Genetic Architecture of a Morphological Shape Difference Between Two Drosophila Species

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Manuscript received November 28, 1998
Accepted for publication September 29, 1999

ABSTRACT

The size and shape of the posterior lobe of the male genital arch differs dramatically between *Drosophila simulans* and *D. mauritiana*. This difference can be quantified with a morphometric descriptor (PC1) based on elliptical Fourier and principal components analyses. The genetic basis of the interspecific difference in PC1 was investigated by the application of quantitative trait locus (QTL) mapping procedures to segregating backcross populations. The parental difference (35 environmental standard deviations) and the heritability of PC1 in backcross populations (>90%) are both very large. The use of multiple interval mapping gives evidence for 19 different QTL. The greatest additive effect estimate accounts for 11.4% of the parental difference but could represent multiple closely linked QTL. Dominance parameter estimates vary among loci from essentially no dominance to complete dominance, and *mauritiana* alleles tend to be dominant over *simulans* alleles. Epistasis appears to be relatively unimportant as a source of variation. All but one of the additive effect estimates have the same sign, which means that one species has nearly all plus alleles and the other nearly all minus alleles. This result is unexpected under many evolutionary scenarios and suggests a history of strong directional selection acting on the posterior lobe.

IN recent years, advances in molecular biology have stimulated a great resurgence of interest in the genetic and developmental bases of morphological diversity. Most of the work in this area is concerned with fundamental differences in the body plans of organisms that diverged from one another many millions of years ago (*e.g.*, Shubin *et al.* 1997). The basic approach is classical comparative biology of the patterns of expression of genes known to play an important role in the development of model organisms. Such studies have suggested hypotheses about how morphological diversity may evolve through changes in patterns of regulatory gene expression (*e.g.*, Carroll *et al.* 1995; Averof and Patel 1997). However, these hypotheses are difficult to test and it is likely that a multitude of complex genetic changes underlie macroevolutionary differences such as variation in numbers and types of appendages.

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An alternative approach to the study of morphological diversity is direct genetic analysis of phenotypic differences between closely related species. This type of microevolutionary analysis has the potential to reveal the underlying genetic architecture of morphological differences in considerable detail. An understanding of the numbers and types of gene substitutions responsible for species differences eventually can provide insight into how morphology evolves in terms of population genetic processes.

A pair of closely related allopatric species of Drosophila, *Drosophila simulans* and *D. mauritiana*, differ dramatically in size and shape of the posterior lobe of the male genital arch (Figure 1). *D. simulans* females hybridize readily with *mauritiana* males in the laboratory, producing fertile female and sterile male F_1's with an intermediate posterior lobe morphology. When F_1 females are backcrossed to parental males, a continuous series of morphologies is produced, suggesting polygenic inheritance. We have previously shown that both the size and shape variation (which are highly correlated) can be quantified by a morphometric descriptor (PC1) based on elliptical Fourier and principal components analyses. We have also reported a preliminary investigation of the genetic architecture of this trait using composite interval mapping (Liu *et al.* 1996). Here we greatly improve the resolution of the quantitative trait locus (QTL) analysis through increases in sample size and

Figure 1.—Camera lucida outlines of a posterior lobe from one specimen each of *D. simulans* and *D. mauritiana*.

marker number as well as the application of a new interval mapping procedure.

MATERIALS AND METHODS

Experimental design and data acquisition: The experimental design and morphological data acquisition methods were described previously in detail (Liu *et al.* 1996). Briefly, females from an inbred line of *D. simulans* (13w JJ) were crossed to males of an inbred line of *D. mauritiana* (Rob A JJ) to produce an F_1 population. The parental lines are fixed for different alleles at 45 marker loci. The F_1 females were backcrossed to each parental line to produce two populations, referred to as *mauritiana* backcross (BM) and *simulans* backcross (BS). These crosses were repeated at two different times to produce two independent samples of males from each backcross population, BS1 (n = 186), BS2 (n = 288), BM1 (n = 192), and BM2 (n = 299). The phenotypic value of an individual is the average over both sides of the first principal component (PC1) of the Fourier coefficients of the posterior lobe.

The genotype of males from BS1 and BM1 was determined at each of 45 marker loci, which are listed in Table 1. The same markers were scored also on BS2 and BM2, except for *prd*, *eve*, and *plu*. Cytological positions in Table 1 are from FlyBase (1997) and genetic map positions are estimated from gametes produced by F_1 females in this study.

Molecular markers were developed by using *D. melanogaster* sequence in FlyBase (1997) to design PCR primers that amplify a specified region from both *mauritiana* and *simulans* parental lines. These PCR products were sequenced to identify interspecific differences to be used as markers. In some cases, the sequence difference alters a restriction site and was detected by restriction digestion and electrophoresis as described by Liu *et al.* (1996). In most cases, an allele-specific oligonucleotide (ASO) hybridization assay was used to genotype flies [modified after Maekawa *et al.* (1995) and Saiki *et al.* (1986)]. In the ASO assay, two 15-base probes were designed to cover the nucleotide sequence difference, with one matching each allelic form. PCR products from single-fly genomic DNA were blotted onto Hybond N+ filters in a 96-well format and hybridized with each probe in succession. At discriminating wash temperatures, the mismatched probe washes off and the perfectly matched probe remains hybridized, revealing the fly's genotype. PCR and hybridization conditions, along with primer and probe sequences, are given in Table 1. The genetic and phenotypic data of this experiment are available through a link on the QTL Cartographer web site (http://statgen.ncsu.edu/qtlcart/cartographer.html).

QTL analysis: Two types of interval mapping analyses were applied to the backcross data sets, composite interval mapping (CIM; Zeng 1994) and multiple interval mapping (MIM; Kao and Zeng 1997; Kao *et al.* 1999).

The CIM analysis involves a single QTL model with multiple regression on marker loci outside of the interval under consideration. In this case the model is

$$y_{ijk} = \mu_{ij} + b_{ij}^* x_{ijk}^* + \sum_l b_{ijl} x_{ijkl} + e_{ijk}, \quad (1)$$

where i indexes backcross, j indexes sample within backcross, k indexes individuals within sample, l indexes markers, y_{ijk} is the phenotypic value, μ_{ij} is the mean, b_{ij}^* is the effect of a putative QTL, x_{ijk}^* is a QTL indicator variable, b_{ijl} is the partial regression coefficient of y_{ijk} on x_{ijkl}, x_{ijkl} is an indicator variable for the lth marker, Σ is over all markers except those flanking the interval containing the putative QTL, and e_{ijk} is the residual. The CIM likelihood function and hypothesis testing for this data set were described previously (Zeng 1994; Liu *et al.* 1996).

For CIM, several different likelihood ratios corresponding to different hypotheses were calculated and results are presented as LOD = $-\log_{10}(L_0/L_1)$. Figure 2a shows joint mapping with both samples from both backcrosses (H_0, $b_{ij}^* = 0$; and H_1, $b_{ij}^* \neq 0$ for i,j = 1 and 2) and joint mapping with both samples in one backcross (H_0, $b_{i1}^* = 0$ and $b_{i2}^* = 0$; and H_1, $b_{i1}^* \neq 0$ and $b_{i2}^* \neq 0$). In addition, two types of interactions were tested, a QTL × backcross interaction (H_0, $b_{11}^* = b_{21}^*$ and $b_{12}^* = b_{22}^*$; and H_1, $b_{11}^* \neq b_{21}^*$ and $b_{12}^* \neq b_{22}^*$) and a QTL × sample interaction (H_0, $b_{11}^* = b_{12}^*$ and $b_{21}^* = b_{22}^*$; and H_1, $b_{11}^* \neq b_{12}^*$ and $b_{21}^* \neq b_{22}$). The critical value of LOD score used is $(0.217\chi^2_{f,0.05/g})$, where f is the number of parameters and g is the number of marker intervals. For joint mapping of both samples and both backcrosses, f = 5, g = 42, and LOD_c = 4.4; for joint mapping of both samples in one backcross, f = 3, g = 42, and LOD_c = 3.5. As interactions were only tested on previously identified QTL, f = 1, g = 14, and LOD_c = 1.8.

