

Quantitative Trait Loci Affecting the Difference in Pigmentation Between *Drosophila yakuba* and *D. santomea*

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ABSTRACT

Using quantitative trait locus (QTL) mapping, we studied the genetic basis of the difference in pigmentation between two sister species of *Drosophila*: *Drosophila yakuba*, which, like other members of the *D. melanogaster* subgroup, shows heavy black pigmentation on the abdomen of males and females, and *D. santomea*, an endemic to the African island of São Tomé, which has virtually no pigmentation. Here we mapped four QTL with large effects on this interspecific difference in pigmentation: two on the *X* chromosome and one each on the second and third chromosomes. The same four QTL were detected in male hybrids in the backcrosses to both *D. santomea* and *D. yakuba* and in the female *D. yakuba* backcross hybrids. All four QTL exhibited strong epistatic interactions in male backcross hybrids, but only one pair of QTL interacted in females from the backcross to *D. yakuba*. All QTL from each species affected pigmentation in the same direction, consistent with adaptive evolution driven by directional natural selection. The regions delimited by the QTL included many positional candidate loci in the pigmentation pathway, including genes affecting catecholamine biosynthesis, melanization of the cuticle, and many additional pleiotropic effects.

DURING the modern synthesis, the dominant view of the genetics of species differences was that of Ronald FISHER (1930), who believed that such differences were almost invariably due to the accumulation of many genes, each of small phenotypic effect. Tests of this proposition, however, were limited by the lack of genetic markers in most crossable but differentiated species, although some data suggested that species differences could occasionally be due to genes of large effect (ORR and COYNE 1992).

Recently, however, the advent of molecular techniques has improved our ability to study the genetics of species differences. Two innovations have been crucial. First, quantitative trait locus (QTL) mapping enables us to localize genes responsible for species differences by determining their association with molecular markers at known sites. Second, molecular techniques such as germline transformation enable us to determine directly whether a candidate gene affects a species difference. ORR (2001) describes these innovations and

the results of recent genetical studies using them. Although most data derive from a small number of organisms (Orr's study describes only 13 analyses, 6 from *Drosophila* and 4 from the monkeyflower genus *Mimulus*), the results show that while differences in traits between species can be polygenic, genes of large effect are involved more frequently than previously suspected. Here we describe a genetic analysis of a striking character difference—the degree of abdominal pigmentation—between two sister species of *Drosophila*: *Drosophila yakuba* and *D. santomea*.

D. yakuba is widely distributed in open habitats across sub-Saharan Africa and the islands near the continent (including Madagascar). In contrast, *D. santomea* is endemic to the 860-km² volcanic island of São Tomé, in the Gulf of Benin 255 km west of the coast of Gabon (LACHAISE *et al.* 2000). On the mountain Pico de São Tomé, *D. yakuba* occurs at elevations below 1450 m, while *D. santomea* occupies the mist forests at elevations between 1153 and 2024 m. (*D. yakuba* is also widespread throughout lowland São Tomé, probably as a result of a secondary invasion after the initial common ancestor evolved into *D. santomea*.) Between ~1100 and 1450 m in elevation, the ranges of the two species overlap, with the ratio of *D. yakuba*/*D. santomea* shifting from 2:1 to 1:20 as one moves upward through this zone. The species show substantial sexual isolation when tested in the laboratory (LACHAISE *et al.* 2000), and, using morphological

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criteria, one finds a low frequency ($\sim 1\%$) of hybrids in the zone of overlap.

Molecular evidence puts the divergence between *D. yakuba* and *D. santomea* at $\sim 400,000$ years ago (LLOPART *et al.* 2002a). In interspecific crosses, F₁ male hybrids are sterile but female hybrids are fertile and thus can be crossed to either parental species (LACHAISE *et al.* 2000; CARIU *et al.* 2001). This fertility permits genetic analysis using backcross individuals.

The diagnostic differences between the species include male genital morphology and sex-comb tooth number (LACHAISE *et al.* 2000; COYNE *et al.* 2004), but the most striking difference involves abdominal pigmentation. Among the nine species in the *D. melanogaster* subgroup, eight of them, including *D. yakuba*, have similar patterns of dark pigmentation: males possess thin black stripes along the posterior portions of tergites 2, 3, and 4, while tergites 5–7 are completely black. Females of these species have stripes along the posterior portions of all tergites, and tergites 5–7 show substantial but not complete black pigmentation. In contrast, *D. santomea* males show virtually no pigmentation, and females show only very light striping on the posterior parts of tergites 2–5, with no pigmentation on other tergites. (Photographs of these differences are given in LACHAISE *et al.* 2000, Figures 1 and 2, and in LLOPART *et al.* 2002a, Figure 1). Given the dark pigmentation in all but one species in the subgroup, including the outgroup species *D. orena* and *D. erecta*, it is nearly certain that the absence of dark pigmentation in *D. santomea* is a novel derived trait.

The adaptive significance of this pigmentation difference, if any, is unknown. Although these species show strong sexual isolation (COYNE *et al.* 2002), this does not diminish when flies are tested in the dark, suggesting that the pigmentation difference is not a cue for mate discrimination (LLOPART *et al.* 2002a,b). Moreover, the relationship between pigmentation and temperature is opposite to that expected from other studies of *Drosophila*: individuals within a species or closely related species living under colder conditions are almost invariably darker (*e.g.*, DAVID *et al.* 1985; GIBERT *et al.* 1998), yet *D. santomea*, which lives at higher altitudes than *D. yakuba*, is lighter.

In previous genetic analyses using three morphological markers and eight molecular markers, we determined that at least three genes were involved in the pigmentation difference between *D. santomea* and *D. yakuba* females and five genes between males. In each case, the genes resided on all three major chromosomes, with the X chromosome having a particularly strong effect in males (LLOPART *et al.* 2002a).

In this study we extend and refine our previous analysis, using a more accurate method of measuring pigmentation as well as a QTL analysis employing 32 molecular markers, which enables us to map “pigmentation genes” more accurately. Our goals are to determine

the number of genes involved in this morphological difference, their chromosomal locations, whether the same genetic regions affect the pigmentation difference in both males and females, and whether QTL from a given species tend to affect the character in the same direction, implying that the species difference evolved by natural selection (ORR 1998). Finally, the mapping of QTL to fairly restricted regions of the genome may eventually allow us to identify specific loci involved in this species difference.

MATERIALS AND METHODS

Drosophila strains: All flies were maintained in 8-dram vials containing standard cornmeal-agar-Karo media on a 12 hr:12 hr light:dark cycle at 24°. One isofemale strain was used from each species. The *D. yakuba* Tai 18 strain was derived from a female collected by D. Lachaise in 1983 in the Tai rainforest on the border between Liberia and the Ivory Coast. The *D. santomea* STO.4 stock was derived from a female collected in March 1998 in the Obo Natural Reserve on São Tomé Island. These two strains were used (and further described) in our previous work on the genetics of pigmentation in these species (LLOPART *et al.* 2002a). The strains are homosequential in chromosome banding pattern except for the right arm of the second chromosome: the *D. yakuba* Tai 18 strain is polymorphic for inversion 2*Rn*, which covers $\sim 40\%$ of the right arm of chromosome 2 (LEMEUNIER and ASHBURNER 1976).

Crosses: Backcross (BC) hybrids were produced by crossing 4-day-old virgin *D. yakuba* Tai 18 females to virgin *D. santomea* STO.4 males, and then backcrossing virgin F₁ females to males from both species. Genetically, female BC hybrids to *D. yakuba* are either homozygous *D. yakuba* or heterozygous *D. yakuba/santomea* and have mitochondrial DNA from *D. yakuba*. Similarly, female BC hybrids to *D. santomea* are either homozygous *D. santomea* or heterozygous *D. yakuba/santomea* and have mitochondrial DNA from *D. yakuba*. Male BC hybrids have the same autosomal and mitochondrial genotypes as females, but the X-linked loci are either pure *D. santomea* or *D. yakuba*, with the Y chromosome from the parental male used in the backcross. We scored pigmentation in ~ 50 males and 50 females from each of the pure species and the reciprocal F₁ hybrids and in between 73 and 544 males and females from each of the two backcrosses (Table 1). To improve the precision of QTL mapping, we selected backcross individuals with extreme and intermediate pigmentation phenotypes for subsequent pigmentation scoring and genotyping. For each of the first three BC genotypes listed in Table 1, we selected flies of each sex from a sample of $\sim 12,500$ individuals (~ 50 bottles, each containing ~ 250 flies of each sex). One-third of the total individuals chosen were judged by eye to have very dark pigmentation, one-third to have very light pigmentation, and the remaining third were chosen randomly from individuals with intermediate phenotypes. (Equal numbers of all three classes were chosen from each bottle inspected until we had accumulated ~ 500 flies of each sex in each backcross. Thus for each sex we selected $\sim 4\%$ of total individuals inspected; this stringent selection facilitates the precision of mapping.) However, females from the backcross of F₁ individuals to *D. santomea* showed little variation in pigmentation, and so all of these were chosen randomly.

