

AN EPISTATIC GENETIC BASIS FOR FLUCTUATING ASYMMETRY OF MANDIBLE SIZE IN MICE

LARRY J. LEAMY,¹ ERIC J. ROUTMAN,² AND JAMES M. CHEVERUD³

¹*Department of Biology, University of North Carolina, Charlotte, North Carolina 28223*

E-mail: ljleamy@email.uncc.edu

²*Department of Biology, San Francisco State University, San Francisco, California 94132*

E-mail: routman@sfsu.edu

³*Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, Missouri 63110*

E-mail: cheverud@pcg.wustl.edu

Abstract.—The genetic basis of fluctuating asymmetry (FA), or nondirectional variation in the subtle differences between left and right sides of bilateral characters, continues to be of considerable theoretical interest. FA generally has been thought to arise from random noise during development and therefore to have a largely or entirely environmental origin. Whereas additive genetic variation for FA generally has been small and often insignificant, a number of investigators have hypothesized that interactions between loci, or epistasis, significantly influence FA. We tested this hypothesis by conducting a whole-genome scan to detect any epistasis in FA of centroid size in the mandibles of more than 400 mice from an F₂ intercross population formed from crossing the Large (LG/J) and Small (SM/J) inbred strains. Genotypic deviations were imputed at each site 2 cM apart on all 19 autosomes, and these and centroid size asymmetry values were used in canonical correlation analyses for each of the 171 possible pairs of 19 autosomes to identify the most probable sites for epistasis. Epistasis for centroid size asymmetry was abundant, occurring far more often than was expected by chance alone (there were 30 separate instances of epistasis at the 0.001 significance level, when only two were expected by chance alone). The contributions of epistasis from 30 pairwise combinations of loci tended to suppress the additive and dominance genetic variance, but greatly increased the epistatic genetic variance for FA in centroid size given the intermediate allele frequencies of an F₂ intercross population.

Key words.—Epistasis, fluctuating asymmetry, mandible centroid size, mice, quantitative trait loci.

Received March 29, 2001. Accepted November 26, 2001.

Several years ago we (Leamy et al. 1997) conducted a quantitative trait locus (QTL) study with mice to ask whether there is a genetical basis for fluctuating asymmetry (FA), that is, nondirectional variation in the subtle differences nearly always found between left and right sides of bilateral characters (Van Valen 1962). We were unable to detect a significant number of QTLs for FA in interlandmark distances between coordinate points on the mandibles in these mice and therefore concluded that there was little or no genetical variance for FA (Leamy et al. 1997). More recently, Klingenberg et al. (2001) reanalyzed these same data using geometric morphometric methods; they also found very little statistical support for QTLs affecting asymmetry of either size or shape in these mandibles. These results were anticipated for FA because of previous studies showing that the heritability of FA in a number of characters is usually quite low in magnitude, if not zero (Leamy 1997, 1999; Møller and Thornhill 1997; Whitlock and Fowler 1997).

The presumed environmental origin for FA is consistent with the general view that FA arises primarily from random noise during development (Palmer 1994; Zakharov 1994) and thus is a direct reflection of developmental stability. Developmental stability is the ability of organisms in populations to consistently produce ideal phenotypes in a given environment (Zakharov 1989) and is a useful indicator of the level of resistance to environmental stress (Parsons 1990). For this reason, FA has become increasingly popular as a measure of stress in populations, with more stressed populations expected to exhibit increased levels of FA (Clarke 1992; Graham 1992; Møller and Pominakowski 1993; Zakharov 1994; Markow 1995).

If FA is little governed by additive effects of genes, however, perhaps nonadditive genetic effects, including both dominance and epistasis, play an important role in controlling levels of FA. Dominance of genes at various loci often has been hypothesized to explain the results of studies that have shown significant differences in FA levels between heterozygotes and homozygotes or between hybrid species and their parents (Thoday 1958; Leamy 1984; Livshits and Kobylansky 1991; Clarke 1994; Livshits and Smouse 1994). These results also may be explained by epistasis, however, because genetic coadaptations present in parents tend to be broken down in their hybrid offspring (Clarke 1993). In addition, Klingenberg and Nijhout (1999) generated epistatic effects in a theoretical model that simulated character variation and developmental noise (Klingenberg and Nijhout 1999), so there also may be developmental reasons to expect epistasis for FA.

