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Natural Genetic Variation in Cuticular Hydrocarbon Expression in Male and Female *Drosophila melanogaster*

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ABSTRACT

Cuticular hydrocarbons (CHCs) act as contact pheromones in *Drosophila melanogaster* and are an important component of several ecological traits. Segregating genetic variation in the expression of CHCs at the population level in *D. melanogaster* is likely to be important for mate choice and climatic adaptation; however, this variation has never been characterized. Using a panel of recombinant inbred lines (RILs) derived from a natural population, we found significant between-line variation for nearly all CHCs in both sexes. We identified 25 QTL in females and 15 QTL in males that pleiotropically influence CHC expression. There was no evidence of colocalization of QTL for homologous traits across the sexes, indicating that sexual dimorphism and low intersex genetic correlations between homologous CHCs are a consequence of largely independent genetic control. This is consistent with a pattern of divergent sexual and natural selection between the sexes.

CUTICULAR hydrocarbons (CHCs) are important for diverse functions in insects and have been studied extensively for their roles in mate and species recognition and ecology (Tillman *et al.* 1999). Of particular interest in Drosophila has been the role of CHCs in sexual signaling, and in *Drosophila melanogaster* the biosynthesis, genetic regulation, and sex specificity of CHC expression has been well described. Much work has been conducted describing the role of CHCs in sex recognition (Savarit and Ferveur 2002a) and the genetic basis of CHC-mediated sexual isolation between closely related species in the *D. melanogaster* species subgroup (*e.g.*, Coyne 1996) and between intraspecific races of *D. melanogaster* (*e.g.*, Fang *et al.* 2002). Although segregating genetic variation for CHC expression has been demonstrated in other species of Drosophila and is associated with variation in mate choice (Blows and Higgie 2002) and traits involved in ecological adaptation such as desiccation resistance (Rouault *et al.* 2004), genetic variation for CHC expression within populations of *D. melanogaster* has not yet been characterized.

In many Drosophila, including *D. melanogaster*, the mate choice system is composed of several distinct elements, including a courtship dance, wing song, and the assessment of the CHCs of a potential mate through olfaction, gustation, and chemoreceptors in the front legs (for a general review, see Greenspan and Ferveur 2000). Most of the interracial and interspecies work in Drosophila mate recognition has focused on the importance of CHCs as a signal (Ferveur 2005), although the genetics of wing song and its role in mate choice has also been studied (for instance, Ritchie and Kyriacou 1996). CHCs have been implicated in mediating assortative mating between the "countryside" and "urban" strains of *D. melanogaster* in the Congo (Haerty *et al.* 2002). They are also correlated with behavioral isolation between the two major cosmopolitan and African races of *D. melanogaster* (Takahashi and Ting 2004), although the precise causative role of CHCs in this isolation is unclear (Coyne *et al.* 1999). At the among-species level, variation in CHC expression appears to contribute to reproductive isolation between *D. melanogaster* and its sister clade of *D. simulans*, *D. sechellia*, and *D. mauritiana* (Coyne *et al.* 1994). CHC differences between species within the *D. melanogaster* species subgroup also vary geographically in a manner that suggests the operation of reinforcement (Cobb and Jallon 1990).

CHCs are highly sexually dimorphic in *D. melanogaster*, with many of the compounds present in one sex absent in the other, while shared compounds often differ between the sexes (Jallon and David 1987). Sexual dimorphism, such as that observed in the CHCs of *D. melanogaster*, is expected to result from sex-specific selection. When considering sexual display traits, the classic quantitative genetic model describing the evolution of sexual dimorphism regards sex-specific sexual selection as the primary mechanism driving evolutionary divergence between the sexes (Lande 1980). Several studies have used mapping approaches to investigate within-species patterns of genetic variation in sexual traits of

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D. melanogaster other than CHCs (Wayne *et al.* 2001; Gleason *et al.* 2002; McMahon *et al.* 2002; Drnevich *et al.* 2004; Moehring and Mackay 2004), but these studies have generally utilized established laboratory stocks or lines derived from laboratory stocks, rather than examining standing genetic variation within populations.

The large differences between races of *D. melanogaster* and between other closely related species have often been studied using mutant approaches (Ferveur and Jallon 1993; Savarit *et al.* 1999; Marcillac *et al.* 2005) or mapping and introgression techniques (Coyne 1996; Takahashi *et al.* 2001; Greenberg *et al.* 2003). It remains to be seen whether these genes are important in mediating population-level variation. Of the genes and genomic regions identified by these studies, the genetic factors identified have been generally expressed in a single sex. For instance, *desat2* in females (Fang *et al.* 2002) corresponds to a major difference in CHC expression between the African and cosmopolitan races of *D. melanogaster*, and *smoq* plays a similar role in males (Ferveur and Jallon 1996). While these sex-specific genetic factors act to modify the products of CHC biosynthesis and result in strong qualitative sexual dimorphism in *D. melanogaster*, the presence of these loci does not indicate to what extent genetic variance for CHC is sex specific and whether shared compounds are highly genetically correlated between the sexes.

Work in natural populations of a closely related species, *D. serrata*, of the *montium* subgroup, indicates that CHCs are sexually selected as a consequence of mate choice within populations when measured on either lab-reared (Blows *et al.* 2004) or field-collected phenotypes (Hine *et al.* 2004; Petfield *et al.* 2005). The consequence of sexual selection acting on CHCs in Drosophila can also be seen in the patterns of variation in CHC expression between species and races of Drosophila (Jallon and David 1987), which have been shown to be important in mate recognition (Cobb and Jallon 1990), but naturally occurring genetic variation in CHCs at the population level has yet to be characterized in *D. melanogaster*. Given the importance of CHCs in generating sexual isolation between races of *D. melanogaster* and other species, and their importance in sexual selection in the closely related *D. serrata*, we focus here on the genetic basis of CHC expression in *D. melanogaster* recently derived from a natural population.

First, we assay naturally occurring genetic variation for the entire *D. melanogaster* CHC phenotype (31 compounds in males and 53 compounds in females) using a panel of recombinant inbred lines (RILs). Second, after finding substantial genetic variation for >95% of compounds, we mapped QTL for CHC expression in both sexes. Finally, segregating variation in CHC expression was compared with previous descriptions of genetic differences among races of *D. melanogaster* and between *D. melanogaster* and closely related species.

MATERIALS AND METHODS

Line development and marker analysis: The Winters lines are a panel of 144 lines derived from a cross of a single male progeny and a single female progeny of two field-caught, inseminated females. The F_2 single-pair families were established, further isogenized by 25 generations of full-sib inbreeding, and then scored for marker state and retained for phenotypic analysis (described in detail by Kopp *et al.* 2003) although, due to attrition of inbred lines during transport and lab maintenance, not all traits were subsequently measured in all lines. The lines were genotyped for 152 variable *roo* inserts by *in situ* hybridization for the presence, absence, or polymorphism of a marker at a given cytological locus, giving a map coverage of ~1/2 cM (Kopp *et al.* 2003). Any marker that was scored as segregating in a line was dropped from any further analyses. There was a single inversion on chromosome 3 (3R Payne, ~89EF, 96A). Given the design of the cross, there were potentially four parental haplotypes for each of the autosomes and three for the X chromosome. However, when the probable parental haplotypes were reconstructed (Kopp *et al.* 2003), two of the parental third chromosomes appear to have been homologous, except for a small region near the tip. All linkage groups subsequently described represent these parental haplotypes.