The MIM analysis is quite different, because it involves a multiple QTL model, which may include both main effects and epistatic interactions. The model is

$$y_{ijk} = \mu_{ij} + \sum_{r=1}^{m} \alpha_{ir} x_{ijkr}^* + \sum_{r \neq s \subset (1,\ldots,m)}^{t} \beta_{irs}(x_{ijkr}^* x_{ijks}^*) + e_{ijk}, \quad (2)$$

where i indexes backcross, j indexes sample within backcross, k indexes individuals within sample, m is the number of putative QTL, α_{ir} is the effect of a putative QTL r in backcross i (assuming that the effect is the same for both samples within a backcross), x_{ijkr}^* is an indicator variable denoting the genotype of QTL r (= $+\frac{1}{2}$ or $-\frac{1}{2}$), β_{irs} is an epistatic effect between QTL r and s, $\Sigma_{r \neq s \subset (1,\ldots,m)}$ is the subset of QTL pairs that show a significant epistatic effect, t is the number of significant pairwise epistatic effects, and e_{ijk} is the residual.

The likelihood of this MIM model is

$$L(\mathbf{E}, \mu, \sigma^2 \mid \mathbf{Y}, \mathbf{X}) = \prod_{i=1}^{2} \prod_{j=1}^{2} \prod_{k=1}^{n_{ij}} [\sum_{l=1}^{2^m} p_{ijkl} \phi(y_{ijk} \mid \mu_{ij} + \mathbf{D}_{ijkl}\mathbf{E}_i, \sigma_{ij}^2)], \quad (3)$$

where $\mathbf{Y}$ denotes the quantitative trait data, $\mathbf{X}$ denotes the marker data, p_{ijkl} is the probability of each multilocus genotype (conditional on marker data), $\mathbf{E}_i$ is a vector of QTL parameters (α's and β's), and $\mathbf{D}_{ijkl}$ is a vector specifying the configuration of x^*'s associated with each α and β for the lth genotype. The term in brackets is the weighted sum of a series of normal density functions, one for each of the 2^m possible multiple-QTL genotypes. The procedure to obtain maximum-likelihood parameter estimates was described by Kao and Zeng (1997). A large fraction of the 2^m possible genotypes have zero or very small probability given marker genotypes and do not need to be evaluated in the analysis.

Selection of the number and map positions of putative QTL to be included in the MIM model followed a stepwise procedure described by Kao *et al.* (1999). Briefly, the procedure consisted of the following steps: (1) selection began with a model containing m QTL at positions determined from CIM

and included no epistatic effects; (2) the genome was scanned to find the best position of an $(m + 1)$th QTL (*i.e.*, that resulting in the greatest increase in likelihood), denoted θ^F_{m+1}; (3) the model with $(m + 1)$ QTL was reevaluated to find the one QTL having the least significant effect, denoted θ^B_{m+1}; (4) if $\theta^F_{m+1} \neq \theta^B_{m+1}$, θ^B_{m+1} was removed, θ^F_{m+1} was kept, and the process returned to step (2). If $\theta^F_{m+1} = \theta^B_{m+1}$, then θ^F_{m+1} was retained in the model if it made a significant contribution (*i.e.*, if the likelihood ratio for models with and without θ^F_{m+1} exceeds a prescribed critical value). If θ^F_{m+1} was retained, all QTL locations were reevaluated and the process returned to step (2). In the positional reevaluation, the entire genome was scanned repeatedly until the positions of maximum LOD score were stationary. If θ^F_{m+1} was not retained, selection of QTL number and position terminated.

It is not clear what critical value should be applied to this type of analysis. Initially, the threshold appropriate for a CIM analysis (LOD score 4.4) was used. Later, a residual permutation (or bootstrap) test was used to guide the final model selection (Zeng *et al.* 1999).

The residual permutation test is a model-dependent, resampling method. After a series of model-fitting cycles, a test is needed to determine whether the least significant QTL in the current model (or the last added QTL) in the model is a statistically significant addition to the model. In this case the test consists of comparing a model of k QTL (null hypothesis) with an alternative model of $k + 1$ QTL (which consists of the k QTL model plus one additional QTL). The test consists of several steps: (1) the estimated genotypic value for each individual is obtained under the null hypothesis and the corresponding residual is calculated as the difference between the observed phenotypic and estimated genotypic values; (2) a permuted sample is obtained by randomly shuffling the residuals among individuals; (3) the permuted sample is used in a search for a new QTL, conditional on the k QTL model, and the maximum test statistic is recorded; (4) the resampling and testing in (2) and (3) are repeated a number of times to obtain an empirical 95% significance threshold for the test; and (5) finally, this threshold is compared with the test statistic for the $(k + 1)$th QTL in the original data.

After establishing a model with main effects, the combined forward/backward procedure was applied to identify significant epistatic effects between pairs of identified QTL. A likelihood-ratio test was performed on each fitted epistatic effect, using a threshold adjusted by the number of tests performed in each cycle. In the first cycle, the LOD threshold was set to 3.0 ($\cong 0.217\chi^2_{1,0.05/171}$). An attempt was also made to search for significant epistatic effects between one QTL identified through its marginal effect and another unoccupied position on the genetic map. This was done by searching for the largest epistatic effect between an identified QTL and all unoccupied positions at 1-cM intervals.

For a single QTL, genotypic values may be defined as a for QQ, d for Qq, and $-a$ for qq. With these definitions, the QTL main effect in one backcross is $(a + d)$ and in the other backcross is $(a - d)$. Thus, a and d can be estimated separately. Variances and covariances can also be estimated. The total phenotypic variance (σ^2_p) can be estimated from the total sum of squares, the total genotypic variance (σ^2_g) from the model sum of squares, and the environmental variance (σ^2_e) from the residual sum of squares. The contribution of a single QTL effect to the total genotypic variance can be expressed as the sum of its variance contribution and ½ of its covariances with other QTL effects. This quantity is

$$\hat{\sigma}^2_{ir} = \frac{1}{n_{i1} + n_{i2}} \sum_j \sum_k \sum_l \sum_s \hat{\pi}_{ijkl}(D_{ijklr} - \bar{D}_{ir})(D_{ijkls} - \bar{D}_{is})\hat{E}_{ir}\hat{E}_{is}, \quad (4)$$

where $\hat{\pi}_{ijkl}$ is the probability of the lth QTL genotype conditioned on the marker genotype and the trait phenotype, D_{ijklr} is the indicator variable (x*) associated with the genotype at a locus or a product of two indicator variables associated with a bilocus combination, $\hat{E}_{ir}$ is the main (α) or epistatic (β) effect associated with a particular QTL locus or a particular bilocus combination, n_{ij} is the size of the jth sample in the ith backcross, and $\bar{D}_{ir} = \Sigma_j \Sigma_k \Sigma_l \hat{\pi}_{ijkl} D_{ijklr}/(n_{i1} + n_{i2})$.