Might epistasis be a crucial component of the genetic architecture of FA? Unfortunately, we do not yet know the answer to this, because direct tests for epistasis are not common for most characters and are even rarer for FA (but see Blows and Sokolowski 1995). This is primarily due to the statistical difficulties inherent in the traditional approaches, which typically rely on covariances among relatives (Falconer and Mackay 1996). Recently, however, a new approach to testing for epistasis has been developed that takes advantage of the genetic and genotypic values estimated in QTL studies (Cheverud and Routman 1995, 1996; Routman and Cheverud 1997). In the study reported here, we took this approach to test for epistasis in FA of the mandibles of the same mice we used for the QTL analyses (Leamy et al. 1997;

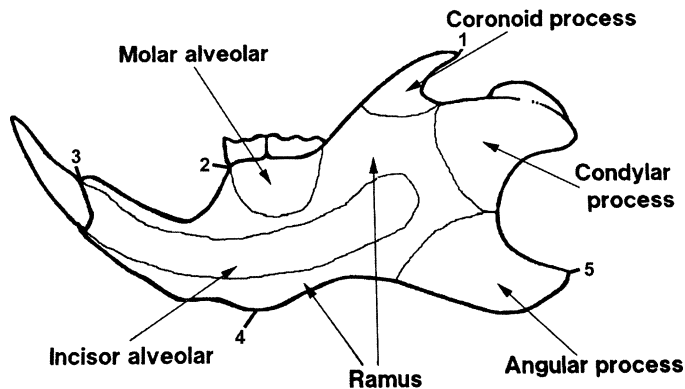


FIG. 1. Outline of a mouse mandible showing the five landmark points that were digitized.

Klingenberg et al. 2001). A whole-genome scan was conducted that made use of all possible pairs of chromosomes; despite the previous negative results in searches for QTLs directly influencing FA in these mice, the results of this scan uncovered evidence of an abundance of epistasis for FA.

MATERIALS AND METHODS

The Population and Measurements

The mice used in this study were 70-day-old F_2 progeny produced from an intercross of the Large (LG/J) and Small (SM/J) inbred strains originally obtained from the Jackson Laboratory (Bar Harbor, ME; see Cheverud et al. 1996). DNA extracted from the spleen in each mouse was used to genotype a total of 76 polymorphic microsatellite loci on the 19 autosomes using standard polymerase chain reaction (PCR) techniques (Routman and Cheverud 1994, 1995). We eliminated one marker, *D2Mit1*, from the analysis because recombination percentages between this marker and the next marker on chromosome 2 (*D2Mit17*) approached 50% (Cheverud et al. 1996). The positions of the other 75 microsatellite loci were determined using the program Mapmaker 3.0b (Lander et al. 1987; Lincoln et al. 1992). The distances between markers are given for reference in the Appendix.

The skeletons of these mice were prepared by exposure to dermestid beetles (Cheverud et al. 1997) with both left and right sides of the mandible scanned into a computer. The coordinates of five landmarks around the periphery of each mandible (Fig. 1) were recorded from these scans with the National Institutes of Health program IMAGE. Each mandible was digitized three times, which yielded three sets of coordinates for both sides of the mandibles in each mouse. After removal of a few outliers (Sokal and Rohlf 1995) in a preliminary analysis of these coordinate points as well as individuals for which mandibles were chipped or broken during the skeletonization or measurement process, the final sample size available was 476 mice (244 males, 232 females).

Asymmetry Measures

Klingenberg et al. (2001) used these same mandible coordinate data to generate centroid size (and shape) characters and their asymmetries, all of which were subjected to QTL analyses. Centroid size is defined as the square root of the

sum of squared distances between each landmark of a configuration and its centroid, where the centroid is the point whose x and y coordinates are the means of the x and y coordinates of all landmarks (Dryden and Mardia 1998). Klingenberg et al. (2001) showed that FA in mandible centroid size asymmetry was statistically significant and contributed about 5.5% to the total variance, whereas measurement error was trivial, amounting to less than 1% of the total variance (Klingenberg et al. 2001).