CHC phenotyping: Virgins were collected from the lines within 8 hr of eclosion (female line $n = 124$; male line $n = 126$) and aged in single-sex vials for 4 days. At least five flies of each sex per line were anesthetized with CO_2, washed individually in 100 μl of hexane for 4 min, and vortexed for another minute before being removed (female $n = 697$; male $n = 652$) (Blows and Allan 1998). All samples were run on an Agilent 6890N gas chromatograph. Relative proportions of CHCs were transformed to logcontrasts to remove the unit-sum constraint associated with compositional data (Blows and Allan 1998).

Previous studies of Drosophila CHCs have focused primarily on the most abundant CHCs (*e.g.*, Cobb and Jallon 1990; Coyne *et al.* 1994; Ferveur and Jallon 1996; Dallerac *et al.* 2000). While abundant CHCs may play a role in sexual isolation, less abundant CHCs are often implicated as being under sexual selection in Drosophila (Blows *et al.* 2004; Hine *et al.* 2004). Thus we took a comprehensive approach and analyzed all compounds that could be measured using gas chromatography. In females, 53 compounds were analyzed, while 31 were analyzed in males (Figure 1). For each individual, the area under each CHC peak in a chromatogram was integrated and expressed as a proportion of the total integrated area for all CHCs. These proportions were transformed into logcontrasts, an appropriate transformation when dealing with compositional data such as hydrocarbon blends as they break the unit-sum constraint associated with proportions and also have a covariance matrix that is nonsingular, unlike more commonly used log ratios (Atchison 1986; Blows *et al.* 2004). Calculation of logcontrasts reduces trait number to $n - 1$; therefore, further analyses of traits were conducted on 52 logcontrasts in females and on 30 in males. Of these, only 20 CHCs were shared between the sexes, and a great deal of sexual dimorphism was evident in the relative abundance of shared compounds as well (Figure 1). For the most part, the most abundant compounds in males are those with 23–25 carbons, whereas in females there is a greater proportion of compounds with 25–27 carbons. In this article, linear alkanes are referred to by the abbreviation C*n*, where "*n*" is the carbon number; thus pentacosane is designated C25, for example. Methylated alkanes are referred to with a 2Me prefix, as all methyl groups are attached to the second carbon in the chain; thus 2-methyl-hexacosane is abbreviated 2MeC26. The position of double bonds is referred to before a colon. The compound