This experiment consists of two independent samples for each backcross. Thus, the MIM model can be cross-validated by using parameter estimates from one sample to predict phenotypic values for the other sample. The prediction equation is

$$\hat{y}_{ijk} = \hat{\mu}_{ij}, + \sum_l^{2^m} \sum_r^{m+t} p_{ijklr}\hat{E}_{ij'r} \quad (5)$$

from Equation 2, where p_{ijkl} is the probability of the lth QTL genotype conditioned on marker genotype only. While j indicates the value for one sample, j' is the corresponding value for the other sample. The first summation is over all possible 2^m QTL genotypes and the second summation is over all effects in the model (m main effects and t epistatic effects).

RESULTS

Descriptive statistics: The phenotypic variances within the highly inbred parental lines and within the F_1 populations provide estimates of the environmental variance. A pooled estimate shows that the mean parental line difference in PC1 equals 34.9 environmental standard deviations. In addition, the environmental variance estimate is an order of magnitude less than the phenotypic variances of the backcross populations, indicating high heritability. The variance estimates ($\times 10^{-4}$) are 0.026 environmental *vs.* 0.546 for BS1, 0.499 for BS2, 0.280 for BM1, and 0.263 for BM2 backcross populations. The large parental difference coupled with high heritability provides a very favorable situation for QTL mapping.

In both replicate experiments there is evidence for partial dominance of *mauritiana* alleles. With strictly additive gene action, and assuming some effect of the X chromosome, the F_1 mean should be greater than the parental midpoint (*i.e.*, more *simulans*-like), because all F_1 males have a *simulans* X chromosome. However, in both cases the F_1 mean is significantly less than the parental midpoint. For example, in sample 1, the F_1 mean is 0.0028, while the midpoint is 0.0054 between the *mauritiana* (−0.0230) and *simulans* (0.0337) parents.

Composite interval mapping: Results of the CIM analysis are summarized in Figure 2a. The joint analysis of both samples in both backcrosses provides evidence for at least 14 different QTL at map positions stated in the figure legend. In most cases, these putative QTL exceed the critical value by a substantial margin and clearly indicate different QTL, because they occur in nonadjacent intervals (Zeng 1994). However, two cases are more complicated: (1) although the LOD peak at 2-67 is somewhat less than the critical value, we count it as a putative QTL because it is highly significant when fewer markers are included as cofactors in the CIM analysis; (2) the

TABLE 1

Summary of markers

Gene	Cytological position	Genetic position	PCR annealing (degrees)[a]	PCR primers	ASO wash (degrees)[b]	ASO probes[c]
ewg	1A8	0.0	60	ATAACAGCAACCAGCGGCGG GGGCATCCATCCTCACATTGG	50	S: TGGGTATACCATCGA M: TCGATGGCATACCCA
w	3C2	3.6				
RpS6	7C	14.2	63	CGGTCTTTTTCAACCCTGC GTAGCCCTTCCACTCGTCAC	55	S: GAATTGCAGCGGATA M: TATCCGCAGCAATTC
v	10A	23.4	55	TGTCCCTATGCAGGAAACGG TGAACAGATGCTCATCGTGC	43	S: CGAGCAGCTTGTCCA M: TGGACAAACTGCTGG
Sd	13F	40.6	55	ACGGCGTTCATGGAAATCCAG GGGGAGCGTTGAATGCGATAG	RT	S: AACTACACACATATA M: TTTATGTATGGTTAG
run	19E	59.3	55	AGTGCATACCGAGAATCCGC ATTGATGGCGATTGCGGAGG	55	S: CGACGGTCGCCGGTT M: AACCGGCAACCGTCG
gl	21A–B	0.0	60/55	TATCAGCAGCTCTATTGGCG AAGCACACCTTTTCGATTCC	37	S: AATTGCCAAAAGTAA M: TTATTTTCGGCAATT
Pgk	22D–23C	7.0	57	GGCAAGCGGGTGTTGATGCG TTGGCAGGATCGGCCTTGAC	53	S: CCGCTGAACTGAAGA M: TCTTCAGCTCAGCGG
Cg25C	25C	17.0	55	GATAGAGGTGCCCGACTGTC CTGTGTTGGGATTGGAGGTC	42	S: TTAGATTACGGAAGA M: TCTTCCATAATCTAA
Gpdh	26A	22.0	60/55	CCCCTGTTCACGGCTATTC CTGGTGATTTGATCTATGCGG	40	S: TGTTTACCCTGCTAT M: ATAGTAGGATAAACA
ninaC	28A	28.5	55	GAAGTCCATCTTCCAGGTCC TATGGGCACTGGCAGTGGTC	RT	S: TATGTACCAGGTCCA M: TGCACCTTGTACATA
Glt	29D	34.7	60/55	ACCCAGCCTCACTAGCCAC TGCCCACTGCTTCCAGAG	53	S: TAACATCGACGTTGC M: GCAACATTGACGTTA
prd	33C1-2	55.2	55	GATGCAAGGTGAGTGTCTATCG GCCATGGGATACACGTAGCT		
Mhc	36A	68.0	60/55	TCATTGGTGTACTGGATATTGC CCTCCTTCTTGTATTCCTCTTG	52	S: ACCAAACCACACAAA M: TTTGTGTTGTTTGGT
DoxA2	37C	71.9	55	TGGCTGACGAGAAGAAGAAC CAAGATTCCGGAAGACGACG	56	S: CCTTCGAAGCTACCC M: GGGTAGCCTCGAAGG
DucC	41-42	76.4	60/55	AAGAGGCCACAGAGCAGC TTACCCGAGAAGATGATGGC	40	S: ACTCACACGGAATCT M: AGATTCCTCGTGAGT
eve	46C	83.9	60	TTGTGGACCTCTTGGCCACC AACTCCTTCTCCAAGCGACC		
sli	52D	113.9	50	TTACCAGCTTTAAGGGCTGC CATTTGTTCTCCAGGCAAGG	45	S: TTTAGCTAAAGCCCT M: AGGGCTTAAGCTAAA
plu	56F	130.8	60	AGCTGCGTTGGACAGGAC CGCACATTGCGCTTATCTAA	51	S: ATGATGTCGTTTCCC M: GGGAAACTACATCAT
Egfr	57F	135.1	60/55	CGTAAGCAATATCCGTTGGC CACATGGACGCACTCCTG	40	S: TCAAGATAGGTGGGA M: TCTCACCCATCTTGA
twi	59C	138.8	55	TCCCTGCAGCAGATCATCCC ATCACTCGAGCTGAGCATGC	37	S: TCTACCATTTAATGC M: GCCTTTAATTATAAT
zip	60F	145.9	60/55	TGTGAGTGTGTGTGGGTGC GATATTCGCTTTTCGGATGC	56	S: ACACTATCAGAGCCC M: GGGCTCTAATAGTGT
Lsp1	61A1	0.0	55	GAATGACGAGGGCATCCGAAG TGACCAGGGCTCCGAAAAACTC	40	S: AACTCCACTTTCTTA M: TAAGAAAATGGAGTT
ve	62A	5.0	53	GAGAACCCAACGCAGAATGT ATATCCTCCGACTCCGGAAG	56	S: TGCTGCAATCGGTGT M: ACACCGACTGCAGCA
Acr64B	64B-C	14.3	63	TGGGATGAGGCCGACTAC GTGGGGTGGTTGCTATCG	45	S: CCATATTAATATCTC M: GAGATATCAATATGG
Dbi	65E	21.3	60/55	ACCCAGAATGGTTTCCGAG GCAGGAACTCGTCATCACTG	37	S: GAATAGTTAGTTCGC M: GCGAACAACTATTTT
h	66D	28.7	55	ACTCAAGACTCTGATTCTGG TGTCTTCTCCAGAATGTCGG	41	S: TCTACTTCCAGCCGG M:CCGGCTAAAAGTAGA
CycA	68E	43.2	60/55	ATTTCGCCGTGCTCAATG ACGTCATGGTTCTCTTTGTCG	37	S: AGAAGTAAGTAAGTG M: CACTTCTCCATCTTA