Pigmentation scores: All scoring of pigmentation was done on 4-day-old virgin flies. We scored only the three posterior tergites of each fly (segments 5, 6, and 7) by examining the fly under a dissecting microscope. A pigmentation score was

TABLE 1

Pigmentation scores of pure *D. yakuba* and *D. santomea* and of F₁ hybrids from the reciprocal crosses and backcross individuals

Genotype	Sex	Mean score (SE)	N
<i>D. yakuba</i> Tai 18	M	14.22 (0.30)	51
	F	9.85 (0.15)	53
<i>D. santomea</i> STO.4	M	0.63 (0.05)	50
	F	1.02 (0.06)	56
F ₁ (Y × S)	M	11.92 (0.21)	51
	F	4.62 (0.18)	50
F ₁ (S × Y)	M	3.72 (0.21)	51
	F	4.29 (0.12)	51
Backcross (F ₁ × Y)	M	7.26 (0.18)	544
	F	7.42 (0.09)	544
Backcross (F ₁ × S)	M	4.50 (0.16)	517
	F	2.05 (0.13)	73

Y, *D. yakuba*; S, *D. santomea* (Tai 18 and STO.4 strains were used in all crosses). In all crosses the genotype of the female parent is given first. All F₁ females were produced by crossing *D. yakuba* females to *D. santomea* males.

assigned on the basis of both the percentage of the tergite that was pigmented and the degree of pigmentation. First, the proportion of the tergite covered by black pigment was estimated to the nearest 0.05 (5%). Then, the relative degree of pigmentation was measured within the pigmented area. Using color standards, we assessed the degree of pigmentation within the pigmented area using a five-point scale ranging from 1 (very light pigmentation, slightly darker than background color) to 5 (dark, shiny black), with intermediate numbers representing intermediate degrees of pigmentation. Unpigmented areas were given a score of 0. We limited ourselves to assigning only three shades of black to each tergite. The percentage of the area of each tergite covered by each shade of pigmentation was then multiplied by the intensity of pigmentation, and these areas were summed. This gives each tergite a minimum possible pigmentation score of 0 (no area pigmented) to 5 [tergite completely covered with very dark pigmentation (1.0 × 5)]. These areas were summed for all three tergites, yielding a minimum possible pigmentation score for a given fly of 0 and a maximum possible score of 15. As shown in Table 1, this procedure discriminates well between the pigmentations of these species.

Molecular markers: We identified single nucleotide polymorphisms (SNPs) and insertion/deletion variants (INDELs) that discriminated between the *D. yakuba* Tai18 and the *D. santomea* STO.4 strains for 41 nuclear regions: *y*, *per*, *sog*, *v*, *sn*, *Rux*, *f*, *bnb*, *Hex-A*, *AnnX*, *su(f)*, *l(2)gl*, *Rad1*, *RpL27A*, *Gart*, *salr*, *wkg*, *Rep4*, *Adh*, *His3*, *barr*, *Sara*, *Hex-C*, *Ngp*, *Kr*, *Lsp1-γ*, *RpL14*, *dib*, *sfl*, *Sod*, *Est6*, *Ss11*, *hb*, *Xdh* (*ry*), *Rpn5*, *AP-50*, *Mlc1*, *ymp*, *janB*, *krz*, and *ci*. Newly reported sequences were deposited in GenBank under accession nos. DQ068949 (Gart_T18) and DQ068950 (Gart_sto4); otherwise see LLOPART *et al.* (2005, this issue) for details. To determine nucleotide sites differentially fixed between *D. yakuba* Tai18 and *D. santomea* STO.4, we tested 20 individuals (10 per strain) for each region by direct sequencing of PCR products obtained from single-fly DNA extractions (ASHBURNER 1989), using *D. melanogaster* or *D. yakuba* primers. We purified PCR products using the Wizard Magnesium PCR clean-up system (Promega, Madison, WI) and sequenced them directly with an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA). We edited the

sequences with the Sequencher 3.0 software (Gene Codes, Ann Arbor, MI) and aligned them using the ClustalX program (THOMPSON *et al.* 1997).

In total, we sequenced ~17.5 kb in each of the 20 flies tested. We detected 263 nucleotide differences fixed between Tai18 and STO.4, that is, 70% of the total nucleotide variation. Among these fixed differences we selected 32 that affect a restriction endonuclease site to be used as markers in the genotyping procedure. Table 2 lists the 32 markers, their relative order within the *D. yakuba* chromosomes, and the conditions for genotyping. We inferred the relative order of markers within each chromosome in *D. yakuba/D. santomea* from the *D. yakuba* genome project (<http://www.genome.wustl.edu/projects/yakuba/>; version 040407).

Marker genotypes: All BC individuals from the pigmentation assays were stored at -80° in 0.5-ml Eppendorf tubes. Genomic DNA was extracted from each BC individual using the Puregene (Gentra Systems, Minneapolis) single-fly DNA extraction protocol. The sample consisted of 506 BC *D. santomea* males, 73 BC *D. santomea* females, 537 BC *D. yakuba* males, and 526 BC *D. yakuba* females. The genotypes of the 1642 BC hybrids were determined for all 32 markers (*i.e.*, 52,544 genotypes).

The 32 molecular markers were designed using sequence data from the parental strains of *D. santomea* and *D. yakuba*. The aligned sequences were used to develop PCR primers, using Primer3 (ROZEN and SKALETSKY 2000) and restriction enzyme (RE) digestions. Genotyping was performed using restriction fragment length polymorphism analysis by PCR amplification from genomic DNA, using RedTaq DNA polymerase (Sigma, St. Louis) followed by RE digestion (see Table 2 for primers, RE, and conditions). All REs were purchased from New England Biolabs (Beverly, MA) and primers were purchased from MWG Biotech (High Point, NC). The digested PCR products were run on a 3% agarose gel stained with ethidium bromide, imaged with the Bio-Rad ChemiDoc System PC RS-170 using Quantity One (version 4.2.1) software, and manually genotyped by assigning a "0" (homozygous *D. santomea*), "1" (*D. yakuba/D. santomea* heterozygote), or "2" (homozygous *D. yakuba*) to each marker genotype. A recombination map based on the 1642 BC hybrids (Table 3) was constructed using the Haldane mapping function.

QTL mapping: QTL affecting variation in pigmentation between *D. yakuba* and *D. santomea* were mapped in each BC population using composite interval mapping (CIM; ZENG 1994) and implemented using QTL Cartographer software (BASTEN *et al.* 1999). CIM tests whether an interval between two markers contains a QTL affecting the trait while simultaneously controlling for the effect of QTL located outside the interval using multiple regression on marker cofactors. Marker cofactors were chosen by forward selection-backward elimination stepwise regression. The likelihood-ratio (LR) test statistic is $-2 \ln(L_0/L_1)$, where L_0/L_1 is the ratio of the likelihood under the null hypothesis (*i.e.*, there is no QTL in the test interval) to the alternative hypothesis (there is a QTL in the test interval). LR test statistics were computed every 2 cM with marker cofactors 10 cM or more from the test location. We used permutation analysis to determine appropriate significance thresholds that take into account the multiple tests performed and correlations among markers. 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 that exceed the 50th greatest LR statistic from the permuted data are significant at the experimentwise 5% level under the null hypothesis (CHURCHILL and DOERGE 1994; DOERGE and CHURCHILL 1996). The approximate boundaries of regions containing QTL were determined by taking 2 LOD intervals