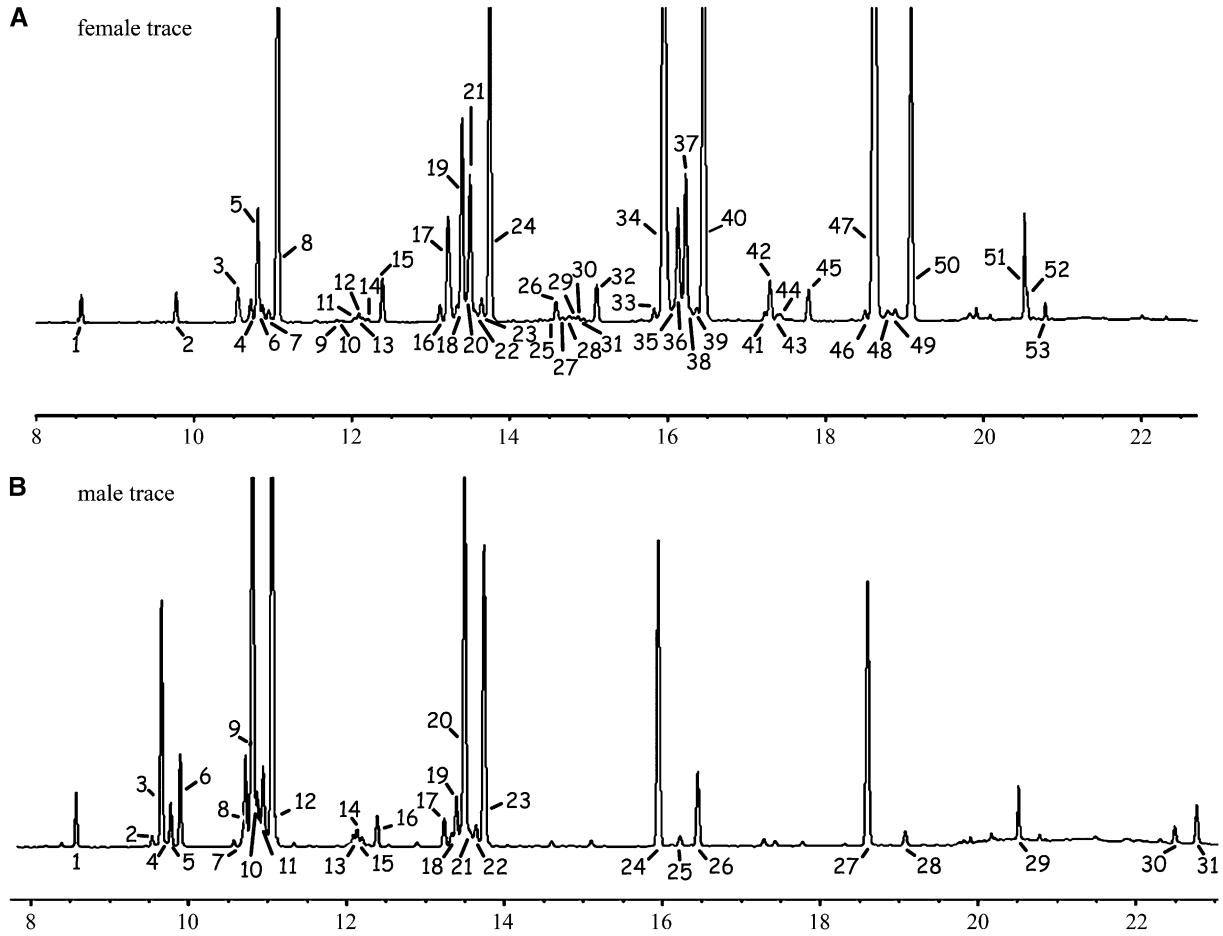


FIGURE 1.—Gas chromatograph traces of cuticular hydrocarbons of *D. melanogaster* for (A) females and (B) males. (A) 1–9:C21; 2–C22; 3–7, 11:C23; 4–9:C23; 5–7:C23; 6–5, 9:C23; 7–5:C23; 8–C23; 9–9, 13:C24; 10–7, 11:C24; 11–9:C24; 12–7:C24; 13–5, 9:C24; 14–5:C24; 15–C24; 16–9,13:C25; 17–7,11:C25; 18–2MeC24; 19–9:C25; 20–6, 10:C25; 21–7:C25; 22–5, 9:C25; 23–5:C25; 24–C:25; 25–9, 13:C26; 26 –7, 11:C26; 27–2MeC25; 28–9:C26; 29–7:C26; 30–5, 9:C26; 31–5:C26; 32–C26; 33–9, 13:C27; 34–7, 11:C27; 35–9:C27; 36–2MeC26; 37–7:C27; 38–5, 9:C27; 39–5:C27; 40–C27; 41–9, 13:C28; 42–7, 11:C28; 43–2MeC27; 44–9:C28; 45–C28; 46–9, 13:C29; 47–7, 11:C29; 48–9:C29; 49–7:C29; 50–C29; 51–7, 11:C31; 52–2MeC30; 53–C31. (B) 1–C21; 2–2MeC20; 3–9:C22; 4–7:C22; 5–5, 9:C22; 6–C22; 7–2MeC22; 8–9:C23; 9–7:C23; 10–5, 9:C23; 11–5:C23; 12–C23; 13–9:C24; 14–7:C24; 15–5, 9:C24; 16–C24; 17–2MeC24; 18–7, 11:C25; 19–9:C25; 20–7:C25; 21–5, 9:C25; 22–5:C25; 23–C25; 24–2MeC26; 25–7:C27; 26–C27; 27–2MeC28; 28–C29; 29–2MeC30; 30–2MeC32; 31–C33.

9-tricosene is referred to by the abbreviation 9:C23, and the compound 7, 11-heptacosadiene is abbreviated 7, 11:C27.

QTL analysis: QTL analysis was performed on the line–mean logcontrasts of 50 CHC traits in females and 30 in males, which exhibited significant among-line variance using ANOVA. The association between individual markers and traits was estimated in SAS (SAS Institute, Cary, NC) using a GLM procedure (DOERGE and CHURCHILL 1996; WANG *et al.* 2004). The null hypothesis of no association between marker and trait was tested for each trait by randomly permuting the marker-trait associations1000 times and recording the most significant probability of association between marker and trait for each of the permutations. The statistic for marker-trait association is above the $P = 0.05$ threshold where it exceeds 95% of these highest permuted values (DOERGE and CHURCHILL 1996).

Composite interval mapping (CIM) was performed on the same traits using the software QTL Cartographer (WANG *et al.* 2001–2004). As has been discussed elsewhere (MEZEY *et al.* 2005), the standard QTL-modeling software is not designed to analyze crosses containing up to four parental haplotypes. However, the two-allele model in QTL Cartographer can be used to analyze the effects of each marker present in a linkage group against the mean of the trait for the other linkage groups (where the *roo* insert is absent). For each of the parental haplotypes (three for the X chromosome, four for the second chromosome, and three for the third chromosome) a separate likelihood function was derived, using recombination distances as calculated by MEZEY *et al.* (2005). The location of QTL for expression of all CHCs in both sexes was estimated by QTL Cartographer, using the CIM option with a walk speed of 2 cM, a window size of 10 cM, and the significance threshold estimated with 1000 permutations.

To determine whether more CHC QTL are shared between the sexes than are expected by chance—which would indicate some level of shared genetic regulation of CHC expression—we performed a resampling test (MACDONALD and GOLDSTEIN 1999). We transposed the position of each of the male CHC QTL onto the female LOD profile and summed the LOD scores of all the 19 homologous CHCs across each of these QTL, both for individual traits and for all homologous CHCs at once. We compared these values with the ranked sum of

LOD scores obtained by randomizing QTL position 10,000 times. The significance level for QTL coincidence is >0.05 in cases for which the observed sum of LOD scores exceeds 95% of the randomized values.

Evaluating agreement in QTL location between traits: For all significant individual-trait QTL, 95% confidence intervals were calculated by dropping down 2-LOD scores along the likelihood function (Van Ooijen 1992; Mangin *et al.* 1994; Lynch and Walsh 1998). As a conservative estimate, we assumed that putative QTL for different traits with overlapping 95% confidence intervals were potentially a single, pleiotropic QTL. Pleiotropic QTL were assumed when CIM found significant LOD maxima for multiple traits at identical locations. Where two single-trait QTL had nonoverlapping confidence intervals, a minimum of two QTL were assumed in the region. While this tends to overestimate the pleiotropy of individual QTL and underestimate the number of QTL, this parsing procedure was generally a straightforward means of resolving an estimate of QTL numbers in a chromosomal region.

For presumed pleiotropic QTL, the likelihood maximum was estimated as the mode of the maxima of contributing-trait QTL. If there were equal numbers of two or more local maxima, the map position was taken to be the mean of those maxima. All QTL were plotted on a centimorgan scale (Figure 2) for each parental haplotype. Confidence intervals were calculated for these QTL using a bootstrapping procedure (Lebreton and Visscher 1998), although these estimates are likely to be conservative (Manichaikul *et al.* 2006). For each sex and linkage group, RILs were sampled with replacement and CIM was performed on each bootstrapped replicate. The computational overhead associated with the bootstrapping procedure was significant; thus we selected a candidate trait from each QTL to perform the bootstrapping procedure. For each new QTL, the associated trait with the highest LOD score was retained, as confidence intervals estimated on strongly supported QTL have been shown to be more accurate and more tightly resolved (Visscher *et al.* 1996; Lebreton and Visscher 1998). For each QTL, 150 bootstrap replicates were used to estimate the confidence intervals, which is sufficient for accurate QTL confidence interval estimation.

Comparison with other studies: Several QTL and deficiency mapping studies have implications for this analysis. Most directly relevant is another study of the Winters lines (Wang *et al.* 2004) examining starvation susceptibility and longevity in both sexes. A deficiency mapping study of differences in female CHC production between *D. melanogaster* and *D. simulans* (Coyne 1996) was considered, as well as a QTL study of female reproductive success and ovariole number (Wayne *et al.* 2001), given the association between ovariole production and CHC expression (Wicker and Jallon 1995). All published confidence intervals of QTL and deletion intervals were already expressed in a cytogenic scale. For comparative purposes, all LOD maxima and confidence interval endpoints of CHC QTL were plotted onto the cytological scale according to their relative position between flanking markers.

A number of genetic studies of *D. melanogaster* CHC expression have been conducted. These have focused mainly on mutagenesis of lab stocks and genetic differences between the cosmopolitan and Zimbabwean races of *D. melanogaster* (Dallerac *et al.* 2000; Wicker-Thomas and Jallon 2000, 2001; Savarit and Ferveur 2002a). We considered genes identified in these studies to be potential candidates. As well as examining genes that have already been identified as contributing to genetic variation in CHC expression in Drosophila, we also queried FlyBase for genes that might be directly involved in CHC biosynthesis, using the key words "fatty acid biosynthesis," "very-long-chain-fatty acid biosynthesis," "very-long-chain-fatty acid metabolism," "regulation of fatty acid biosynthesis," and "fatty acid desaturation" (supplemental Table 3 at http://www.genetics.org/supplemental/). Many of the latter genes have not been cited in other studies, but have had functions inferred through sequence similarity with known genes.