(*continued*)

TABLE 1

(Continued)

Gene	Cytological Position	Genetic Position	PCR Annealing (degrees)[a]	PCR primers	ASO wash (degrees)[b]	ASO probes[c]
fz	70D	50.0	55	GTTGCAGGTGCTCACCTTC	51	S: ATCCAACAACCAAGT
				CGCCATGCAACAGAAGTAG		M: ACTTGGTAGTTGGAT
Eip71CD	71C–D	53.5	55	CCTGTATGGAGCCACCCG	45	S: AATTTCCCCGTTTGC
				GGGGCTGAGATTTAGCGATG		M: GCAAACGTGGAAATT
tra	73A	59.9	56	GAACAAGCGAGAGGGATAGC	56	S: TTCGAGAACAGGATC
				CTTTGGCGGTGGATTATACC		M: GATCCTGCTCTCGAA
rdgC	77B	71.7	63	CAAAGACATCGACTCAGCTACG	51	S: CACCACCTTTCACCA
				CGAACTCTCCACGATGCC		M: TGGTGAACGGTGGTG
5-HT2	82C	76.3	60/55	TGACGATTCCCCTCCTCC	48	S: AATGGCATACTCGTT
				CGCCCACTGATAGGAATTTG		M: AACGAGTCTGCCATT
Antp	84B	83.2	60	ACGGACGTTGGAGTTCCCGA	RT	S: CACCTACTTCAACTT
				ACATGCCCATGTTGTGATGG		M:AAGTTAAAGTAAGTG
ninaE	92B	89.5	55	CTACATCTGGTTCATGTCGAGC	51	S: CTGTAAGCGGCGACG
				CCACAGCGTGATGGTGAC		M: CGTCGCCACTTACAG
Fas1	89	101.3	60/55	GCGACTCTTTGAGTATCCGC	41	S: ATGTGGTATACTTAC
				GGCCAAGATGATGCAAATG		M: GTAAGTACACCACAT
Mst	87F	114.2	50	TCCTTTGCCTCTTCAGTCCG	37	S: TTCTCCCGTCGAACC
				TCCACAGGCATAGCATGGTC		M: GGTTCGCCGGGAGAA
Odh	86D	123.3	55	CAGTGGTGTTGGGACATGAG	51	S: TGAAATGAGGCGTTT
				TGGCCAGCTCAAACTTGTC		M: AAACGCCACATTTCA
Tub85E	85E	126.6	60/55	ACCAGTGCTAGGATTTTCGTG	37	S: TGGATCCCTGAAAGT
				TCCAGTCTCGCTGAAGAAGG		M: ACTTTTAAGGATCCA
hb	85A3–B1	134.6	53	TCTGCCCATCTAATCCCTTG	37	S: CCCTGGCTTATTTTT
				CTGCGTCGAGTTTTTTCCTC		M: AAAAATACCCCAGGG
Rox8	95D	147.7	60/55	ACAACAGCTCTGACCGCC	37	S: GTTATCATATGGCCA
				GACTTCCCTTTACACCGGAC		M: TGGCCACCTGATAAC
Ald	97A–B	157.7	55	ATGGGCCCTCACCTTCTC	37	S: ACCAATCGTGCATAG
				GTGGTCATCCACATGCAAAG		M: GTATGCACAATTGGT
Mlc1	98B	161.4	63	ACCCTGTTCGCTGACTGC	42	S: GTGCATAAACGATTT
				AAGTAGACATCCAGAGATCGGC		M: AAATCGTATATGCAC
jan	97A–B	171.2	55	CGCATTGAACACAATCCCGA	37	S: TTCCAAAACCCTTAA
				CTCATCGGAGATTTCGATCG		M: TTAAGGGCTTTGGAA
Ef1d2	100E	174.6	55	GACTGGTCTCCTCAAGCCAG	37	S: CCAATTCAGCAGAAT
				AGCCTCGTGGTGCATCTC		M: ATTCTGCAGAATTGG

[a] All PCR reactions followed this format: 94° for 3 min, 1 cycle; 94° for 45 sec, annealing temperature for 30 sec, and 72° for 15 sec, 40 cycles, unless two annealing temperatures are given; then the first was used for 10 cycles and the second for 30 cycles.
[b] Done for 15 min at designated temperatures.
[c] S, *simulans*-specific probe; M, *mauritiana*-specific probe.

region from 3-70 to 3-84 has a peak in each of two adjacent intervals. One peak is significant in the *simulans* backcross only, while the other is significant in the *mauritiana* backcross only. This region may contain two separate QTL with backcross-specific effects or it may contain a single QTL, in which case the difference in LOD peak positions in the two backcrosses is due to sampling error. These alternatives were tested using the procedure of Jiang and Zeng (1995), which is designed to distinguish pleiotropy and close linkage.

Figure 3 shows a two-dimensional likelihood profile surface for distinguishing between a single QTL with significant effects in both backcrosses and two QTL, each with a significant effect in only one backcross. The two-dimensional surface represents all possible combinations of two QTL positions between 3-70 and 3-84. The diagonal elements represent null hypotheses of just one QTL (*i.e.*, effects at the same map position in both backcrosses), while the off-diagonal elements represent alternative hypotheses of two QTL. A comparison is made between the maximum value on the two-dimensional surface (20.0 LOD score) and the maximum value on the diagonal (17.2 LOD score). The difference between the two likelihoods is asymptotically χ^2-distributed with 1 d.f. under the null hypothesis (one QTL). Thus the 95% significance threshold for the test is $0.217\chi^2_{1,\,0.05} = 0.83$ in LOD score, while the observed difference in LOD score is 2.82. Therefore, the test is significant and the hypothesis of two QTL with backcross-specific effects is favored.