TABLE 2
Molecular polymorphisms discriminating *D. yakuba* and *D. santomea*

Marker	Cytological location	Primer sequence (5'-3')	Type	PCR T_A (°)	Restriction endonuclease
<i>y</i>	1A5	CGCTGCGTGTGTTGTTTATT GCGAATGTTCAAAGAATAATTC	S	55	<i>Ava</i> II
<i>per</i>	3B1-2	TTCCAGTTCTCCGAATCAGC CCTTAGGGCTGAGCCACTCT	S	55	<i>Bbv</i> I
<i>sog</i>	13E1	GCTGGCGTACAACATTGAAA CTCGGTGGCCACATTCAC	S	57	<i>Xho</i> I
<i>v</i>	9F11	AGACTCCCTTCTGCCTTTC TGAGAGCTCCAGTCCGACT	S	55	<i>Ssp</i> I
<i>rux</i>	5D2	CATTTGCTCATCCGTTTCCT GTGCTTGTAGCGCGTTGTC	S	55	<i>Hpy</i> CH4IV
<i>f</i>	15F4-7	CTCGCCGAATGGCAGCAT AATGTACGTCCGCCTGGAT	S	55	<i>Hpa</i> II
<i>bmb</i>	17D6	TTCTTCTCCTGCTCCTTGA CCGAGAAGAAGTCCATCGAG	S	55	<i>Pvu</i> II
<i>Hex-A</i>	8E10	GGTACCCAGCTCTTCGATCA GGCAATGGCATCCTTTAGAA	S	57	<i>Hha</i> I
<i>AnnX</i>	19C1	AAACCAGAGAGCTGCCTTCA ATTCTCCTTGCGACGTCCTG	S	55	<i>Taq</i> I ^α
<i>su(f)</i>	20E	TGGTGGGCAAAAGTCAAAT AAAATCTTAGCCGCTGGAC	ID	57	<i>Nco</i> I
<i>l(2)gl</i>	21A5	TGACGTCGCTGAAGTTCTTG GATGGGCCAGCTTATATTGC	S	54	<i>Mse</i> I
<i>Rad1</i>	23A1	ATGAATGTGCTGTCCGAGTG GTTCCGTGGAACACCTTCGAT	S	55	<i>Hpy</i> CH4IV
<i>RpL27A</i>	24F3	ATCAAGCGGAAGAAGACCAG GACCTTGCCGAAGTAACCAG	S	55	<i>Nco</i> I
<i>salr</i>	32E4-F1	AGCTGACTGATCCCAACCAG GATGATGCCGTTGGAGAACT	S	60	<i>Scr</i> F1
<i>Rep4</i>	34B4	TCACGGAGTACGAACACCAA TACGGGTCAGTTCCTCCTTG	S	57	<i>Taq</i> I ^α
<i>His3</i>	39D3-E1	TTTCAGGACCACAAAACCACA CCGTTTGCCCTTATAAACA	ID	57	<i>Mfe</i> I
<i>barr</i>	38B1-2	GCAGTGCAGGATGAAGATCA TTGGAGTCCACCTCCAGAAC	S	53	<i>Hae</i> III
<i>Sara</i>	57E6	CGACCACAAAACCTGAATTT CATGTTATCCGGCACCCATA	S	55	<i>Hph</i> I
<i>Kr</i>	60F5	ACCAGCCATGAGTGGAGATT CTACAGAGCTGGCTCCATCC	S	56	<i>Mly</i> I
<i>Lsp1γ</i>	61A6	CAAAAACCCACCACAAGCAG CCTTGTACTCCTTCTCGTACATGAT	S	52	<i>Alu</i> I
<i>dib</i>	64A5	AGTCCTTTTCTCCCCAGGAA ATTGGGCCTGGCTGAGTT	S	52	<i>Nru</i> I
<i>sfl</i>	65B3-4	GGGTAATCCCTGTGACGATG TTCCGATGAAAAGAAGTCCA	S	52	<i>Nsi</i> I
<i>Est-6</i>	69A1	TCCTGCCTACGCTTTTGTCT AAAAGTAGTCGTCGCCATGC	ID	52	<i>Mse</i> I
<i>SslI</i>	80B2	GGTGCCAGTAGTGGTGAGT GACGCACATTTTCGAGATCA	S	52	<i>Bsu</i> I
<i>ry</i>	87D9	CGCTTTGAGCAAAAATCCA GAAGAACAAGCTCACCACCA	S	52	<i>Sac</i> I
<i>Rpn5</i>	83C4	TACCGAGGGCAAGATTTACG TGCTGATCTTCTTGGCAATG	S	57	<i>Mse</i> I
<i>AP-50</i>	94A15-16	AGTGCAAGTTCGGCATCAA GAATGGCAGCGAAATGTCTT	S	57	<i>Hae</i> III
<i>Mlc1</i>	98A14-15	TGCAAACAGAGTTCGTCCAG AACGGGCATTATCAGCATGT	S	52	<i>Tsp</i> 5091
<i>ymp</i>	96E	CCTCGAGACCCGCAGTAGT	S	53	<i>Hae</i> III

(continued)

TABLE 2
(Continued)

Marker	Cytological location	Primer sequence (5'-3')	Type	PCR T_A (°)	Restriction endonuclease
<i>janB</i>	99D3	CACCTCGCACTTCTGATTGA CATGGCTTCACGAAATACGG CTTACCCTGGAGGTGCCATA	S	57	<i>SalI</i>
<i>krz</i>	100E3	CGCATGTTGTCAAATAAAATCG TTTTTGGGATAACCCATTATTCA	S	55	<i>MseI</i>
<i>ci</i>	102A1-3	AGCCCTTGCAGTGAAGACTC TGGTAGGTCTGCTACGTCC	S	50	<i>HpaI</i>

Cytological locations are given on the basis of *D. melanogaster* cytology (LEMEUNIER and ASHBURNER 1976). The order of the markers in the first column reflects their relative positions in *D. yakuba*/*D. santomea* chromosomes inferred from the *D. yakuba* genome project (<http://www.genome.wustl.edu/projects/yakuba/>). Marker types: S, SNP; ID, insertion/deletion. The PCR protocol for all markers is 1 cycle at 94° for 2 min; 35 cycles at 94° for 30 sec, annealing temperature (T_A) for 30 sec, and 72° for 30 sec; and 1 cycle at 72° for 4 min, where the T_A is listed. PCR products were digested with a restriction endonuclease, run on a 3% agarose gel stained with ethidium bromide, imaged with the Bio-Rad Chemi Doc System PC RS-170 using Quantity One (version 4.2.1) software, and manually genotyped.

(9.22 LR) surrounding the point of greatest significance and interpolating the cytological location of the interval on the basis of the observed amount of recombination between flanking markers.

We estimated the effects of each QTL as the difference between the appropriate homozygous genotypes and heterozygous *D. santomea*/*D. yakuba* genotypes at the peak LR, scaled by the phenotypic standard deviation. The effects in females and autosomes of both sexes are thus estimates of $a - d$ in the cross to *D. yakuba* and $-a - d$ in the cross to *D. santomea*, where $-a$ and a are, respectively, the genotypic values in *D. santomea* and *D. yakuba*, and d is the heterozygous effect (FALCONER and MACKAY 1996). The effects of X-linked QTL in males are estimates of $2a$.

We evaluated pairwise epistatic interactions between all possible marker pairs by running ANOVA models to account for the main effects of all significant markers and of one pairwise interaction between markers (DILDA and MACKAY 2002). The ANOVAs were performed with the PROC GLM procedure, using SAS (Cary, NC) 8.02 software. Interactions with P -values < 0.0001 are significant on the basis of a Bonferroni correction for 496 tests per BC population. We estimated the effects of significant two-locus interactions from the least-squares means of the four marker locus classes as $[(\bar{x}_{11} + \bar{x}_{22}) - (\bar{x}_{12} + \bar{x}_{21})]$, where the first subscript is 1 if the marker has a homozygous genotype for either parental species and 2 if the marker has a heterozygous genotype, and the second subscript takes on the same values for the other marker in the interaction. Standard errors of the interaction effects were estimated as described by DILDA and MACKAY (2002).

Candidate genes: Cytological bands in *D. melanogaster* of the markers that define the interval under the QTL peak were obtained using FlyBase (DRYSDALE and CROSBY 2005). Using the known cytological positions of these markers in *D. melanogaster*, we determined the corresponding positions in *D. yakuba* (LEMEUNIER and ASHBURNER 1976; ASHBURNER 1989). This defined an interval both in *D. melanogaster* and in *D. yakuba* that allowed us to search for candidate genes within that interval. We obtained a complete list of candidate genes involved in pigmentation in *D. melanogaster* from FlyBase (DRYSDALE and CROSBY 2005). Candidate genes were identified on the basis of the markers that delimit each QTL and the presence of pigmentation genes between these two markers (Table 6).

RESULTS

Pigmentation of pure species and F₁ hybrids: Table 1 gives the mean pigmentation scores, standard errors, and sample sizes for the pure species, the reciprocal F₁ hybrids, and the backcross hybrids used for genotyping. The difference between the pure species is substantial: the mean pigmentation scores of *D. yakuba* males and females are 14.22 and 9.85, respectively, and for *D. santomea* males and females 0.63 and 1.02, respectively. As seen in our previous analysis (LLOPART *et al.* 2002a), reciprocal F₁ hybrid males show a large effect of the X chromosome on pigmentation: these males have pigmentation scores fairly close to those of males from the species of the maternal parent. The difference in pigmentation scores between the two classes of F₁ males is highly significant ($t = 27.1$, 100 d.f., $P < 0.001$). The relative effect of the X chromosome in male pigmentation can be judged as the percentage of the total difference between males of the two species explained by the difference between the reciprocal F₁ males; this effect is ~60%. This effect is much larger than the relative size of this chromosome [constituting ~21% of the haploid genome (Table 3)] and suggests that either the X chromosome carries a disproportionate number of genes affecting pigmentation or individual X-linked genes have disproportionately large effects. (The QTL analysis below shows that the second explanation is most likely to be correct.)

In contrast to males, the F₁ females do not differ significantly in pigmentation scores (Table 1; $t = 1.55$, 99 d.f., $P = 0.12$). There is thus no evidence for a maternal or mitochondrial effect affecting pigmentation of these females, who are identical in nuclear genotype. The mean score of all F₁ females (4.45) is slightly lighter than the average score of females for the two species (5.43), showing a small amount of dominance for the *D. santomea* phenotype.