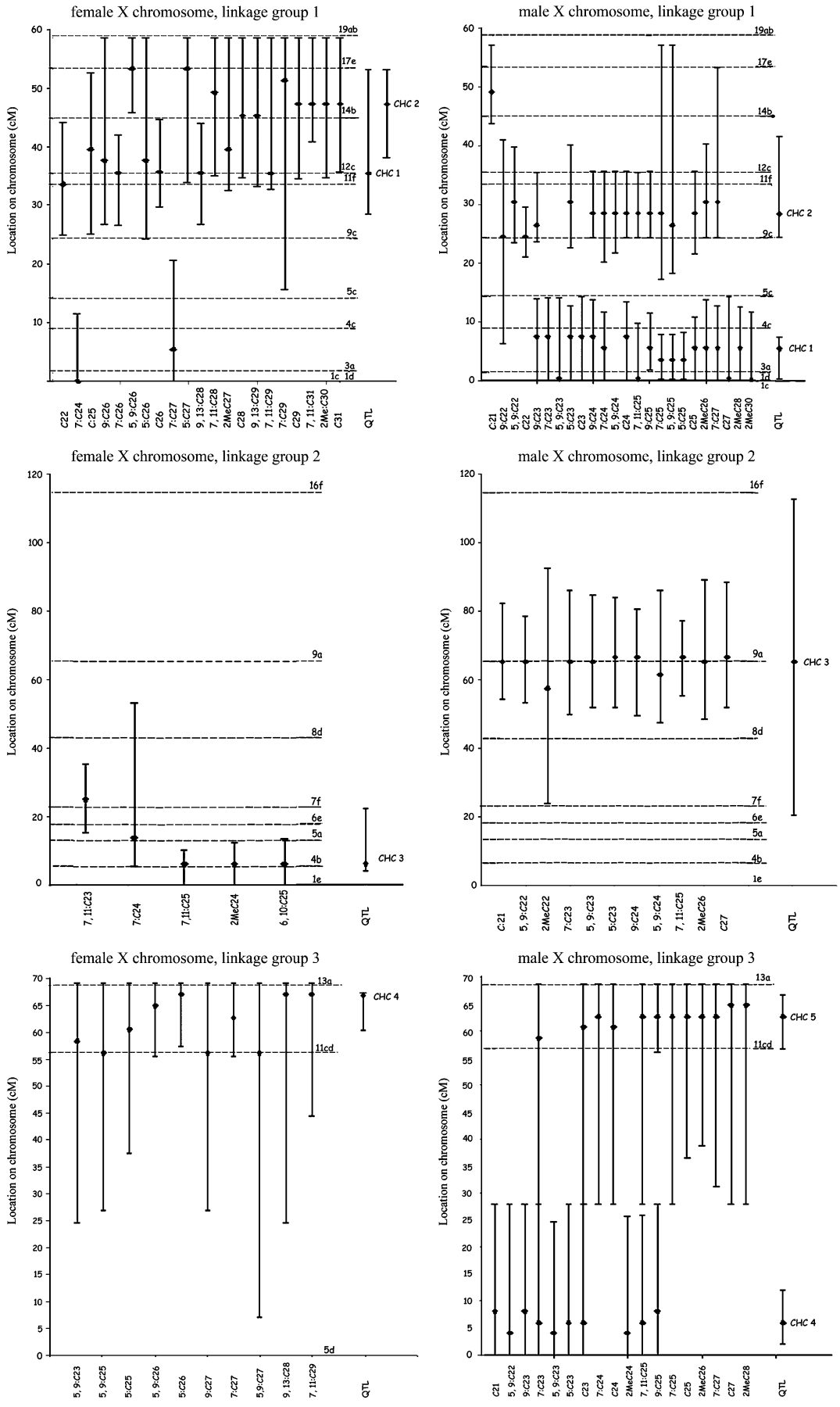
RESULTS

In males, all CHCs showed significant among-line variance with univariate analysis of variance (corrected for multiple tests using the sequential Bonferroni) except for 7C:22 and 2MeC32. In females, all CHCs, apart from six (C23; 9, 13:C24; 9C24; C24; 9C25; and 7, 11:C27), showed significant among-line variance after correcting for multiple tests.

QTL analysis: Of 30 CHCs in males, 28 were found to have significant QTL associations exceeding the significance threshold estimated by bootstrap, and of 52 CHCs in females where logcontrasts were calculated, 48 were associated with QTL (Figure 2). This slightly exceeds the number of CHCs found in females that have significant between-line variance after correction for multiple tests because, although all CHCs included in the analysis had significant between-line variance when tested by one-way ANOVA, the sequential Bonferroni was not used to exclude CHCs from QTL analysis. The additive effects for all QTL are given in supplemental Table 1 at http://www.genetics.org/supplemental/. The tight colocalization of CHC QTL in most cases (within the 2-cm limit of precision specified in QTL Cartographer) as well as overlapping 95% confidence intervals (Figure 2) is consistent with pleiotropic regulation of CHCs. The results for the CIM analysis for CHC expression largely agreed with the single-marker analysis as well, such that significant flanking marker-trait associations often supported local CIM maxima (supplemental Table 1 at http://www.genetics.org/supplemental/). As a result, most QTL reported are strongly supported by both analyses and are presumed to be significant for several traits. QTL were found for nearly all CHCs in both sexes. Only two CHCs with significant variance in expression in females (CHCs 9:C24 and C25) were not convincingly linked with any QTL.

A substantial number of QTL were found in each sex. In females, 25 CHC QTL were found, and 15 were found

Figure 2.—Linkage maps of *D. melanogaster* showing the confidence intervals of individual-trait QTL as estimated by 2-LOD drop-down. The estimated locations of the pleiotropic CHC QTL as determined by CIM in QTL Cartographer are also shown, with confidence intervals estimated by bootstrapping. Likelihood ratio statistic maxima are indicated. The position of markers is noted by a broken line, and cytological positions are provided. A dot on the broken line indicates that a significant association between marker and CHC expression was found in the single-marker analysis.


