes. For example, darker pigmentation can in principle be due either to increased expression or activity of enzymes required for the synthesis of dark pigments (*e.g.*, *Ddc*, *yellow*, or *tan*) or to decreased expression or activity of enzymes involved in the synthesis of light pigments (such as *ebony*, *black*, or *Dat*). Increased or decreased expression of these enzymes can in turn be caused either by mutations in the regulatory regions of these loci or by changes in the expression of their upstream regulators such as *bab*, *omb*, and *Abd-B*. Thus, the "mutational target" for producing any given phenotypic change is quite extensive, so that similar phenotypic adaptations can take distinct genetic paths in different evolutionary lineages.

Since few other traits have been studied in as much detail and in as many different species as Drosophila pigmentation, it is too early to say whether a different genetic basis of similar phenotypic changes is the rule or an exception. Perhaps the closest example comes from vertebrate pigmentation, where the same gene, *Mc1R*, is implicated in the evolution of color patterns in many distant taxa (Mundy 2005; Hoekstra 2006). However, *Mc1R* is not the only gene that contributes to this variation (Steiner *et al.* 2007), and different loci may control similar phenotypes in different populations (Hoekstra and Nachman 2003). The loss of dorsal cuticular projections in Drosophila larvae appears to be caused by changes at the same locus, *ovo/svb*, in at least two different lineages (Sucena *et al.* 2003; McGregor *et al.* 2007). Independent mutations in the same gene, *Oca2*, are responsible for the convergent origin of albinism in different cavefish populations (Protas *et al.* 2006), while the loss of eyes in the same species is apparently controlled by different genes in different populations (Jeffery 2005). Although the same loci contribute to skeletal changes in different populations of sticklebacks (Colosimo *et al.* 2004; Cresko *et al.* 2004; Shapiro *et al.* 2006), this may be due in part to independent fixation of the same ancestral alleles in different lakes (Colosimo *et al.* 2004). The extent of "genetic convergence" may in fact be overestimated due to ascertainment biases, since many studies tend to focus on the same candidate genes.

An intriguing possibility is that the genetic basis of trait evolution is biased by the structure of the regulatory pathways that control the development of these traits. For example, in pathways where a single gene integrates multiple upstream inputs and regulates multiple downstream targets, this "nexus" gene may be predisposed to convergent genetic changes when the pathway as a whole is placed under similar selective pressure in different species. The *ovo/svb* locus may be an example of such a nexus gene in the pathway that controls larval cuticular patterns in Drosophila (Chanut-Delalande *et al.* 2006). On the other hand, gene networks with more complex topologies may favor evolutionary changes distributed over multiple loci. One of the key goals of the continuing synthesis between evolutionary and developmental genetics should be to elucidate how the intrinsic structure of developmental pathways affects the fixation of genetic variation under different combinations of selective and demographic forces.

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