TABLE 3
Molecular markers and map positions

Marker	Marker name	Cytological location	<i>D. santomea</i>		<i>D. yakuba</i>	
			<i>r</i>	Genetic distance (cM)	<i>r</i>	Genetic distance (cM)
Chromosome X						
1	<i>y</i>	1A5	0.0432	0.0	0.0245	0.0
2	<i>per</i>	3B1-2	0.1572	4.5	0.1468	2.5
3	<i>sog</i>	13E1	0.1883	23.4	0.1364	19.9
4	<i>v</i>	9F11	0.3005	47.0	0.2690	35.8
5	<i>rux</i>	5D2	0.0570	93.0	0.0913	74.4
6	<i>f</i>	15F4-7	0.0777	99.0	0.1110	84.5
7	<i>bnb</i>	17D6	0.0363	107.5	0.0329	97.2
8	<i>Hex-A</i>	8E10	0.0760	111.2	0.0593	100.6
9	<i>AnnX</i>	19C1	0.0501	119.5	0.0254	106.9
10	<i>su(f)</i>	20E	0.0000	124.7	0.0000	109.5
Chromosome 2						
11	<i>l(2)gl</i>	21A5	0.0639	0.0	0.0865	0.0
12	<i>Rad1</i>	23A1	0.1054	6.8	0.0922	9.5
13	<i>RpL27A</i>	24F3	0.1364	18.7	0.1665	19.7
14	<i>salr</i>	32E4-F1	0.1002	34.6	0.1001	39.9
15	<i>Rep4</i>	34B4	0.2073	45.8	0.2023	51.2
16	<i>His3</i>	39D3-E1	0.0345	72.6	0.0132	77.1
17	<i>barr</i>	38B1-2	0.1140	76.1	0.1477	78.4
18	<i>Sara</i>	57E6	0.2694	89.1	0.2653	96.0
19	<i>Kr</i>	60F5	0.0000	127.8	0.0000	133.8
Chromosome 3						
20	<i>Lsp1γ</i>	61A6	0.2729	0.0	0.1966	0.0
21	<i>Dib</i>	64A5	0.2712	39.5	0.1345	25.0
22	<i>sfl</i>	65B3-4	0.2314	78.5	0.2700	40.6
23	<i>Est-6</i>	69A1	0.3057	109.6	0.2493	79.5
24	<i>SsII</i>	80B2	0.2211	156.9	0.1637	114.0
25	<i>ry</i>	87D9	0.2159	186.1	0.1797	133.8
26	<i>Rpn5</i>	83C4	0.0967	214.3	0.1176	156.1
27	<i>AP-50</i>	94A15-16	0.2297	225.1	0.1844	169.5
29	<i>Mlc1</i>	98A14-15	0.1036	255.8	0.1072	192.5
30	<i>ymp</i>	96E	0.2107	267.4	0.1110	204.6
28	<i>janB</i>	99D3	0.2055	294.8	0.1326	217.1
31	<i>krz</i>	100E3	0.0000	321.3	0.0000	232.5
Chromosome 4						
32	<i>ci</i>	102A1-3	0.0000	NA	0.0000	NA

r is the recombination rate between two adjacent markers. The genetic distance *d* was inferred from *r* using the Haldane map function, $d = -\frac{1}{2}\ln(1 - 2r)$. Cytological locations are given on the basis of *D. melanogaster* cytology (LEMEUNIER and ASHBURNER 1976).

QTL affecting variation in pigmentation in BC hybrids: We mapped four QTL with large effects on pigmentation (Table 4, Figure 1). The same four QTL were detected in male hybrids in the backcrosses to both *D. santomea* and *D. yakuba*: two QTL were on the X chromosome (between markers 1 and 2 and markers 6 and 10), one QTL was on the second chromosome (between markers 15 and 18), and one QTL was on the third chromosome (between markers 23 and 26). The magnitude of the QTL effects ranged from 0.49 to 1.42 phenotypic standard deviations in the backcross to *D. santomea* and accounted for 67% of the total phenotypic variation. Similarly, the QTL effects ranged from 0.62 to 1.62 phenotypic standard deviation in the backcross to *D. yakuba* and accounted for 58% of the

total phenotypic variation. In both backcrosses, the sum of the estimated QTL effects equaled or exceeded that expected from the difference between the parental genotypes. In the backcross to *D. santomea*, the expected difference in pigmentation is -11.29 [*i.e.*, the difference in pigmentation between *D. santomea* males (0.63) and ($Y \times S$) F_1 males (11.92)], whereas the sum of the QTL effects was -11.64 . In the backcross to *D. yakuba*, the expected difference in pigmentation is 10.5 [*i.e.*, the difference in pigmentation between *D. yakuba* males (14.22) and ($S \times Y$) F_1 males (3.72)], whereas the sum of the QTL effects is 16.15 . Thus, it is likely that we have detected all of the QTL affecting variation in pigmentation in this hybridization and that our selective genotyping protocol led to overestimation of effects

TABLE 4
QTL affecting variation in pigmentation between *D. yakuba* and *D. santomea*

Backcross population	Sex	QTL	Peak LR ^a	LR ^a	Effect (SE) ^b	Effect/ σ_p ^c	R ^{2d}
F ₁ females × <i>D. yakuba</i> males	F	1A5–13E1	1A5	26.71	0.68 (0.18)	0.31	0.0169
		15F4–20E	8E10	87.08	1.44 (0.56)	0.66	0.0584
		34B4–57E6	34B4	441.74	1.81 (0.76)	0.83	0.4382
		69A1–83C4	80B2	130.57	1.69 (0.69)	0.78	0.1135
	M	1A5–13E1	3B1-2	65.13	2.60 (0.65)	0.62	0.0327
		15F4–20E	17D6	525.94	6.75 (2.24)	1.62	0.3186
		34B4–57E6	34B4	193.19	3.29 (0.70)	0.79	0.1060
		69A1–83C4	80B2	216.23	3.51 (0.91)	0.84	0.1227
F ₁ females × <i>D. santomea</i> males	F	15F4–20E	19C1	53.485	−1.39 (0.68)	−1.27	0.4280
		1A5–13E1	1A5	67.88	−2.16 (0.45)	−0.61	0.0409
	M	15F4–20E	17D6	450.81	−5.03 (1.84)	−1.42	0.3964
		34B4–57E6	34B4	199.18	−1.73 (0.61)	−0.49	0.1397
		69A1–83C4	80B2	133.46	−2.72 (0.76)	−0.77	0.0966

^a QTL regions are estimated from 2 LOD support intervals ($P \leq 0.05$). The peak is the cytological location with the highest likelihood ratio (LR). Cytological locations are given on the basis of *D. melanogaster* cytology (LEMEUNIER and ASHBURNER 1976).

^b Effects were estimated from the least-squares means of the two marker locus classes as: $[\bar{x}_1 - \bar{x}_2]$, where the subscript is 1 if the marker has a homozygous genotype and 2 if the marker has a heterozygous or hemizygous genotype. The standard error (SE) is listed in parentheses.

^c Effect divided by the phenotypic standard deviation. See footnote ^a for the calculation of the effect.

^d The proportion of variance explained by the QTL and estimated by $R^2 = (s_0^2 - s_1^2)/s^2$, 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.* 1999).

(LYNCH and WALSH 1998). It is also possible that estimates of main effects have been biased by epistatic interactions (see below).

Four QTL in the same positions affected variation in pigmentation in the female *D. yakuba* BC hybrids. The magnitude of the QTL effects ranged from 0.31 to 0.83 phenotypic standard deviations and accounted for 63% of the total phenotypic variance. The sum of the estimated QTL effects slightly exceeded that expected from the difference between the parental genotypes in this backcross, again suggesting that we have detected all of the QTL affecting variation in pigmentation in this hybridization. The expected difference in pigmentation is 5.39 [*i.e.*, the difference in pigmentation between *D. yakuba* females (9.85) and F₁ females (4.46 on average)], whereas the sum of the QTL effects is 5.62. We observed only a single X chromosome QTL (between markers 6 and 10) in the *D. santomea* BC females, accounting for 43% of the total phenotypic variance. This could be attributable to a lack of power to detect QTL in this cross, since only 73 flies were assessed for genotype-phenotype associations, compared to >500 individuals in each of the other crosses. Indeed, this QTL accounted for only 40% of the expected difference in pigmentation [−3.44; *i.e.*, the difference in pigmentation between *D. santomea* females (1.02) and F₁ females (4.46 on average)].