female X chromosome, linkage group 1
Location on chromosome (cM)
19ab
17e
14b
12c
11f
9c
5c
4c
3a
1c
1d
CHC 2
CHC 1
C22
7:C24
C:25
9:C26
7:C26
5, 9:C26
5:C26
C26
7:C27
5:C27
9, 13:C28
7, 11:C28
2MeC27
C28
9, 13:C29
7, 11:C29
7:C29
C29
7, 11:C31
2MeC30
C31
QTL
male X chromosome, linkage group 1
Location on chromosome (cM)
19ab
17e
14b
12c
11f
9c
5c
4c
3a
1d
1c
CHC 2
CHC 1
C:21
9:C22
5, 9:C22
C22
9:C23
7:C23
5, 9:C23
5:C23
C23
9:C24
7:C24
5, 9:C24
C24
7, 11:C25
9:C25
7:C25
5, 9:C25
5:C25
C25
2MeC26
7:C27
C27
2MeC28
2MeC30
QTL
female X chromosome, linkage group 2
Location on chromosome (cM)
16f
9a
8d
7f
6e
5a
4b
1e
CHC 3
7, 11:C23
7:C24
7,11:C25
2MeC24
6, 10:C25
QTL
male X chromosome, linkage group 2
Location on chromosome (cM)
16f
9a
8d
7f
6e
5a
4b
1e
CHC 3
C:21
5, 9:C22
2MeC22
7:C23
5, 9:C23
5:C23
9:C24
5, 9:C24
7, 11:C25
2MeC26
C27
QTL
female X chromosome, linkage group 3
Location on chromosome (cM)
13a
11cd
5d
CHC 4
5, 9:C23
5, 9:C25
5:C25
5, 9:C26
5:C26
9:C27
7:C27
5,9:C27
9, 13:C28
7, 11:C29
QTL
male X chromosome, linkage group 3
Location on chromosome (cM)
13a
11cd
CHC 5
CHC 4
C21
5, 9:C22
9:C23
7:C23
5, 9:C23
5:C23
C23
7:C24
C24
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7:C27
C27
2MeC28
QTL