Centroid size provides a very useful overall measure of size in the mandible, and we therefore decided to use the asymmetry of centroid size for the epistasis analysis (see below). The mean of the three repeat measurements for centroid size first was calculated for both left and right sides of each mandible, and the distribution of signed differences between left and right sides was examined. This distribution was found to be normal, suggesting that there was no apparent antisymmetry, a kind of asymmetry detected by significant platykurtosis (Palmer and Strobeck 1992). The mean of the right minus left differences of centroid size was subtracted from the signed difference between the sides to statistically correct for directional asymmetry, and the absolute values of these differences were used to measure FA (Leamy 1984; Hutchison and Cheverud 1995; Leamy et al. 1997). The distribution of unsigned differences between sides for centroid size, however, was half-normal as expected (Palmer 1994), and these values therefore were subjected to Box-Cox transformations (Swaddle et al. 1994) of the form $(FA + 0.0005)^{0.33}$, which were successful in achieving normality. These unsigned differences also were adjusted for potential effects of sex, dam, experimental block, and litter size (see Leamy et al. 1999) and, finally, regressed on centroid size left-right means to test for any scaling effects. No significant scaling was detected, and therefore no further adjustment of these asymmetry characters was necessary prior to the epistasis analysis.

Analysis of Epistasis

Epistasis generally has been viewed in a statistical sense, where deviations from additive combinations of single-locus effects contribute to an interaction or epistatic variance typically assessed in an analysis of variance (Falconer and Mackay 1996). This statistical epistasis is a population phenomenon that depends on allele frequencies at the relevant loci (Cheverud and Routman 1995; Routman and Cheverud 1997). Various models such as diallel crosses and line crosses have been used to estimate the interaction variance in an attempt to assess the importance of statistical epistasis (Lynch and Walsh 1998). These approaches actually are designed to generate relatives of various sorts (such as half- and full-siblings), because covariances between these relatives can then be used to estimate genetic components of interest, including epistatic ones.

We tested for epistasis in the unsigned asymmetry of centroid size using a different approach that depends on estimates of effects of individual QTLs (Cheverud and Routman 1995). In this approach, epistasis is viewed in a physiological sense, where the phenotypic differences among various genotypes depend on genotypes at other loci. Physiological epistasis

therefore is measured by genotypic values only and is independent of allele frequencies at the loci considered (Cheverud and Routman 1995; Routman and Cheverud 1997). Although we used the physiological epistasis approach here, this approach also allowed us to estimate the contribution of physiological epistasis to the genetic variance components familiar to quantitative geneticists (Cheverud and Routman 1995).

As a first step in assessing epistasis in asymmetries of centroid size in the F_2 mouse population, we set additive genotypic deviations (here denoted X_{aij} where the i and j subscripts denote the two alleles at a locus) to -1 , 0 , and $+1$ and dominance genotypic deviations (X_{dij}) to 0 , $+1$, and 0 , respectively, for SM/J homozygotes, heterozygotes, and LG/J homozygotes, at the locations of all genetic markers on each chromosome. We then imputed all additive and dominance genotypic deviations in 2-cM steps between flanking microsatellite markers using recombination frequencies calculated with the program Mapmaker 3.0 (Lincoln et al. 1992) and the formulas in Haley and Knott (1992). Once all genotypic deviations were obtained for all chromosomes in each individual mouse, they were merged with centroid size asymmetry values and subjected to canonical correlation analysis via the CANCELL procedure in SAS (SAS Institute 1989). For each of the 171 possible pairs of 19 autosomes, canonical correlations were run for every pair of locations 2 cM apart, generating more than 290,000 separate analyses.

Each canonical correlation analysis consisted of centroid size asymmetry as a single character in one group, and all four interactions of the additive and dominance genotypic deviations from the two chromosomes ($X_{aij}X_{akt}$, $X_{aij}X_{dkt}$, $X_{dij}X_{akt}$, $X_{dij}X_{dkt}$) as the characters in the other group. All analyses were run by holding the main effects associated with the additive and dominance genotypic deviations (X_{aij} , X_{dij} , X_{akt} , X_{dkt}) constant. At each position 2 cM apart on a given chromosome, these analyses generated F approximations to Rao's statistic with their associated probabilities. For each pair of chromosomes, the pair of positions associated with the lowest probability within valleys of probabilities of 10% or less was taken to be a likely combination for potential epistasis. Where the two-chromosome plots of probabilities yielded two or more valleys clearly separated by ridges (areas with probabilities greater than 10%), each valley was viewed as a potential separate instance of epistasis.