The QTL effects were largely additive within loci. In the BC to *D. yakuba*, the effects of the two X chromosome QTL in females ($a - d$) were approximately half that

of the effects in males (2*a*), consistent with $d = 0$. In addition, the effects of the chromosome 3 QTL in males from the backcrosses to *D. yakuba* and *D. santomea* were equal and opposite, as expected if $d = 0$. The second chromosome QTL had a larger effect in the BC to *D. yakuba* than to *D. santomea*, suggesting partial dominance of the *D. santomea* genotype. Since dominance of *D. santomea* QTL reduces the power to detect QTL in the backcross to *D. santomea*, this could also account for our failure to detect this QTL in females from this backcross.

Epistatic interactions: We assessed all possible epistatic interactions between pairs of markers within each cross and sex (Table 5, Figures 2–4). We observed significant epistasis (after correcting for multiple tests) in males from both backcrosses between markers in regions encompassed by the QTL, but not between QTL regions and regions without main effects or between two regions with no main effects on pigmentation (Figure 2). The significant interactions were between the two X chromosome QTL, between the X chromosome QTL between markers 6 and 10 and the chromosome 2 QTL, between the X chromosome QTL between markers 6 and 10 and the chromosome 3 QTL, and between the chromosome 2 and chromosome 3 QTL (Figure 2, Table 5). The nature of these interactions is illustrated in Figure 3, where the effect of a Y-S substitution at the second locus is shown in the form of reaction norms, conditional on the genotype of the first locus (where Y denotes a *D. yakuba* allele and S denotes a *D. santomea* allele at the QTL). In the absence of epistasis, the effect

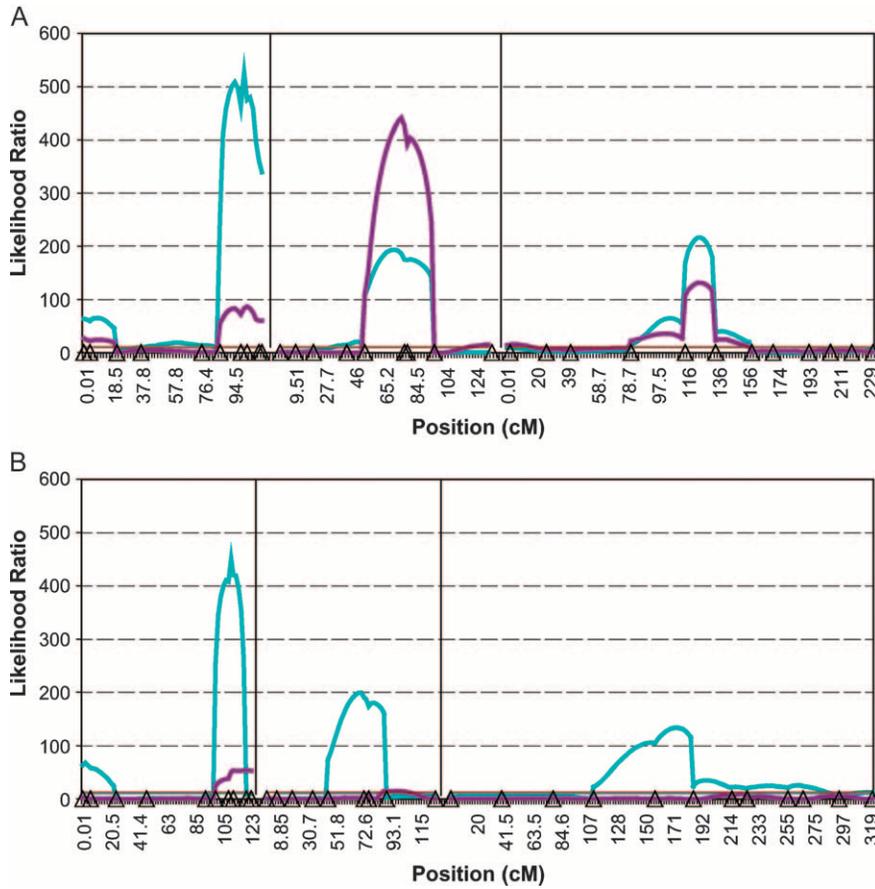


FIGURE 1.—QTL affecting variation in pigmentation between *D. yakuba* and *D. santomea*. (A) F₁ females [from *D. yakuba* (males) × *D. santomea* (females)] backcrossed to *D. yakuba* males. (B) F₁ females [from *D. yakuba* (females) × *D. santomea* (males)] backcrossed to *D. santomea* males. Molecular markers are indicated as triangles on the *x*-axis. Plots are likelihood-ratio (LR) test statistics for pigmentation differences between males (teal) and females (magenta) as determined by composite interval mapping. Significance thresholds for each cross were determined by permutation and are \sim LR = 10 for each cross, denoted by the dashed horizontal line.

of the substitution at the second locus would be independent of the genotype of the first, and the reaction norms would be parallel.

In the backcross to *D. santomea*, we expect the hemizygous Y or heterozygous SY genotype at the second locus to be more pigmented than the hemizygous S or homozygous SS genotype at this locus. However, for all the interacting markers in this backcross, this is true only if the genotype at the first locus is Y (or SY). Either there is no difference between the genotypes at the

second locus if the first is S (SS), or, for the case of the interaction between the second X chromosome QTL and the chromosome 2 QTL, the SS genotype at the chromosome 2 QTL is actually more pigmented than the SY genotype at this QTL when the X chromosome QTL is S (Figure 3). In other words, the effect of a YS substitution in an otherwise S background is smaller than the effect of an S-Y substitution at each QTL in the Y background. Equivalently, the effect of Y-S substitutions at two interacting loci in the homozygous S

TABLE 5

Epistatic effects of QTL affecting variation in pigmentation between *D. yakuba* and *D. santomea*

Backcross population	Sex	Markers	Effect (SE) ^a	Effect/ σ_p ^b	<i>P</i> -value
F ₁ females × <i>D. yakuba</i> males	M	1A5 × 19C1	1.38 (0.30)	0.33	<0.0001
		8E10 × 57E6	−1.54 (0.32)	−0.37	<0.0001
		8E10 × 87D9	−2.50 (0.33)	−0.60	<0.0001
		57E6 × 87D9	−1.33 (0.17)	−0.32	0.0001
	F	8E10 × 39D3–E1	−1.04 (0.25)	−0.48	<0.0001
F ₁ females × <i>D. santomea</i> males	M	1A5 × 19C1	1.29 (0.30)	0.36	0.0001
		8E10 × 57E6	2.59 (0.58)	0.73	<0.0001
		8E10 × 87D9	2.17 (0.47)	0.61	<0.0001
		57E6 × 87D9	1.64 (0.36)	0.46	<0.0001

^a See text for details. The standard error (SE) is given in parentheses.

^b The QTL effect divided by the phenotypic standard deviation.