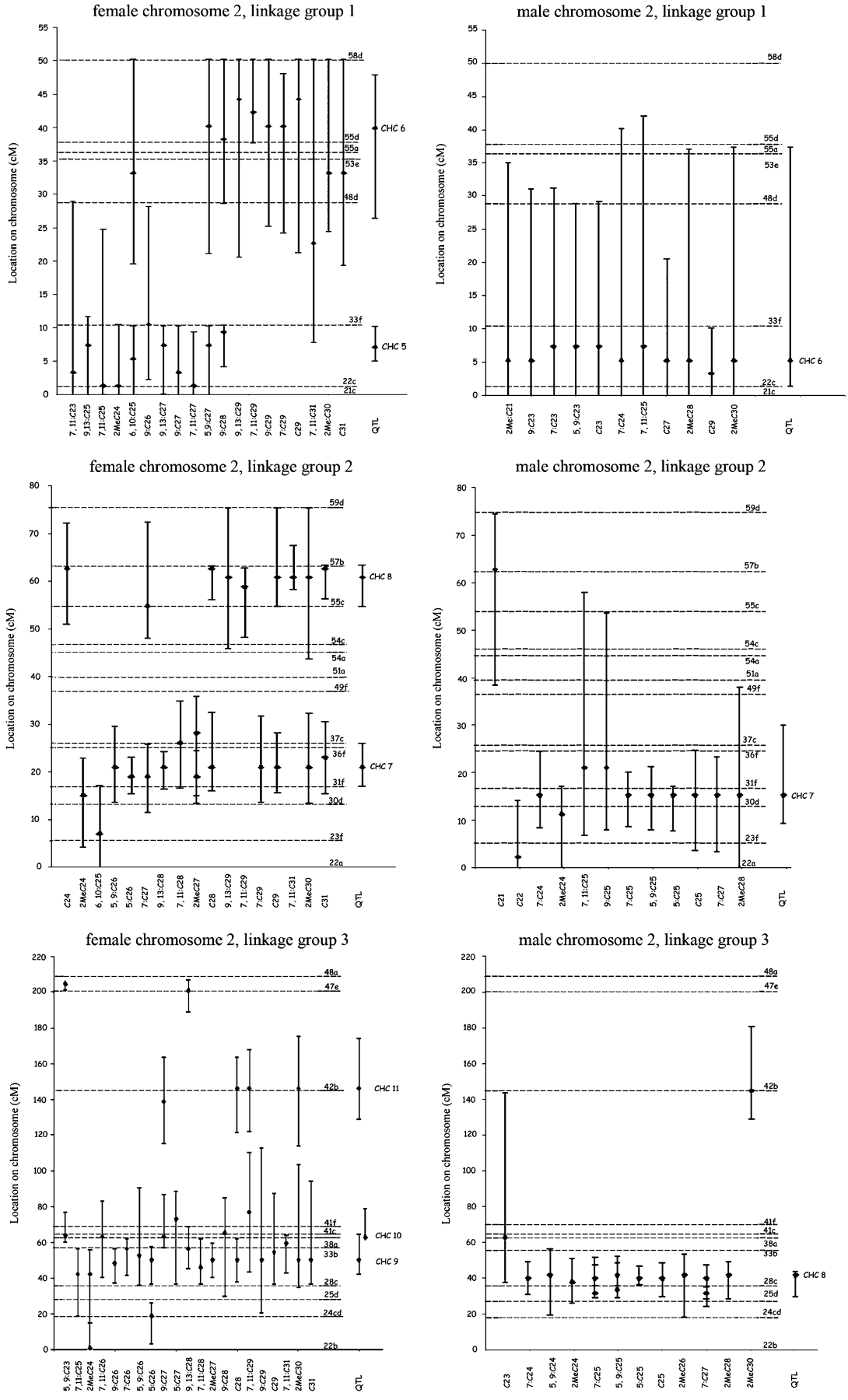

FIGURE 2.—*Continued.*

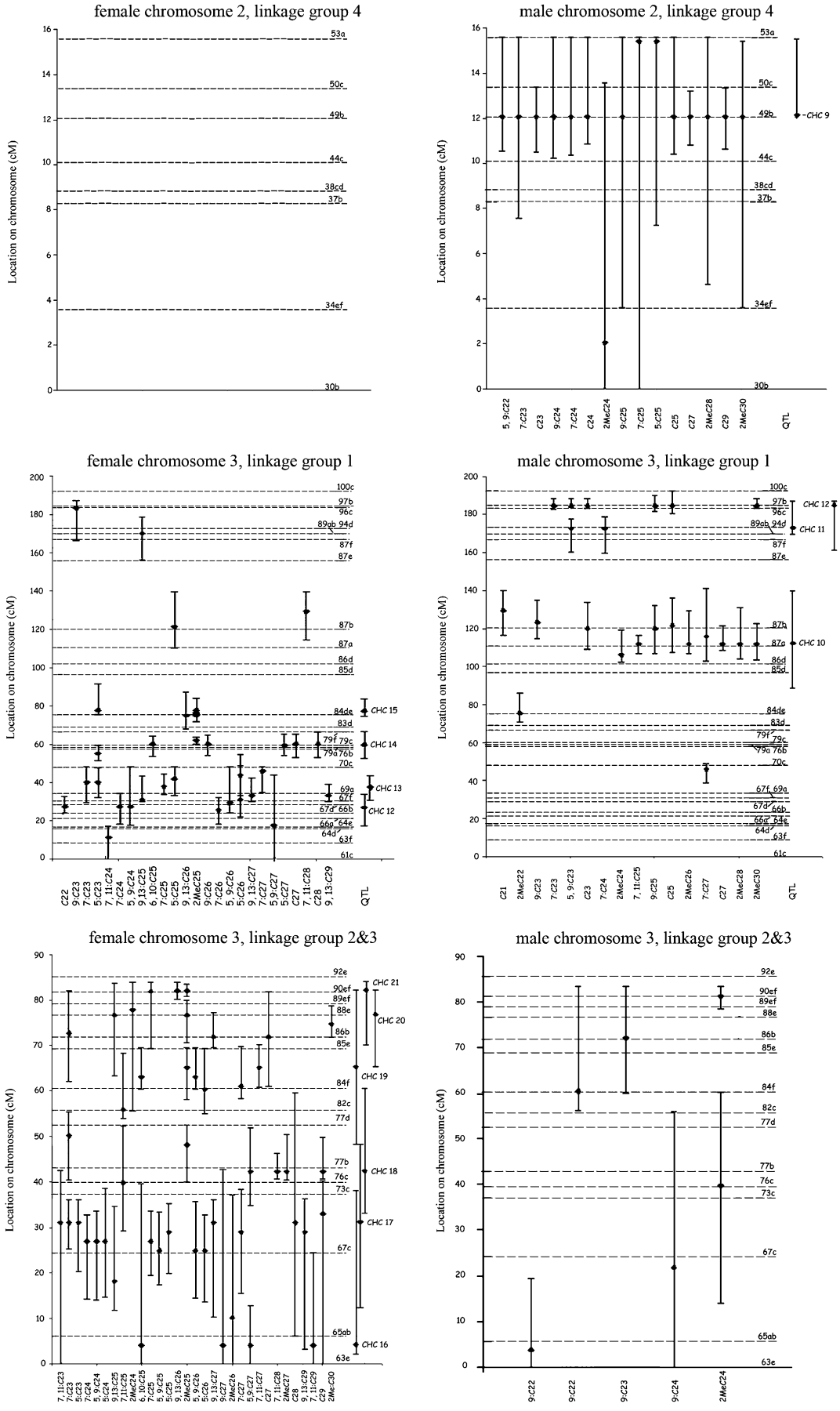

FIGURE 2.—*Continued.*

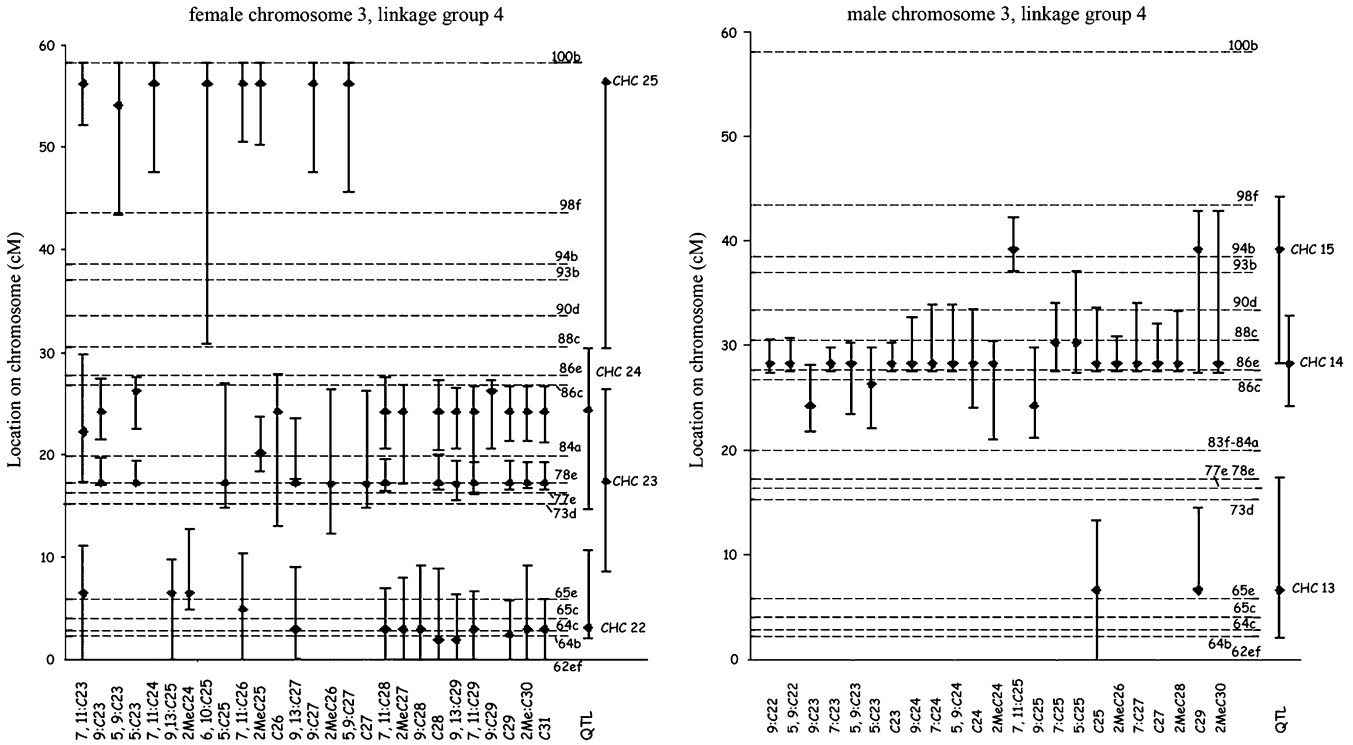

FIGURE 2.—*Continued.*

in males across all linkage groups (Table 1), although this might be an overestimate of the number of loci as QTL on one linkage group of a chromosome are potentially homologous with those on another linkage group on the same chromosome. While the extensive overlap of bootstrap confidence intervals suggests that several apparently adjacent QTL may be identical, this may be due to conservative bootstrap confidence intervals (MANICHAIKUL *et al.* 2006) as many of the 2-LOD confidence intervals in these regions are nonoverlapping. QTL were distributed across all chromosomes and most linkage groups for both sexes; however, there was little direct evidence for shared genetic regulation of homologous traits. Considering homologous traits individually, only one, 7:C27, demonstrated significant coregulation ($P = 0.021$), a level that does not retain significance under a sequential Bonferroni correction for multiple testing. For all traits considered simultaneously, the summed LOD exceeds only 13.5% of the summed LOD scores of the simulations ($P = 0.135$). This level of independent genetic control of CHC expression between the sexes reflects the low correlations among line–means for the 19 homologous CHCs for which logcontrasts were calculated. Although 9 of these 19 CHCs displayed significant positive correlations between the sexes (data not shown), no homologous pair of traits displayed a line–mean (genetic) correlation of >0.5. No chromosome seems to have a disproportionate effect on CHC expression in either sex, given the percentage of the genome represented by each chromosome (ADAMS *et al.* 2000; FITZPATRICK 2004) (female $\chi^2 = 1.456$, d.f. $= 2$, $P = 0.483$; male $\chi^2 = 2.340$, d.f. $= 2$, $P = 0.310$) (Table 2).