We conducted multiple regression analyses of centroid size asymmetry values on the imputed genotypic deviations and all four of their pairwise products at each of the combinations of sites identified as described above. For each of the two separate loci (e.g., A and B), these regressions estimated the average effect of a gene substitution (α_A and α_B) as well as the dominance deviations (δ_A and δ_B) and the pairwise interactions of these effects ($\alpha_A\alpha_B$, $\alpha_A\delta_B$, $\delta_A\alpha_B$, and $\delta_A\delta_B$; see Cheverud and Routman 1995). These genetic values, in turn, were converted to the desired additive (a_A , a_B) and dominance genotypic values (d_A , d_B) for each locus and their epistatic interactions (a_Aa_B , a_Ad_B , d_Aa_B , and d_Ad_B) using the equations in Routman and Cheverud (1997; eq. 10), which are repeated here for reference:

$$a_A = \alpha_A + \alpha_A\delta_B/3, \quad (1a)$$

$$d_A = \delta_A + \delta_A\delta_B/3, \quad (1b)$$

$$a_B = \alpha_B + \delta_A\alpha_B/3, \quad (1c)$$

$$d_B = \delta_B + \delta_A\delta_B/3, \quad (1d)$$

$$aa = \alpha_A\alpha_B, \quad (1e)$$

$$ad = 2\alpha_A\delta_B/3, \quad (1f)$$

$$da = 2\delta_A\alpha_B/3, \quad \text{and} \quad (1g)$$

$$dd = 4\delta_A\delta_B/9. \quad (1h)$$

Additive-by-additive epistasis (a_Aa_B) implies that the single-locus additive genotypic value for the A locus (a_A) differs depending on what genotype is at the B locus and vice versa. Additive-by-dominance epistasis (a_Ad_B) implies that the single-locus additive genotypic value for locus A (a_A) differs depending on what genotype is at the B locus, whereas the single-locus dominance genotypic value at the B locus (d_B) differs depending on the locus A genotype. Dominance-by-additive epistasis (d_Aa_B) is the same except for a reversal of these roles at each locus. Dominance-by-dominance epistasis (d_Ad_B) implies that the single-locus dominance genotypic value at locus A (d_A) changes depending on the genotype at locus B and vice versa (Cheverud 2000). The relative importance of all four of these epistatic genotypic values depends in part on the frequencies of the alleles at both loci (for a detailed description of this, see Cheverud and Routman 1996).

For each combination of loci, we tested for the overall significance of epistasis with an F -statistic. This statistic (with 4 and $n - 9$ df, where n is the total sample size) was calculated by the mean of the squared t -values associated with the four individual genotypic epistasis terms (Routman and Cheverud 1997). It should be noted that this test has considerably more statistical power to detect true epistasis, if present, than conventional tests based on the interaction variance (Routman and Cheverud 1997). This is because this test assesses the total effects of epistasis, including those that contribute to additive and dominance genetic variance (Cheverud and Routman 1996). Once significant overall epistasis was discovered for any given combination of sites on two chromosomes, significance for each of the four genotypic epistasis terms (a_Aa_B , a_Ad_B , d_Aa_B , d_Ad_B) was tested with t -tests (Routman and Cheverud 1997).