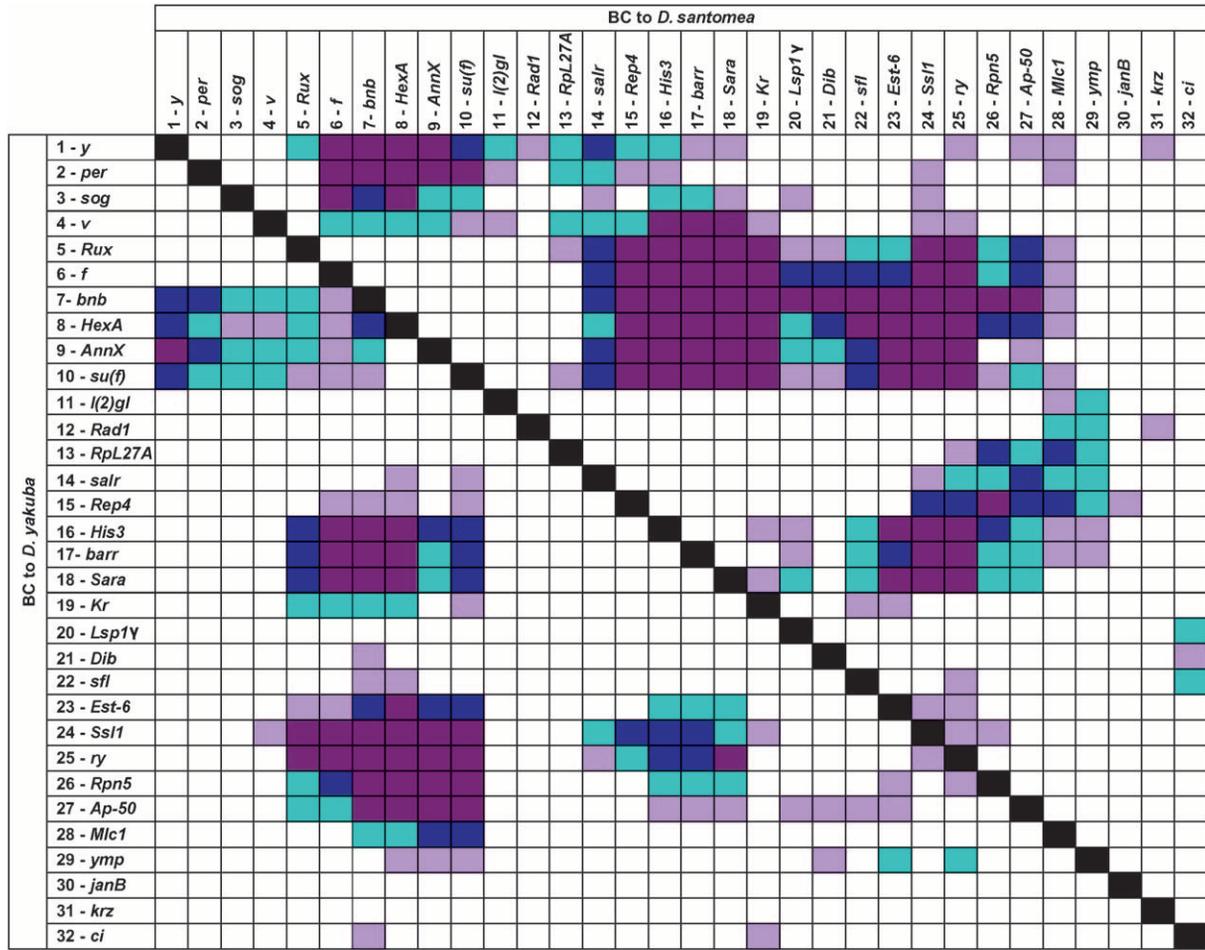


FIGURE 2.—Pairwise epistasis between markers. The significance of all pairwise interactions between markers for males from the backcross to *D. santomea* is indicated above the diagonal, and for interactions for males from the backcross to *D. yakuba* it is shown below the diagonal. (Magenta box) $P < 0.0001$ (Bonferroni correction); (blue box) $0.0001 < P < 0.001$; (teal box) $0.001 < P < 0.01$; (light purple box) $0.01 < P < 0.05$.

background is greater than additive, and the effect of S-Y substitutions at two interacting loci in the heterozygous SY background is less than additive. This is illustrated in Figure 4, where the sum of the effects of substituting single Y alleles at each QTL in the S background would yield a predicted pigmentation score of 4.09 for the YYYY haplotype, whereas the observed score is 9.43.

The epistatic interactions are more complicated in the backcross to *D. yakuba*. Here we expect the hemizygous Y or homozygous YY genotype at the second locus to be more pigmented than the hemizygous S or heterozygous SY genotype at this locus. In the interaction between the two X chromosome QTL (marker 1 × marker 9), this is true if marker 1 is Y. On the other hand, in the interactions with the chromosome 3 QTL (marker 8 × marker 25 and marker 18 × marker 25), this is true if marker 8 is S and marker 25 is SY (Figure 3). However, the interaction between the second X chromosome QTL and the chromosome 2 QTL (marker 8 × marker 18) is in the opposite direction to that expected: the SY genotype at marker 18 is actually more pig-

mented than the YY genotype at this marker, but only if marker 8 is Y. Overall, the effect of Y-S substitutions at two interacting loci in the heterozygous SY background is less than additive, and the effect of S-Y substitutions at two interacting loci in the homozygous YY background is greater than additive. Figure 4 shows that the sum of the effects of substituting single Y alleles at each QTL would yield a predicted pigmentation score of 15.15 for a YYYY haplotype, whereas the observed score is 13.43.

A single epistatic interaction was observed in females from the backcross to *D. yakuba*, between the second X chromosome QTL (markers 6–10) and the chromosome 2 QTL (data not shown). The direction of the epistatic effects between these QTL is the same as that in males from this hybridization. The effect of Y-S substitutions at the two interacting loci in the heterozygous SY background is less than additive, and the effect of S-Y substitutions at two interacting loci in the homozygous YY background is greater than additive. Figure 4 shows that the sum of the effects of substituting single Y alleles

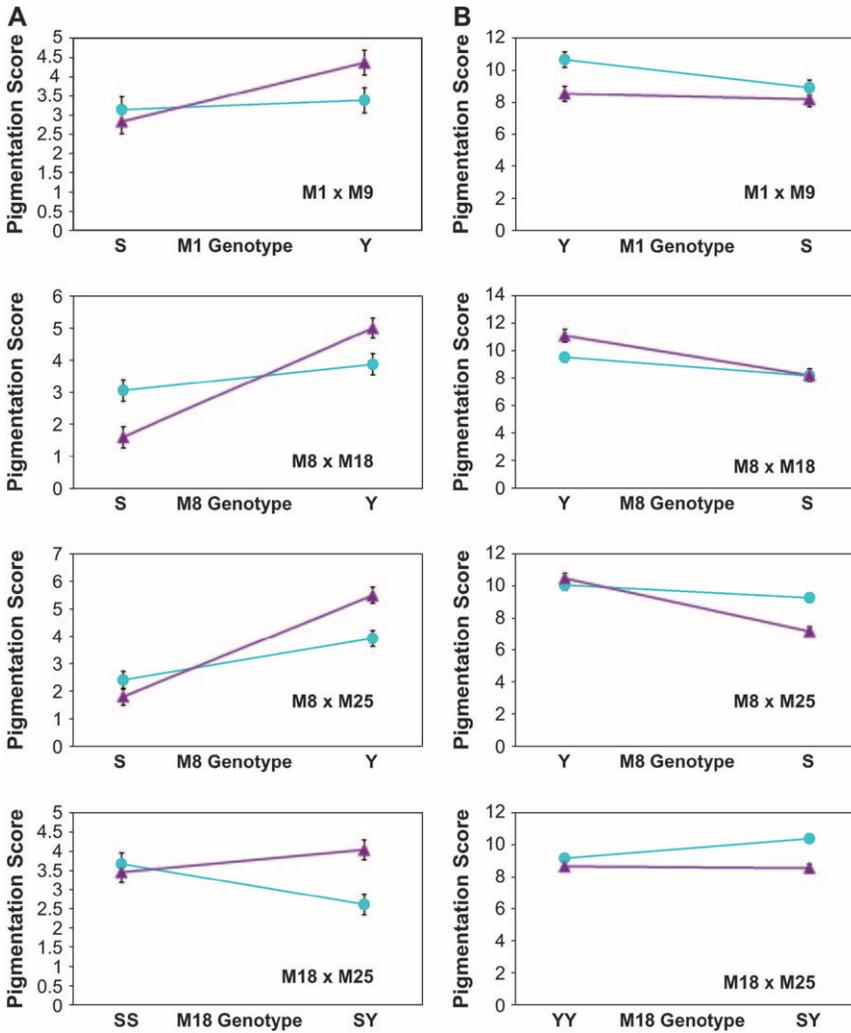


FIGURE 3.—Significant epistatic interactions between QTL, depicted as reaction norms. Values shown are the mean pigmentation scores (y-axis) for a particular genotype in the background of another genotype. (A) Backcrosses to *D. santomea* males. (B) Backcrosses to *D. yakuba* males. Epistatic interactions are shown for the significant markers common to both backcross populations, as shown in Figure 2 (M1 × M9, M8 × M18, M8 × M25, and M18 × M25). See Table 3 for marker definitions. Marker genotypes are indicated as *D. santomea* (A) or *D. yakuba* (B) homozygote (blue circle) and *D. santomea/D. yakuba* heterozygote (purple triangle).

at each QTL would yield a predicted pigmentation score of 11.17 for a YYYY haplotype, whereas the observed score is 9.49.

DISCUSSION

We have mapped at least four QTL with large effects associated with the variation in pigmentation between *D. yakuba* and *D. santomea*. The QTL mapped to the same locations in both males and females in the backcrosses to *D. yakuba* and in males in the backcross to *D. santomea*: two QTL mapped to the X chromosome and one each to the second and third chromosomes. Thus, the loss of pigmentation in *D. santomea* involved evolutionary changes in several genes, which probably affected both sexes. (Although only a single QTL was detected in females in the backcross to *D. santomea*, it mapped to the same location as one of the X chromosome QTL, and the small sample size of this population and narrow range of pigmentation conspire to reduce the power to detect QTL with small effects.)