Comparison with other studies: The comparison with the candidate gene and QTL/deficiency studies are shown in supplemental Table 3 after conducting the searches as detailed in supplemental Table 4 at http://www.genetics.org/supplemental/. There was some correspondence between CHC QTL and previously identified QTL for ovariole number (WAYNE *et al.* 2001). Of the four QTL identified by WAYNE *et al.* (2001), one of the ovariole QTL corresponds with chromosomal regions dense with CHC QTL. There was also a strong correspondence between starvation resistance/longevity (as measured in a separate study in the Winters RILs) (WANG *et al.* 2004) and CHC expression in both sexes.

Of the nine independent QTL identified by WANG *et al.* (2004) in both sexes, five fell within the confidence intervals of CHC QTL in this study. There is a suggestion of a trade-off between the production of long-chain CHCs (requiring a greater input of energy and carbon resources) and starvation resistance in females. QTL 7 and 10 in females correspond to a general decrease in long-chain CHC production and an increase in starvation resistance. Both QTL 13 in males and QTL 23 in females correspond with a QTL that has a negative relation with longevity in both sexes (Table 1). Some of these associations might be due to poorly resolved QTL and confidence intervals, particularly along female linkage groups 3-2&3 and 3-4 where nearly the entire

TABLE 1

Major pleiotropic QTL for CHC expression identified in male and female *D. melanogaster*

QTL	Linkage group	95% C.I.[a]	LOD maximum	Maximum LOD score[b]	Maximum trait[c]
			Females		
CHC 1	X-1	10A-17E	12E	3.975	7:C26
CHC 2	X-1	13B-17E	14F-15A	4.057	7, 11:C31
CHC 3	X-2	3BC-7E	4A	2.937	2MeC24
CHC 4	X-3	12A-12EF	12EF	3.678	7:C27
CHC 5	2-1	28A-34A	30B	4.501	9:C28
CHC 6	2-1	46D-57F	56C	3.459	7, 11:C29
CHC 7	2-2	32A-37C	35B	5.296	5:C26
CHC 8	2-2	55C-57B	56EF	5.941	7, 11:C31
CHC 9	2-3	28D-35A	31C	5.533	2MeC27
CHC 10	2-3	37A-41F/42A	41F	3.947	5, 9:C23
CHC 11	2-3	42A-44B	42B	2.924	7, 11:C29
CHC 12	3-1	64EF-69B	67B	3.951	C22
CHC 13	3-1	68A-70C	69D	7.219	9,13:C25
CHC 14	3-1	74A-79E	79C	9.58	2MeC25[d]
CHC 15	3-1	84C-84F	84E	9.206	2MeC25[d]
CHC 16	3-2&3	64B-73F	64DE	2.941	9:C25
CHC 17	3-2&3	66A-77B	70C	9.083	6, 10:C25
CHC 18	3-2&3	71C-84E	76F	3.786	7, 11:C26[d]
CHC 19	3-2&3	77C-90F	85C	3.931	7, 11:C26[d]
CHC 20	3-2&3	85C-90F	88D	4.341	7, 11:C26[d]
CHC 21	3-2&3	85E-91DE	90F	5.712	7, 11:C26[d]
CHC 22	3-4	64B-69C	64DE	3.274	9,13:C25
CHC 23	3-4	67C-86B	79AB	6.078	9, 13:C29
CHC 24	3-4	73A-88A	85C	5.492	C29
CHC 25	3-4	88B-99F	99F	4.935	7, 11:C26
			Males		
CHC 1	X-1	1E-4A	3E	5.28	5, 9:C25
CHC 2	X-1	9CD-13F	10C	5.058	2MeC24
CHC 3	X-1	7C-16D	9A	4.657	2MeC24
CHC 4	X-3	5F-7C	6E	2.205	5, 9:C23
CHC 5	X-3	10C-12F	12B	2.264	C25
CHC 6	2-1	22C-55C	28A	3.15	C29
CHC 7	2-2	28B-47D	31D	4.847	7:C25
CHC 8	2-3	27C-30B	29EF	9.585	7:C27
CHC 9	2-4	43BC-52A	49B	3.218	C24
CHC 10	3-1	85AB-87C	87A	5.739	C27
CHC 11	3-1	88F-98A	93D	2.509	7:C24
CHC 12	3-1	87F-98A	97B	2.832	C23
CHC 13	3-4	64B-79B	66B	2.299	C25
CHC 14	3-4	85D-90A	87A	5.225	2MeC26
CHC 15	3-4	87A-98F	94F	3.093	2MeC24

[a] Determined by bootstrapping.
[b] The highest single-trait LOD score associated with the QTL.
[c] The trait with the highest LOD score associated with the QTL.
[d] In two cases involving QTL in females, the same CHC returned the highest LOD score for adjacent QTL along a linkage group. In these cases, we used the trait with the second-highest LOD to calculate bootstrapped C.I.'s because it was impossible to verify homology of these multiple QTL among bootstrap replicates. For CHC 14 and CHC 15 on linkage group 3-1, 5:C23 and 9, 13:C26 were used, respectively, while along linkage group 3-2&3 the following substitutions were required: CHC 18–9,13:C25; CHC 19–9:C25; CHC 20–C26; CHC 21–C:25.

extent of these linkage groups falls within the confidence intervals of one or more QTL. Apart from the association between the longevity QTL in females and CHC QTL 23, however, all of these associations remain even when these two poorly resolved haplotypes are excluded.

There is also some indication that genomic regions that are important for differences in CHC expression between species are highly variable within populations. The region that is most important for CHC differences between *D. melanogaster* and *D. simulans* females (Coyne 1996) is also dense with CHC QTL in the Winters lines,

TABLE 2

Observed and expected number of CHC QTL per chromosome for male and female *D. melanogaster*

Chromosome	Proportion of genomic euchromatin[a]	Female		Male	
		QTL found	QTL expected	QTL found	QTL expected
X	0.184	4	4.60	5	2.76
2	0.374	7	9.35	4	5.61
3	0.442	14	11.05	6	6.63

The number of QTL expected is calculated from the proportion of genomic euchromatin on each of the major *D. melanogaster* chromosomes. There was no evidence for preferential location of CHC QTL on any chromosome in females ($\chi^2 = 1.456$, d.f. = 2, $P = 0.483$) or males ($\chi^2 = 2.340$, d.f.=2, $P = 0.310$).

[a] Data in this column are from Table 1, Fitzpatrick (2004).

and four of these regions (between 65F and 72D), identified through deficiency mapping, span the maxima identified as probable QTL locations. Of 12 genes or gene complexes that have previously been identified as having effects on CHC expression or on the regulation of sexual dimorphism, 10 colocalize with QTL identified in this study. If we again disregard those associations found in female linkage groups 3-2&3 and 4, only the Antennapedia and Bithorax regions are excluded. Of the 20 genes identified as having metabolic functions related to the biosynthesis of long-chain fatty acids through searching the FlyBase database, 14 potentially contribute to variation in CHC expression in the Winters lines, disregarding the associations in the two poorly resolved female linkage groups.