All of these many F -tests performed, however, naturally generated a multiple comparisons problem (Sokal and Rohlf 1995). We addressed this problem via a method described by Cheverud (2000, 2001), which is based on the number of effective markers on each chromosome (see also Cheverud et al. 1983; Wagner 1984; Cheverud 1996). To calculate this number, we first obtained the ratio of the observed variance of the eigenvalues ($V[\lambda]$) of correlations of the additive genotypic deviations at each marker on each chromosome, to the maximum possible variance of the eigenvalues. The maximum eigenvalue variance, M , was simply set equal to the number of markers used (75). The effective number of markers on each chromosome then was calculated as $M_e = M\{1 - [V(\lambda)(M - 1)/M^2]\}$. Finally, the total number of inde-

pendent epistasis tests was calculated by the sum of the cross-products of the effective number of markers for all 171 pairs of chromosomes. This sum was 1850, suggesting that we might expect about 92 tests to be significant at the 5% level, 19 at the 1% level, and two at the 0.1% level, due to chance alone.

Once this assessment of epistasis was completed, it seemed useful as an illustrative exercise to compute the contribution of epistasis between pairs of loci to additive (V_A), dominance (V_D), and epistatic or interaction genetic variance (V_I). Additivity, dominance, and epistasis of genes all contribute to V_A , both dominance and epistasis contribute to V_D , but only epistasis itself contributes to V_I , but it also may contribute to additive and dominance genetic variance (Cheverud and Routman 1995). These genetic variances conventionally reflect the variation across all loci in the genome; here, however, we assess the magnitude of these variances contributed by pairwise combinations of loci.

The contribution of epistasis to the genetic variances was calculated using equation (19) in Cheverud (2000), modified according to the assumption that the allelic frequencies at all relevant loci were 0.5:

$$V_A = 0.5[a_A + 0.5(e_{11..} - e_{12..}) + 0.5(e_{12..} - e_{22..})]^2 + 0.5[a_B + 0.5(e_{11..} - e_{..12}) + 0.5(e_{..12} - e_{..22})]^2, \quad (2a)$$

$$V_D = [0.5d_A - 0.25(e_{11..} - 2e_{..12} + e_{..22})]^2 + [0.5d_B - 0.25(e_{..11} - 2e_{..12} + e_{..22})]^2, \quad (2b)$$

$$V_I = \frac{\epsilon_{1111}^2}{16} + \frac{\epsilon_{1112}^2}{8} + \frac{\epsilon_{1122}^2}{16} + \frac{\epsilon_{1211}^2}{8} + \frac{\epsilon_{1212}^2}{4} + \frac{\epsilon_{1222}^2}{8} + \frac{\epsilon_{2211}^2}{16} + \frac{\epsilon_{2212}^2}{8} + \frac{\epsilon_{2222}^2}{16}. \quad (2c)$$

Although somewhat arbitrary, this allelic frequency was chosen because all polymorphic loci in the F_2 individuals should have frequencies that average about 0.5.

To solve these equations, the additive (a) and dominance genotypic values (d) were obtained as already explained above (see eqs. 1a–h). Two-locus genotypic values were calculated from multiple regression results, and nonepistatic genotypic values were calculated from the marginal single locus genotypic values (Cheverud and Routman 1995). The epistatic genotypic values (e -values) then were calculated from the differences between two-locus genotypic values and nonepistatic genotypic values (see Cheverud and Routman 1995). Terms such as $e_{11..}$ refer to the mean of the epistatic genotypic values across the specified genotype, calculated by multiplying the frequency of each of the three genotypes (0.25 for the homozygotes, 0.5 for the heterozygotes) by the appropriate epistatic genotypic values (Cheverud 2000). Epistatic genetic values (ϵ -values) were calculated as follows:

$$\epsilon_{ijkl} = e_{ijkl} - e_{ij..} - e_{..kl} + e_{....}, \quad (3)$$

where $e_{....}$ is the mean of the epistatic genotypic values weighted by their frequencies. The additive and dominance genetic variances were calculated with and without their ep-

istatic (e) components so that the proportional contribution of epistasis to these variances could be readily assessed.

Beyond centroid size asymmetry, it also seemed worthwhile to test for epistasis in centroid size itself (mean of the left and right sides). To accomplish this, the mean of the left and right centroid size values first were calculated in each mouse (Klingenberg et al. 2001) and adjusted as before for potential effects of the four covariates. Centroid size was then subjected to the same epistasis analyses as already described for centroid size asymmetry. The results from these analyses are presented first so that the extent and location of epistasis for centroid size could be compared to that for its asymmetry.