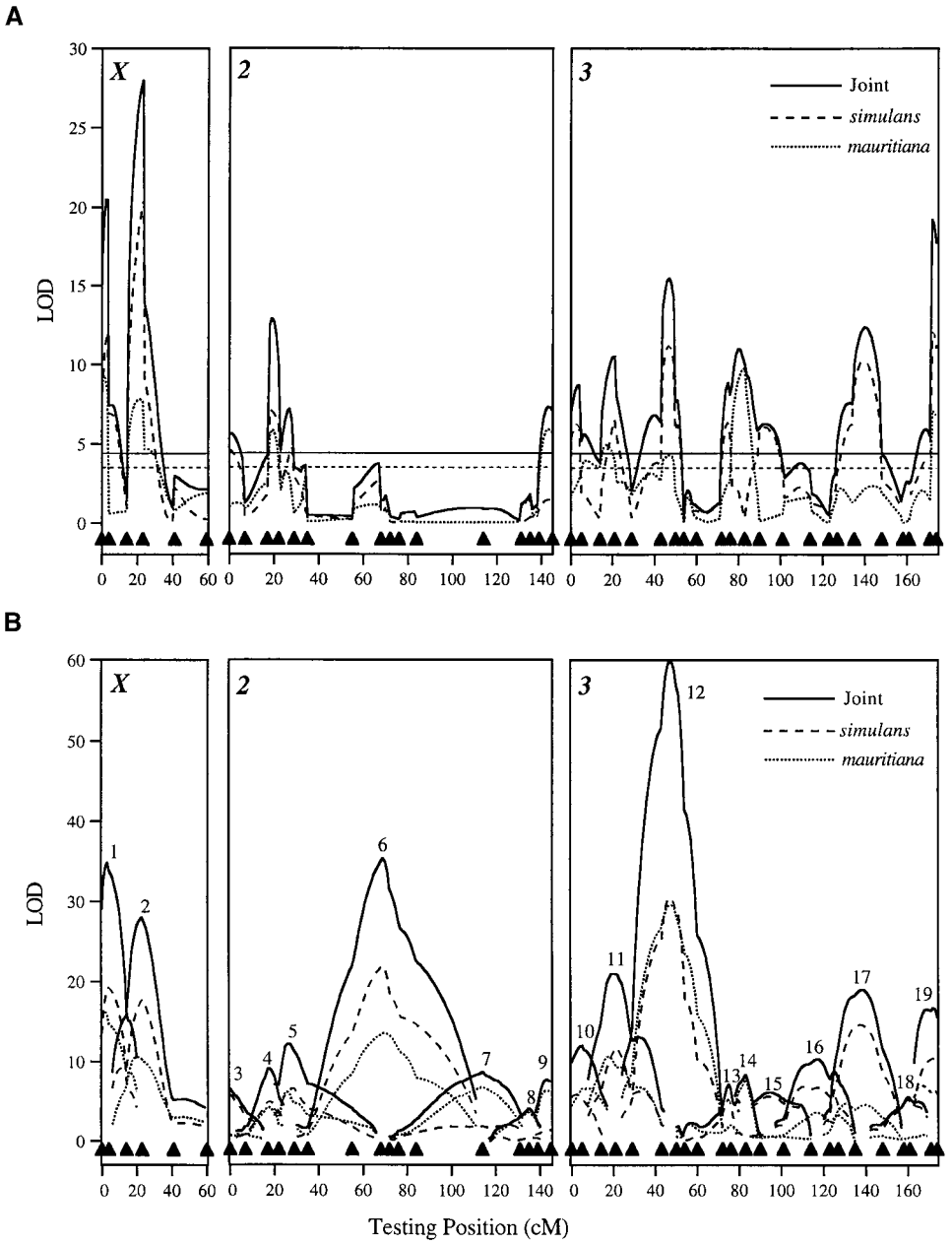

Figure 2.—LOD profiles for chromosomes X, 2, and 3 from composite interval mapping (a) and multiple interval mapping (b). The solid curve represents the joint analysis of both backcrosses, while the dashed and dotted curves represent separate analysis of each backcross. In a, the horizontal lines represent the critical values, solid for the joint analysis and dashed for the individual backcrosses. Putative QTL identified in a occur at map positions 1-3, 1-23, 2-1, 2-19, 2-67, 2-143, 3-4, 3-21, 3-47, 3-75, 3-83, 3-94, 3-140, and 3-172. In b, putative QTL are numbered for reference to information in Tables 2 and 3. Marker positions are given by triangle symbols.

Testing for QTL × sample interactions at each of the 14 putative QTL positions gave a significant LOD score only at position 3-75. Therefore, the mapping results for each backcross are generally consistent across the two independent samples. Testing for QTL × backcross interactions at each of the 14 positions gave significant LOD scores at three positions, 3-75, 3-83, and 3-94, and a nearly significant score at 3-140. These cases provide evidence of nonadditive inheritance (*i.e.*, dominance and/or epistasis).

An analysis of sample 1 (n = 378 over both backcrosses) with 18 markers was published previously (Liu *et al.* 1996). It is interesting to compare the results of the earlier, low resolution analysis with the present analysis, where the sample size is more than doubled (n = 965 over both backcrosses) and the marker number is increased to 45. This comparison is provided in Figure 4. Although the power to detect QTL effects is increased with larger sample size, the LOD scores in the full analysis ('99 CIM in Figure 4) are less than those in the smaller experiment ('96 CIM) because more background markers are included in the model to control for variation outside of the test interval. Although there is generally a good correspondence between the two analyses, it is clear that increases in sample size and marker number have improved the resolution. Some of the broad peaks in the '96 CIM analysis are resolved in the '99 analysis into two different peaks in nonadjacent intervals, clearly indicating multiple underlying QTL. This comparison suggests that further increases in sample size and marker number might lead to further changes in the estimated number and positions of QTL.

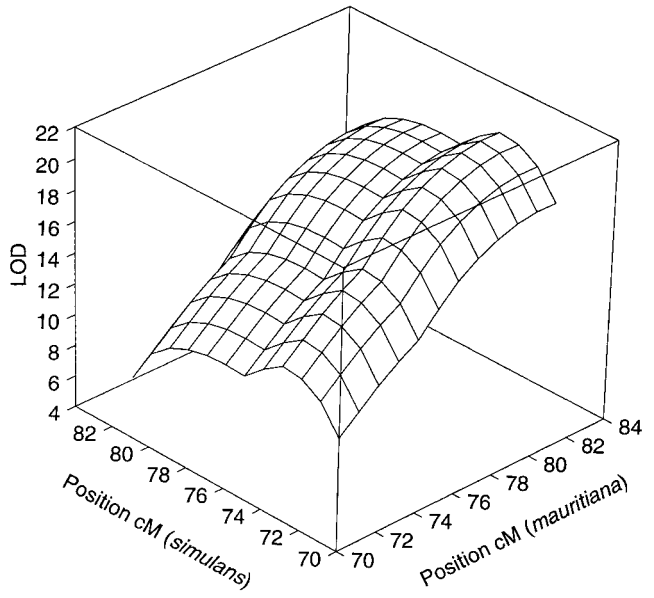

Figure 3.—Two-dimensional likelihood profile surface for the test to distinguish between a single QTL with significant effects in both *mauritiana* and *simulans* backcrosses and two QTL, each having a backcross-specific effect. See text for further explanation.

Multiple interval mapping: In the MIM analysis, model selection began with an initial genetic model suggested by the CIM results and continued in a search for additional QTL through several cycles of a backward/forward selection procedure. In each cycle, estimates of QTL position were readjusted for the model selected. In the joint analysis of both backcrosses and both samples, a total of 18 putative QTL were detected with a LOD value exceeding the CIM threshold of 4.4. A 19th QTL (number 8 at 2-135 cM) has a LOD value of 3.6, just below the CIM threshold. Figure 2b shows, for each of the putative QTL, a LOD profile that spans the region from one neighboring QTL to the other. The estimated positions and main effects are given in Table 2 and estimates of *a* and *d* are plotted in Figure 5.

A residual permutation test was performed under the null hypothesis of 18 QTL to determine the significance of adding the 19th QTL (*i.e.*, QTL 8 at 2-135). In a joint analysis of all four samples, a threshold of 4.6 LOD score was obtained. With this test, QTL 8 would not be significant. However, the evidence for this QTL comes mainly from sample BS2 (Table 3), so a residual permutation test was performed for BS2 only. In this case, the threshold value is 2.3 and the comparable test statistic for QTL 8 is 3.9. Given these 19 QTL, no other position shows a significant effect based on either joint or separate analyses of the four samples. Therefore it appears reasonable to include QTL 8 in the model, which brings the total number of QTL to 19.