This study not only largely expands and refines the earlier results of LLOPART *et al.* (2002a), in which pigmentation differences were assessed in backcross hybrids using eight molecular markers, but also provides the first accurate chromosomal locations of genetic factors associated with these differences. In LLOPART *et al.* (2002a), the QTL of largest effect was also associated with *AnnX* at the base of the X chromosome, with a second QTL with smaller effect at the tip of the X chromosome associated with *y*. The locations of the autosomal QTL are also concordant between the two studies. The QTL with the smallest effect detected by LLOPART *et al.* (2002a) was associated with the marker at *bric-à-brac 1* (*bab1*) at the tip of 3L, which is only marginally significant in the backcross to *D. yakuba* females in this study (LR = 14 between the *Lsp1γ* and *dib* markers). One possible explanation for this small discrepancy is that the *bab1* marker is in linkage disequilibrium with the chromosome 3 QTL mapped in this study. This, however, is not likely because the map distance between the major QTL detected on chromosome 3 and the *bab1* region is >100 cM. It is possible that

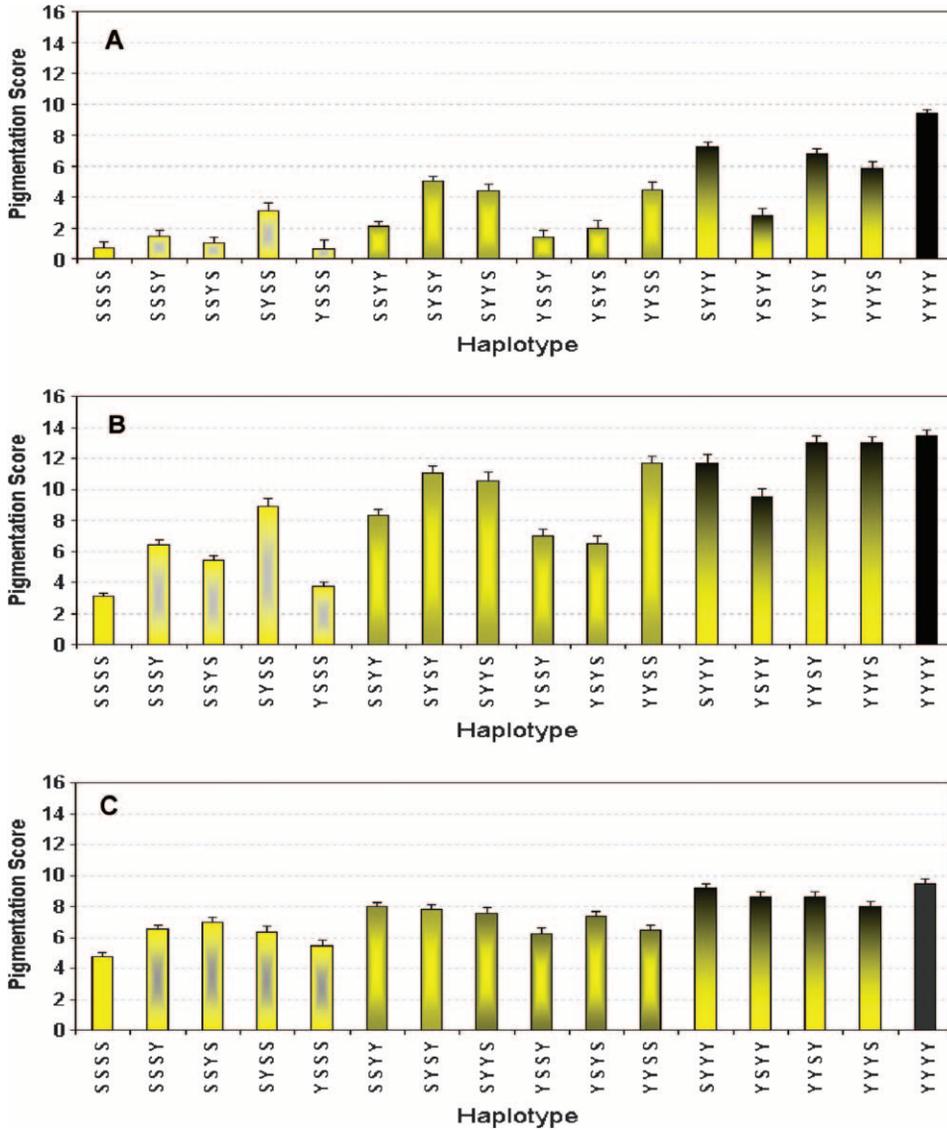


FIGURE 4.—Mean pigmentation scores (y-axis) of the eight marker haplotypes derived from the four QTL with large effects on pigmentation. The letters denote the genotype of the QTL allele (S, *D. santomea*; Y, *D. yakuba*), and the order of the letters indicates the genotype for the QTL at the tip of the X chromosome, the QTL at the base of the X chromosome, the second chromosome QTL, and the third chromosome QTL, respectively. Haplotypes refer to the markers at the peak LR in the respective QTL analyses. See Table 3 for marker descriptions. Haplotypes for male backcross hybrids are hemizygous *D. yakuba* and/or *D. santomea* for the two X chromosome QTL. The X chromosome QTL in female backcross hybrids and all autosomal QTL are heterozygous or homozygous; the marker genotype of the nonrecurrent parent is indicated. (A) Males from the backcross to *D. santomea*. Haplotypes are for markers M1, M7, M15, and M24. (B) Males from the backcross to *D. yakuba*. Haplotypes are for markers M2, M7, M15, and M24. (C) Females from the backcross to *D. yakuba*. Haplotypes are for markers M1, M8, M15, and M24. The bar graphs are color coded (increasing ratio of black to yellow) to indicate increasing number of *D. yakuba* alleles and pigmentation scores.

the discrepancy could be due to the fact that the methods used to score abdominal pigmentation in both studies, although correlated, are different.

The results presented here raise the interesting possibility that the genetic basis of pigmentation differences between *D. yakuba* and *D. santomea* is fairly simple. We infer that we have detected all major QTL accounting for variation in pigmentation in these backcross hybrids (with the exception of females in the backcross to *D. santomea*), since the sum of the QTL effects equals or exceeds that expected from the difference between parental strain means. Further high-resolution mapping is required to determine whether single genes or multiple closely linked loci are responsible for the large QTL effects. Nevertheless, all QTL from the same species affected pigmentation in the same direction, suggesting that the species difference might have arisen by natural selection (ORR 1998). We were not able to formally test this hypothesis, since a minimum of six QTL are required to reject the null hypothesis (ORR

1998). However, sequencing of the relevant loci may show, by the ratio of coding *vs.* noncoding substitutions, whether selection was involved in their divergence.

Only two other studies have investigated the genetic basis of pigmentation differences between closely related species of *Drosophila*. HOLLOCHER *et al.* (2000b) studied two Caribbean species in the *D. cardini* group having extremely different pigmentation patterns: *D. arawakana* (a light-colored sexually dimorphic species) and *D. nigrodunni* (the darkest sexually monomorphic species of the *D. dunni* subgroup). Using quantitative measures of abdominal pigmentation in F₁ hybrids and backcross flies, the authors conclude that, at least for the posterior segment of the abdomen (HOLLOCHER *et al.* 2000a, Figure 2, “area 3”), which is roughly equivalent to the area scored in our analysis, there are paternal and maternal effects, with no particular effect of the X chromosome. The second study mapped QTL for the difference in pigmentation between *D. americana* and *D. novamexicana* (WITTKOPP *et al.*

2003), using 23 molecular markers. Five genes (*y*, *e*, *Ddc*, *omb*, and *bab*) previously implicated in the development and evolution of abdominal pigmentation were used as markers. The authors indicate that this species difference is polygenic with no significant effect of the X chromosome but with significant effects of three of the five autosomes. There is little genetic commonality between the results reported by these two studies and our results. Of course, unless there are a very limited number of genes that could be potentially responsible for differences in pigmentation, one does not expect the genetic architecture to be shared among distantly related species.

Our observations of epistatic interactions between QTL with main effects on pigmentation are consistent with genes corresponding to the QTL that are in the same pathway(s). In the absence of high-resolution mapping, however, we can only speculate about what candidate genes might correspond to the QTL. An obvious candidate for the QTL at the tip of the X chromosome is *yellow* (*y*) itself, and complementation tests to *D. santomea* using a *y* mutation in *D. yakuba* are consistent with a very small contribution of mutations at the *y* locus in the pigmentation difference between these species (LLOPART *et al.* 2002a). In addition, two enhancers of *y* are located in the region embraced by the QTL at the base of the X chromosome; these could contribute to the interactions between the two X chromosome QTL.

Several candidate genes affecting body pigmentation have been identified by mutagenesis in *D. melanogaster* (DRYSDALE and CROSBY 2005) and colocalize to the regions containing QTL affecting pigmentation differences between *D. yakuba* and *D. santomea* (Table 6). Catecholamines are required for proper melanization and sclerotization of the *Drosophila* cuticle (WRIGHT 1987; WALTER *et al.* 1996). The *Ddc* gene cluster on chromosome 2 [genetically defined by *Df(2L)TW130; 37B9–C1,2;D1–2*] contains at least 18 functionally related genes involved in the catecholamine pathway, including *Catsup*, *Ddc*, *Dox-A2*, *amd*, and *l(2)37Ca* (STATHAKIS *et al.* 1995). Mutations in 11 of the loci in this complex (including *Ddc* and *amd*) produce melanotic pseudotumors, indicating abnormal catecholamine metabolism (WRIGHT 1996), and mutations in 14 of the loci affect the formation, sclerotization, or melanization of the cuticle (WRIGHT 1996). The *Ddc* cluster colocalizes with the QTL on chromosome 2. *Pu* encodes GTP cyclohydrolase, the rate-limiting step in the synthesis of tetrahydrobiopterin, the cofactor required for the phosphorylation of tyrosine hydroxylase, which is in turn the rate-limiting step in the synthesis of dopamine (STATHAKIS *et al.* 1999). *Pu* also colocalizes with the QTL on chromosome 2. The *silver* (*svr*) gene, which encodes proteins that are members of the carboxypeptidase family (SETTLE *et al.* 1995), colocalizes with the QTL at the tip of the X chromosome. Mutations in *svr*

affect pigmentation, wing shape, and catecholamine pools (WRIGHT 1987). *tan* (*t*) is an excellent candidate gene corresponding to the QTL at the base of the X chromosome. *t* is probably the structural gene for β -alanyl dopamine hydrolase activity; *t* mutants have reduced dopamine levels (WRIGHT 1987).