RESULTS

Epistasis for Centroid Size

The results of the two-way genome scan for centroid size produced 219 combinations of loci that represented potential cases of epistasis. Of these combinations, 189 have F -values with associated probabilities less than 5%, this being more than twice the number (92) expected at this level by chance alone ($\chi^2 = 98.9$, $df = 1$, $P < 0.0001$). At the 1% significance level, there are 85 cases of overall epistasis, more than four times the number (19) expected by chance alone at this level ($\chi^2 = 231.7$, $df = 1$, $P < 0.0001$). If we restrict the significance level even more to the 0.1% level, there are still 28 cases of overall epistasis, or about 15 times the expected number (two) at this level due to chance alone ($\chi^2 = 338.4$, $df = 1$, $P < 0.0001$). These 28 instances of epistasis are depicted in Figure 2, where the F -statistic for the test of overall epistasis is given for each pair of chromosomes containing the putative QTLs producing this epistasis. As may be seen, all chromosomes except number 16 are represented in these significant combinations, with chromosomes 1 and 5 being the most represented among these combinations. The four combinations with the highest F -statistics reach the experimentwise level of significance of 2.7×10^{-5} .

Figure 2 also depicts the magnitude of the aa , ad , da , and dd epistatic values for each of the 28 combinations with significant overall epistasis. There are six aa , 10 ad , 14 da , and 17 dd epistatic values that reach significance for centroid size. Negative epistatic values predominate over positive values, especially for aa , where all six values are negative. The means for the significant aa , ad , da , and dd values also are negative (-0.0939 , -0.1215 , -0.0233 , and -0.0224 , respectively), and those for aa and ad are significantly different from zero ($P < 0.01$). Eleven of these 28 epistatic combinations are associated with just one significant pure form of epistasis, whereas the other 17 exhibit two or three significant forms and thus a more complex pattern of epistasis.

Figure 3 depicts estimates (all $\times 100$) of the additive (V_A), dominance (V_D), and epistatic genetic variances (V_I) for centroid size generated by each of the 28 combinations of loci for which there was significant ($P < 0.001$) epistasis. Estimates of V_A and V_D with and without their epistatic contribution are depicted to show the importance of epistasis. V_A -values that include epistasis vary from near zero to 0.0087 (recall that all variances are $\times 100$) with a mean of 0.0030. V_A -values calculated without epistatic effects average