The MIM analysis provides evidence for five QTL that were not detected in the CIM analysis. Their positions are 2-27, 2-114, 2-135, 3-117, and 3-160. CIM gives some indication of QTL at 2-27 and 3-117, but the evidence is not conclusive. However, when CIM is performed under relaxed conditions (*i.e.*, fewer markers in the multiple regression), the LOD scores in these two regions are significant.

There is a large difference between CIM and MIM in the LOD score for the putative QTL at 2-69. The reason is that the test under MIM is conditional on all QTL in the model, while the test under CIM is conditional on all markers. In the case of the putative QTL at 2-69, the neighboring QTL are 87 cM apart, while the neighboring markers are only 37 cM apart. A similar difference in LOD is found for the QTL at 3-47. Note that

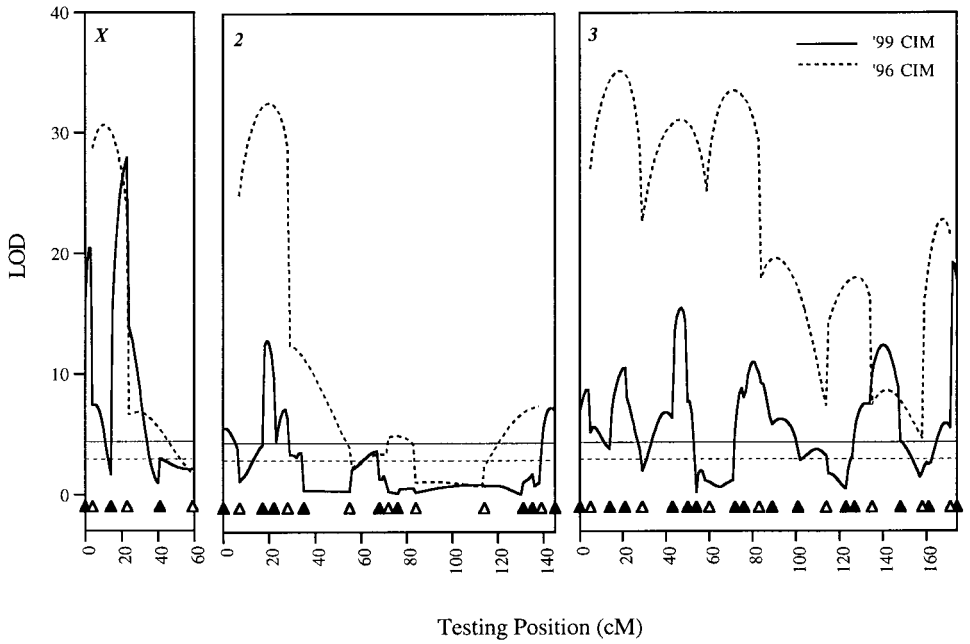

Figure 4.—LOD profiles for chromosomes X, 2, and 3 from composite interval mapping for the joint analysis of both backcrosses. The profile labeled '99 CIM is analysis of the full data set with two samples per backcross and 45 markers, while the profile labeled '96 CIM is a previously published (Liu *et al.* 1996) analysis of a smaller data set (one sample per backcross and 18 markers). Horizontal lines represent the critical values. Marker positions are given by triangle symbols; open triangles represent the 18 markers used in the '96 CIM analysis; all markers were used in the '99 CIM analysis.

TABLE 2

QTL positions, effects, and variance components estimated by multiple interval mapping

QTL	Position (Chromosome-cM)	Effect (%)		Variance component (%)[c]	
		$(a + d)$[a]	$(a - d)$[b]	BM	BS
1	1–3	8.4[d]	—[e]	4.4	4.4
2	1–23	8.3[d]	—[e]	4.5	3.0
3	2–0	−0.6	4.3	0.1	2.8
4	2–17	5.1	6.5	3.8	5.9
5	2–27	9.0	7.0	6.7	5.7
6	2–69	4.6	7.9	3.3	5.0
7	2–114	4.7	2.4	2.5	0.9
8	2–135	−2.6	0.3	−0.7	0.3
9	2–143	5.9	3.1	3.2	0.9
10	3–5	5.0	5.1	4.5	3.5
11	3–21	8.0	7.7	7.7	6.8
12	3–47	10.2	12.3	12.7	11.6
13	3–75	0.7	8.4	0.7	9.1
14	3–83	12.4	−1.2	14.9	−0.3
15	3–94	1.7	7.0	2.6	7.6
16	3–117	4.4	5.6	4.3	6.4
17	3–139	4.8	8.3	4.2	8.9
18	3–160	1.6	7.1	1.3	5.5
19	3–172	7.5	7.2	4.4	5.2
Total		99.1	99.0	85.1	93.2

[a]Expressed as percentage of difference between F_1 (0.0028) and *mauritiana* (−0.0230).
[b]Expressed as percentage of difference between *simulans* (0.0337) and F_1 (0.0028).
[c]100 ($\hat{\sigma}^2_{ir}/\hat{\sigma}^2_p$), see Equation 4.
[d]The average of additive effect estimates from the two backcrosses.
[e]QTL in chromosome X do not contribute to the difference between *simulans* and F_1.

the magnitude of LOD score is not strictly proportional to magnitude of effect because the LOD also depends on the proximity of conditioning markers or neighboring QTL.

Once the number and positions of QTL main effects were established in a joint analysis of both backcrosses, the MIM proceeded to select QTL pairs that have significant epistatic effects in each backcross separately. No significant interactions were detected in the *simulans* backcross, while six were detected in the *mauritiana* backcross. It is notable that the main effect of QTL at 2-0 was significant only in the *simulans* backcross, yet it shows significant interactions with two other QTL in the *mauritiana* backcross. However, no completely new QTL were detected by their interaction with QTL identified through marginal effects in one backcross or the other. The six effects detected in the *mauritiana* backcross together account for only 6.5% of the phenotypic variance. Therefore, epistasis appears to be relatively unimportant in these backcross populations.

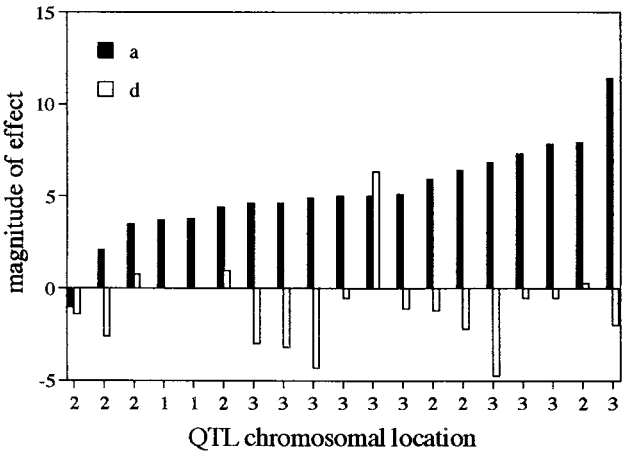

Figure 5.—The distribution of additive (*a*) and dominance (*d*) effect estimates of putative QTL, arranged in rank order according to the estimate of *a*. The effects are expressed as a percentage of half the difference between the parental lines.