DISCUSSION

Line–mean analysis of the Winters panel of *D. melanogaster* RILs indicated the presence of a substantial level of genetic variance in almost all male and female CHCs. A large number of genetic loci for CHC expression were found in both sexes (20 in males and 29 in females), many of which contributed to pleiotropic regulation of more than one CHC. QTL were found to be distributed among all linkage groups.

Candidate genes: Despite the potential importance of metabolism and resource allocation to variation in CHC expression, most of the previously identified candidate genes and regions, apart from *sept* and *ecdysoneless*, were potentially associated with QTL (supplemental Table 3 at http://www.genetics.org/supplemental/). Some, like the *desaturase* 1 and 2 genes and *smoq* (Ferveur and Jallon 1996), have been demonstrated to be important for racial and species differences in CHC expression and mate recognition. Similarly, differences in CHC expression between *D. melanogaster* and *D. simulans* (Coyne 1996) match well with the locations of CHC QTL found here, and it is possible that genes associated with racial and species differences are implicated in within-population variation as well. It may be that these genes, which are important sources of variation at a population level, respond well to selection under situations favoring the evolution of isolating mechanisms between species.

Many of the CHC QTL detected in both sexes did not correspond with the location of candidate CHC biosynthesis genes identified through interracial or interspecific studies. The colocalization of an ovariole QTL (Wayne *et al.* 2001) with a QTL affecting CHC expression is consistent with previously identified correspondences between ovariole production and CHC expression (Schal *et al.* 1994; Wicker and Jallon 1995). The colocalization of several starvation and longevity QTL in both sexes (Wang *et al.* 2004) with CHC QTL suggests that a large proportion of variation in CHC expression may reflect variation in a number of biological processes that require the expenditure of energy or lipid resources. Genes identified through a search in FlyBase for genes involved in fatty acid metabolism or biosynthesis also correspond with loci identified in this study and may be candidates for common variation in CHC expression in *D. melanogaster*.

Biosynthesis and expression of CHCs: The biosynthesis of CHCs in Drosophila is well characterized and results from a simple pathway, and several of the genes regulating CHC expression have been characterized by the effects of their alleles on ratios of different classes of CHCs, such as *smoq* (Ferveur and Jallon 1996) and *desaturase* 2 (Dallerac *et al.* 2000). In *D. melanogaster*, CHCs range in length from ~21 carbons to 31. These are synthesized by chain lengthening with the serial addition of acetate onto myristate or palmitate (Howard and Blomquist 2005), after which double bonds are selectively created by various desaturases. Branched (methyl group) hydrocarbons are also present and are synthesized in an entirely different pathway by the addition of carbon to valine, an amino acid (Howard and Blomquist 2005), and males express more methylated alkanes than females (Savarit and Ferveur 2002b). Cosmopolitan females express more CHCs with double bonds at positions 7 and 11 (an abundance of 7:C*n*, 11:C*n*, and 7, 11:C*n* alkenes and alkadienes) rather than with the single double bonds and monoenes (an abundance of 7:C*n*, and C*n* alkenes and alkanes) common in cosmopolitan males (Rouault *et al.* 2001).

Given the simplicity of the system, we might expect to see genetic variants for CHC expression generally increasing the proportion of one class of compounds at the expense of others. There was, however, no evident simple correspondence between QTL and the relative amounts of different classes of compounds, such as 7, 11 dienes or methylated alkanes (supplemental Tables 1 and 2 at http://www.genetics.org/supplemental/). One possible exception might be QTL 7 and 10 in females, which seem to correspond with a trade-off between long and short chain CHCs and to colocalize with a QTL for starvation resistance (Wang *et al.* 2004). This could be suggestive of a negative correlation between long-chain CHC production and starvation resistance, perhaps due to the higher carbon and energy demands of synthesizing larger CHCs. Otherwise, the additive effects of QTL detected in the Winters RILs on CHC expression do not clearly indicate trade-offs between biochemical classes, perhaps indicating some level of control apart from biosynthesis of the blend of CHCs deposited on the cuticle.

Sexual dimorphism: Consistent with the low correlations between homologous CHCs (none >0.5), there was little evidence for coregulation of homologous CHCs across the sexes. It should be noted, however, that it is difficult to assess which compounds to compare between the sexes, for while CHCs are produced from identical starting compounds in both sexes and share a similar biochemical pathway, there are genes for sex-specific processing of fatty acids (Gleason *et al.* 2005). Quite different compounds, varying in chain length or in the number and position of double bonds, may, apart from one or two processing steps late in biosynthesis, be products of identical genetic regulation while identical compounds might result from sex-specific enzymatic modifications of different substrates.

Mutant studies of CHC expression in *D. melanogaster* (Ferveur and Jallon 1993; Coyne *et al.* 1999; Dallerac *et al.* 2000; Wicker-Thomas and Jallon 2001; Fang *et al.* 2002) have also indicated independent genetic control of trait expression between the sexes (although see Labeur *et al.* 2002). While it might be tempting to assume that the qualitative sexual dimorphism in *D. melanogaster*, where almost entirely different compounds are expressed, has led to the low covariance between traits, in *D. serrata*, which does not exhibit qualitative differences in CHC expression between sexes or races (Howard *et al.* 2003), genetic covariance between males and females is likewise low although, in this case, the X chromosome plays a major role (Chenoweth and Blows 2003). In contrast, ecological traits such as longevity (Curtsinger and Khazaeli 2002; Mackay 2002; Forbes *et al.* 2004) and starvation resistance (Wang *et al.* 2004) do not seem to have quite the same degree of independent genetic regulation (but see Nuzhdin *et al.* 1997).

Sex-specific regulation of homologous traits is thought to be the result of sexually antagonistic selection (Lande 1980). The direction of sexual selection is expected to differ between the sexes (Johnstone *et al.* 1996; Bonduriansky 2001) due to their differing roles in reproduction and the differing costs of choosiness in the sexes. This difference in selection favors the evolution of independent genetic regulation of homologous traits, which in turn allows for the evolution of phenotypic sexual dimorphism (Lande and Arnold 1985). In *D. melanogaster* CHCs, a combination of (1) strong qualitative sexual dimorphism, (2) weak intersexual genetic correlations for homologous traits, and (3) little evidence for colocalization of QTL between the sexes all indicate low constraint on independent evolution of the sexes.

Conclusion: Many of the loci important for interracial and between-species differences in *D. melanogaster* identified in previous studies colocalize with loci contributing to variation in CHC expression identified in this study. These loci are largely sex specific and lead to low intersex genetic correlations in CHC expression in this species. What remains to be determined are the selective processes responsible for the evolution of CHC sexual dimorphism in *D. melanogaster*.

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