Additional candidate genes in the pigmentation pathway include *optomotor-blind* (*omb*), *black* (*b*), *Cysteine proteinase-1* (*Cp1*), and *Black cells* (*Bc*) (WRIGHT 1987; WITTKOPP *et al.* 2002, 2003). The developmental gene, *omb*, colocalizes with the QTL at the tip of the X chromosome. *omb* encodes a T-box transcription factor that is necessary for patterning the pigment band in each adult abdominal segment of *D. melanogaster* (KOPP and DUNCAN 1997, 2002). A recent study by BRISSON *et al.* (2004) examined patterns of nucleotide variation at the *omb* locus in *D. polymorpha*, a species highly polymorphic for abdominal pigmentation. Two classes of haplotypes that appear to be under balancing selection were associated with variation in abdominal pigmentation in this species.

b, *Cp1*, and *Bc* all colocalize with the chromosome 2 QTL. *b* encodes a product involved in β -alanine biosynthesis; *b* mutants are heavily pigmented. *Cp1* encodes a product with cathepsin L activity; deletion studies of *Cp1* have shown complete female sterility and reduced pigmentation in abdominal segments 1–5 (GRAY *et al.* 1998). *Bc* encodes a tyrosinase, which catalyzes the *de novo* synthesis of melanin from tyrosine (WITTKOPP *et al.* 2003).

Conspicuously absent from the list of strong potential candidate genes are *bab1* and *bab2*, two closely linked genes at the tip of chromosome 3L that are thought to be repress male-specific abdominal pigmentation in females (KOPP *et al.* 2000) and contribute significantly to variation of abdominal pigmentation in females of *D. melanogaster* (KOPP *et al.* 2003). The expression of *bab* is correlated with pigmentation across a diverse range of *Drosophila* species, such that species in which neither sex is pigmented exhibit similar expression of *Bab* in males and females, but species in which abdominal tergites of males are more pigmented than those of females have female-specific *Bab* expression (KOPP *et al.* 2000). Thus, it was possible *a priori* that overexpression of *Bab* in *D. santomea* could have resulted in loss of pigmentation in both sexes. This is not the case, however, since none of the QTL map in the vicinity of *bab*. Further, *Bab2* protein is expressed in a dimorphic *melanogaster*-like pattern in *D. santomea* (GOMPEL and CARROLL 2003), which is inconsistent with mutations at *bab* affecting the difference in pigmentation between *D. santomea* and *D. yakuba*.

While it is plausible that the loss of pigmentation in *D. santomea* was driven by natural selection, it is also possible that selection acted on pleiotropic effects of genes affecting pigmentation and not pigmentation itself. All of the candidate genes listed in Table 3 have

TABLE 6
Candidate genes

Symbol	Gene name	Cytological location	Function ^a
<i>cin</i>	<i>cinnamon</i>	1A1	Mo- molybdopterin cofactor biosynthesis
<i>y</i>	<i>yellow</i>	1A5	Cuticle pigmentation
<i>a(1)HM26</i>	<i>abnormal abdomen HM26</i>	1B1–5C2	Mutants affect abdominal tergite and sternite
<i>mk</i>	<i>murky</i>	1B1–5C2	Oogenesis
<i>svr</i>	<i>silver</i>	1B5–7	Cuticle biosynthesis
<i>su(b)</i>	<i>suppressor of black</i>	<1B8	Interacts genetically with <i>black</i>
<i>su(s)</i>	<i>suppressor of sable</i>	1B13	Transcriptional repressor activity
<i>dor</i>	<i>deep orange</i>	2B5	Pteridine biosynthesis
<i>A</i>	<i>Abnormal abdomen</i>	3A5	Mutations affect the abdominal tergite
<i>l(1)3B2</i>	<i>lethal(1)3B2</i>	3B2	Mutations affect the tergite
<i>omb</i>	<i>optomotor-blind</i>	4C3–4	Patterning the pigment band
<i>lac</i>	<i>lacquered</i>	4C6	Mutations are body color defective
<i>amb</i>	<i>amber</i>	4C6–D1	Mutations are recessive body color defective
<i>pt</i>	<i>platinum</i>	7F1	Mutations are body color defective
<i>t</i>	<i>tan</i>	8A1–B8	β-Alanyl-dopamine hydrolase activity
<i>s</i>	<i>sable</i>	11F1–12A1	Encodes a product involved in pigmentation
<i>e(y)1</i>	<i>enhancer of yellow-1</i>	16E1	Interacts genetically with <i>y</i> , <i>w</i> , <i>z</i> , <i>ct</i> , and <i>sc</i>
<i>e(y)3</i>	<i>enhancer of yellow-3</i>	18C–D	Interacts genetically with <i>y</i> , <i>w</i> , <i>z</i> , <i>ct</i> , and <i>sc</i>
<i>mel</i>	<i>melanized</i>	19B3–C3	Mutations are recessive body color defective
<i>mal</i>	<i>maroon-like</i>	19D1	Mo-molybdopterin cofactor biosynthesis
<i>mel1</i>	<i>melanized-like</i>	19E1+	Mutations affect the abdominal tergite
<i>vao</i>	<i>varied outspread</i>	19E7	Mutations are body color defective
<i>su(f)</i>	<i>suppressor of forked</i>	20E	Encodes a product with putative poly(A) binding
<i>b</i>	<i>black</i>	34D5	Glutamate decarboxylase activity
<i>yellow-c</i>	<i>yellow-c</i>	35B8	Cuticle pigmentation
<i>Catsup</i>	<i>Catecholamines up</i>	37B11	Regulation of catecholamine metabolism
<i>Dox-A2</i>	<i>Diphenol oxidase A2</i>	37B12	Endopeptidase activity
<i>Ddc</i>	<i>Dopa decarboxylase</i>	37C1	Dopamine biosynthesis; pigmentation patterning
<i>amd</i>	<i>α-methyl dopa-resistant</i>	37C1	Involved in cuticle biosynthesis
<i>l(2)37Ca</i>	<i>lethal(2)37Ca</i>	37C5	Interacts genetically with <i>Ddc</i>
<i>tyr1</i>	<i>tyrosine-1</i>	38A6–C1	Mutations are body color defective
<i>pr</i>	<i>purple</i>	38B3	Involved in pteridine biosynthesis
<i>Bkd</i>	<i>Blackoid</i>	43E18–52D7	Mutations are body color defective
<i>dkb</i>	<i>dark bubbly</i>	<49D7	Mutations are body color defective
<i>Cp1</i>	<i>Cysteine proteinase-1</i>	50C18–20	Mutations affect the abdominal segment 1–5
<i>U</i>	<i>Upturned</i>	53A	Mutations are body color defective
<i>Bc</i>	<i>Black cells</i>	54F6	Involved in melanization defense response
<i>Pu</i>	<i>Punch</i>	57C7–8	Tetrahydrobiopterin biosynthesis
<i>D</i>	<i>Dichaete</i>	70D3	Mutations affect the abdominal segment 3–7
<i>db</i>	<i>dark body</i>	73C1–D2	Mutations are body color defective
<i>Crn</i>	<i>Crown</i>	<77B3	Mutations are dominant body color defective
<i>kkv</i>	<i>krotzkopf verkehrt</i>	83A1	Chitin synthase activity

^a In cases where the function is unclear, mutant phenotypes are listed. All information, including cytological locations, was retrieved from the FlyBase website (<http://www.flybase.org>) (DRYSDALE and CROSBY 2005).

highly pleiotropic effects on traits related to fitness, including reproduction and immune response. For example, *Ddc* catalyzes the final step in the biosynthesis of the neurotransmitters dopamine and serotonin. Dopamine is required in *Drosophila* for normal development (NECKAMEYER 1996); ovarian maturation, fecundity and sexual receptivity in females (NECKAMEYER 1996, 1998a); learning (TEMPEL *et al.* 1984; NECKAMEYER 1998b); locomotion (PENDLETON *et al.* 2002); and aggressive behavior (BAIER *et al.* 2002). Serotonin also regulates or modulates a variety of behaviors in many

animal species, including aggression, feeding, learning, locomotion, sleep, and mood (BLENAU and BAUMANN 2001). Further speculation about the nature of the pleiotropic effects (and sex-specific epistatic effects) of genes affecting variation in pigmentation between these species must await the positional cloning of these genes.

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