A difference in magnitude of effect of a QTL between the two backcrosses may be due to dominance. For example, a case of complete dominance would give a significant effect in one backcross (say, Qq *vs.* qq), but no effect in the other (QQ *vs.* Qq). Estimation of *a* and *d* from the difference between the backcrosses reveals considerable variation among loci in the estimated degree of dominance (Figure 5). On the average, *d* is negative, suggesting that *mauritiana* alleles tend to be dominant, which is consistent with the observation that the F_1 mean is significantly less than the midparent value.

Only one QTL (3-83) appears to have strong dominance of the *simulans* allele.

Figure 5 shows the distribution of additive effect estimates for the 19 putative QTL. The shape of this distribution is probably quite different from the distribution of true values of *a* for two reasons: (1) the true distribution may have a large number of very small effects that cannot be detected with the power of the current experiment; and (2) some of the estimates may represent the combined effects of multiple, closely linked QTL. Nevertheless, Figure 5 contains some important observations. It shows that no one QTL accounts for a large fraction of the parental difference and that nearly all of the effects are positive, which means that *simulans* has plus alleles, and *mauritiana* has minus alleles, at nearly all QTL.

Table 2 gives an estimate of the fraction of the phenotypic variance in the backcross population that is accounted for by each putative QTL ($\hat{\sigma}^2_{ir}/\hat{\sigma}^2_{\mathrm{p}}$). These estimates are likely to be more accurate than the variance components reported in most previous QTL studies, which generally are based on simple or multiple regression of the phenotype on marker genotypes. The sum of ($\hat{\sigma}^2_{ir}/\hat{\sigma}^2_{\mathrm{p}}$) estimates over all putative QTL effects is R^2 for the model, which provides an estimate of broad-sense heritability. The heritability estimates are very high: 0.93 for BS and 0.92 for BM (0.85 from Table 2 plus 0.07 for epistasis). Nevertheless, they are consistent with the observation noted above that the environmental variance is an order of magnitude smaller than the phenotypic variance of the backcross populations.

The MIM model-building procedure was evaluated by comparing models obtained through separate and joint analyses of the four different samples (Table 3). Within each backcross, QTL detected in the smaller samples are also detected in the larger samples and the larger samples detect more QTL, as expected. The estimated positions of QTL are very consistent in different samples within each backcross. These results indicate that the MIM method gives very similar results in the analysis of independent samples.

The MIM results are also generally consistent between the two backcrosses, even though one expects some differences due to dominance effects. Table 3 shows that most QTL are detected at similar positions in both backcrosses. However, in some cases, QTL are found at a certain position in one backcross but not the other. Three such cases (3-75, 3-83, and 3-94) showed significant QTL × backcross interactions in the CIM analysis, which can be interpreted as dominance effects, as noted earlier. Other cases might also be due to dominance effects or possibly just sampling errors. In one case (QTL 7), the result seems to indicate that a single QTL in the joint analysis might be due to two QTL with backcross-specific effects and slightly different positions. However, this interpretation is inconclusive due to the flat likelihood profile in the BS samples in this region.

A cross-validation study was performed separately on each backcross to further assess the MIM model-building procedure and effect estimation. In this study, one sample was analyzed by MIM and then used to predict phenotypic values in the other independent sample. The results (Table 3) show a high level of predictability. For predicting sample 1 from sample 2, the R^2 is 0.83 for BM (Figure 6) and 0.89 for BS. For predicting sample 2 from sample 1, the R^2 is 0.86 for BM and 0.88 for BS. Although the results appear quite impressive, this study may not provide a very sensitive test of the predictive ability of the MIM procedure because of the consistency in direction of most allelic effects and the existence of considerable linkage disequilibrium in the backcross populations.

The MIM analysis was compared with a multiple regression of phenotypic value on marker genotype using a backward stepwise selection procedure. Table 3 shows that the two types of analysis give similar results in terms of the number of QTL in the MIM model and the number of markers in the regression model. They also give similar results for the R^2 of the model and for cross-validation R^2 values. The MIM model gives consistently higher R^2 values, but not by a large margin (*i.e.*, average of 0.92 *vs.* 0.88 for model R^2 and 0.87 *vs.* 0.83 for cross-prediction R^2). Given the high density of markers relative to the level of recombination in the backcross populations analyzed here, the similarity between the two types of model is not unexpected. It is likely that the difference between MIM and multiple regression on markers would be much larger in experiments with lower marker density.

DISCUSSION

This study is one of the first applications of multiple interval mapping, which has several advantages over previous QTL mapping procedures. These advantages stem mainly from the fact that MIM uses a multiple-QTL model, whereas other procedures like CIM use a different, single-QTL model for each interval analyzed. With a multiple-QTL model, the effects of all putative QTL are analyzed simultaneously so that epistatic terms can be included, and variance components and heritability can be estimated. Thus, it is expected that MIM will be more precise and powerful than single-QTL mapping methods, particularly in parameter estimation. In this study, the utility of MIM was analyzed in a cross-validation study, which showed a very high level of predictability. In addition, MIM analysis of replicate samples gave similar estimates for QTL positions. These results are very promising, but further work is needed to assess the efficacy of MIM in dealing with various types of genetic models that involve less coupling linkage disequilibrium.

The difference in posterior lobe morphology between *D. simulans* and *mauritiana* is clearly polygenic. The CIM

TABLE 3

Summary results of separate and joint MIM analyses

	BM1 (n = 192)			BM2 (n = 299)			BS1 (n = 186)			BS2 (n = 288)			Joint analysis (n = 491; 474)		
QTL	$\hat{p}$[a]	LOD	$\hat{\alpha}$[b]	$\hat{p}$	LOD	$\hat{\alpha}$	$\hat{p}$	LOD	$\hat{\alpha}$	$\hat{p}$	LOD	$\hat{\alpha}$	$\hat{p}$	LOD	$\hat{\alpha}$
Chromosome X															
1	0	7.7	1.72	1	9.1	1.95	7	19.5	2.93	3	20.4	2.89	3	36.4	1.76; 2.56
2	20	12.7	2.29	20	12.0	1.52	25	7.3	1.84	23	16.7	2.45	23	28.6	1.82; 2.43
Chromosome 2															
3	—	—	—	—	—	—	—	—	—	0	6.1	1.61	0	7.0	−0.16; 1.32
4	18	44.1	3.63	10	2.8	0.94	19	69.7	5.18	18	5.6	1.89	17	8.2	1.31; 2.01
5	—	—	—	26	21.2	2.50	—	—	—	28	5.7	2.30	27	13.0	2.33; 2.16
6	66	20.5	2.36	69	12.2	1.00	66	20.0	2.28	69	22.8	2.49	69	36.1	1.19; 2.45
7	—	—	—	114	9.3	1.99	125	12.3	2.20	125	9.9	1.20	114	9.1	1.22; 0.74
8	—	—	—	135	3.9	−1.84	—	—	—	—	—	—	135	3.6	−0.68; 0.10
9	139	9.2	1.40	143	7.2	2.34	—	—	—	—	—	—	143	8.2	1.53; 0.97
Chromosome 3															
10	12	4.6	2.04	5	3.0	1.02	5	6.6	2.17	0	6.8	1.54	5	15.1	1.29; 1.57
11	19	3.3	1.30	17	14.4	2.54	21	9.5	2.42	21	16.9	2.51	21	21.1	2.07; 2.37
12	56	24.8	3.60	47	33.2	3.10	51	36.2	4.24	47	29.7	3.37	47	66.3	2.64; 3.80
13	—	—	—	—	—	—	—	—	—	75	14.9	3.45	75	7.4	0.17; 2.61
14	84	37.8	3.46	83	55.5	3.71	—	—	—	—	—	—	83	8.0	3.20; −0.36
15	—	—	—	—	—	—	88	26.7	2.68	97	6.0	2.08	94	6.7	0.44; 2.16
16	—	—	—	117	7.9	.70	118	7.6	1.45	118	6.9	1.93	117	10.1	1.14; 1.73
17	138	22.3	2.36	141	2.6	0.86	134	6.7	2.43	139	16.3	2.82	139	19.5	1.25; 2.58
18	—	—	—	—	—	—	155	10.4	2.90	160	3.9	1.59	160	7.8	0.40; 2.20
19	172	18.5	2.17	168	22.4	2.4	174	13.9	2.13	172	10.8	2.50	172	17.5	1.93; 2.23
Summary of MIM results															
QTL number	11			15			13			16			19		
Epistatic terms	1			5			0			0			6; 0		
R^2	0.91			0.91			0.93			0.93			0.92; 0.93		
Cross-prediction[c] R^2	0.83			0.86			0.89			0.88					
Summary of backward stepwise selection on markers (with α = 0.01)															
Marker number	11			16			14			18					
R^2	0.86			0.83			0.9			0.92					
Cross-prediction[c] R^2	0.82			0.77			0.87			0.85					

[a] Estimated map position in centimorgans.
[b] Estimated effect in PC1 units $\times 10^{-3}$. In the joint analysis, $\hat{\alpha}$ is given for BM and BS separately.
[c] R^2 values are given for prediction of sample 2 from sample 1 (or sample 1 from sample 2) within each backcross.

analysis shows that at least 14 loci contribute to the PC1 trait difference, whereas MIM indicates a somewhat larger number of 19. Effect estimates from both analyses show that no single QTL explains a large fraction of the parental difference. In the MIM analysis, the largest additive effect estimate is 11.4% of the parental difference and 10 of the 19 putative QTL detected have additive effect estimates ≥5.0%. Some of these values may be overestimates if there are multiple, closely linked QTL within a single interval.

We have previously proposed a quantitative definition of a major gene effect as one for which the distributions of alternative homozygotes (on a uniform isogenic background) show little overlap, such that the probability of misclassification is <0.05 (True *et al.* 1997). When both homozygous classes are normally distributed with equal variance, the probability of misclassification is 0.05 when the means are 3.28 standard deviations apart. For PC1, the parental means are 35 environmental standard deviations apart, so a major gene would have an effect

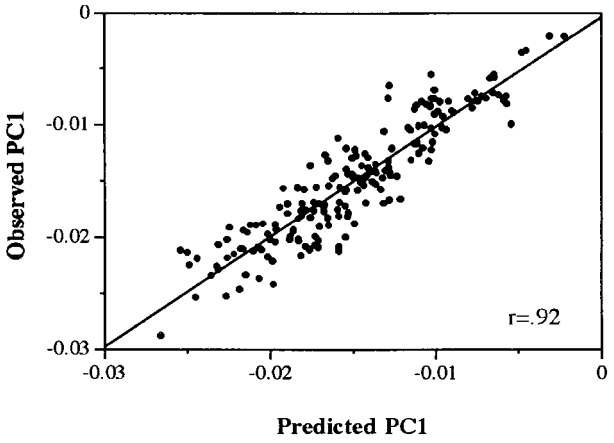

Figure 6.—Plot of cross-validation study results for the *mauritiana* backcross. Sample 2 was analyzed with multiple interval mapping to develop a model and parameter estimates, which were then used to predict the phenotypic values of sample 1 individuals.

that explains 9.4% of the parental difference. Only one putative QTL (3-47) has an additive effect estimate >9.4%. However, seven putative QTL have effect estimates >2.0 environmental standard deviations (5.7%), which can be detected readily with moderate sample sizes. Therefore, identification of individual QTL appears feasible through introgression and other approaches (Alpert and Tanksley 1996; Cormier *et al.* 1997).

Epistatic effects appear to be relatively unimportant for PC1 in the interspecific backcross populations. This observation is difficult to interpret biologically, because an interspecific backcross is segregating for alleles that may never have occurred together in the same population before. However, the lack of strong epistasis between alleles that were fixed in different populations may indicate that such alleles are generally "good combiners." Crow (1957) once suggested that mutations with largely additive gene action may produce advantageous effects on a variety of genetic backgrounds and therefore be selected efficiently, while those with complex interactions may tend to remain at low frequency. On the other hand, Long *et al.* (1995) found strong epistatic effects on Drosophila bristle number for genes that responded to strong artificial selection, which indicates that interacting alleles may respond to directional selection. However, strong artificial selection in a small population may produce different results than natural selection in a large population.

A striking result of the QTL analysis reported here is that all but 1 of the 19 additive effect estimates have the same sign (Figure 5). Orr (1998) provides a statistical test for whether the observed numbers of plus and minus alleles in one line is greater than expected under neutrality, given that the QTL study begins with a certain phenotypic difference. A simplified form of the test assumes that all QTL have equal effects, which appears to be conservative in the present case where the single negative allele has a smaller effect than any of the 18 plus alleles. Using this test, the probability of observing 18 of 19 plus alleles in the high line is 0.0001. Therefore, it is highly unlikely that the interspecific difference in genital morphology evolved by random drift.

The strong preponderance of plus alleles in one species suggests a history of consistent directional selection operating on the trait. *D. mauritiana* and *simulans* diverged from a common ancestor an estimated 0.6–0.9 million years ago (Hey and Kliman 1993), which probably corresponds to ~10 million fly generations. A lot of things can happen in 10 million generations. If there were reverses in the direction of selection or a long period of stabilizing selection, a mixture of plus and minus allelic effects is expected. However, consistently positive directional selection throughout the period of isolation would produce the type of genetic architecture observed. It is also possible that a recent strong episode of directional selection has erased the traces of previous evolutionary changes, but this would require that the recent fixations occurred at nearly all of the same loci involved in previous fixations. This situation seems unlikely because studies of artificial selection in Drosophila have shown that replicate selection lines frequently differ in the contributions of the three major chromosomes to selection response [see appendix of Charlesworth *et al.* (1987)] and therefore in what genes respond at any given time.

For most quantitative traits analyzed previously, there is a mixture of plus and minus alleles in each species, leading to transgressive segregation (Tanksley 1993). For example, in a cross between two tomato species, 36% of QTL detected had effects opposite to those predicted by the parental phenotypes (DeVicente and Tanksley 1993). Even in *D. mauritiana* and *simulans*, QTL analysis of male-specific bristle number traits revealed a mixture of plus and minus alleles (True *et al.* 1997). Therefore, the consistent allelic effects on posterior lobe morphology may be a rare situation that reflects a history of unusually strong directional selection.

Divergent male genital structures, such as the posterior lobe, are thought to evolve by sexual selection through cryptic female choice implemented by postmating mechanisms such as remating and sperm displacement (Eberhard 1996; Price 1997; Arnqvist 1998). A runaway process of sexual selection (Lande 1981) may produce very strong selection pressures that lead to rapid evolution of sexually dimorphic traits. However, under some models, cyclical change in the direction of selection on a male trait is expected (Iwasa and Pomiankowski 1995), which is not consistent with the posterior lobe genetic architecture reported here.

We acknowledge the Duke University Morphometrics Laboratory

for the use of equipment and software. This work was supported by U.S. Public Health Service Grants GM-47292 and GM-45344.

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Communicating editor: A. G. Clark