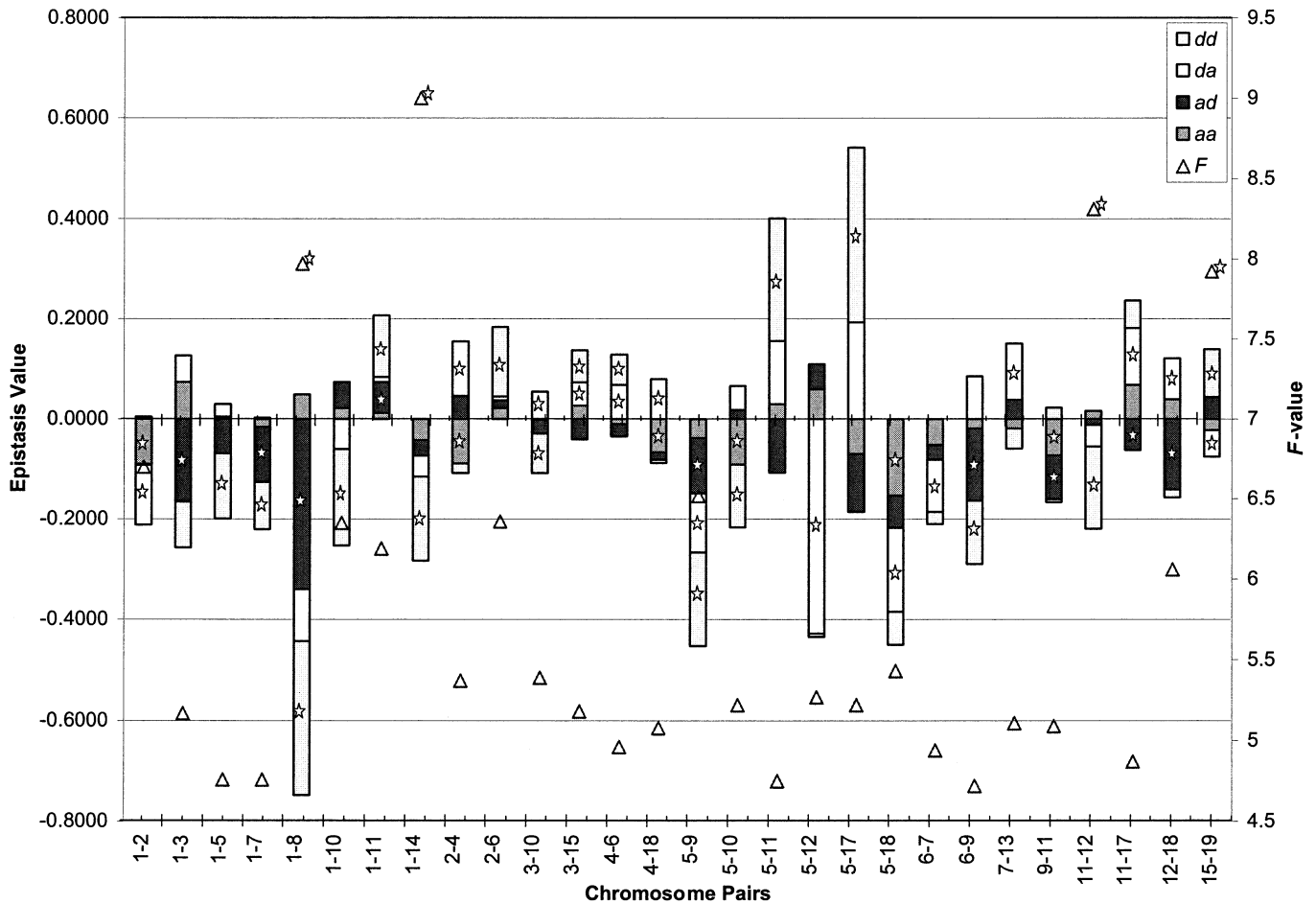


FIG. 2. Epistatic values (*aa*, *ad*, *da*, *dd*) for the 28 pairs of quantitative trait loci affecting centroid size significantly at the 0.1% level in the overall *F*-tests. Stars indicate significance at the 5% level for the epistatic values and at the experimentwise level for the *F*-values.

0.00396, so epistasis generally tends to suppress V_A for centroid size. V_D -estimates for centroid size tend to be lower than those for V_A , averaging 0.00158 when calculated with epistasis and 0.00327 when calculated without epistasis. Epistasis generally suppresses the dominance genetic variance; 25 of the 28 ratios of V_D calculated with and without epistasis are less than 1.0, and average 0.56. Epistatic variance itself (V_I) for centroid size is quite large for these 28 pairwise combinations of loci, averaging 0.0136, which is considerably larger than either the additive or dominance genetic variance. As a consequence, estimates of the total genetic variance that include epistatic contributions average 0.0182, much larger than the comparable values calculated without epistasis (0.00723). Epistasis enhances the genetic variance of centroid size for all 28 combinations of sites, the average ratio for genetic variance calculated with and without epistasis being 2.55. Put another way, epistasis contributes on average about 70% of the total genetic variance of centroid size at these combinations of loci.

Epistasis for Unsigned Asymmetry of Centroid Size

The two-way genome scan for unsigned asymmetry of centroid size yielded 197 combinations of loci that generated *F*-

values with associated probabilities less than 5%, 91 combinations at the 1% significance level, and 30 combinations at the 0.1% significance level. As was the case for centroid size, these all represent far more than the number of combinations expected by chance alone ($\chi^2 = 126.1, 275.6, 392.4$; $df = 1, P < 0.0001$). At the 0.1% level, loci on all chromosomes except number 9 are represented in these significant epistatic combinations, with chromosomes 3 and 5 being the most frequently represented (see Fig. 4). Six combinations have *F*-values exceeding 7.0 and reach significance at the experimentwise Bonferroni level of 2.7×10^{-5} . Four of these six combinations involve a QTL on chromosome 5.

For unsigned asymmetry of centroid size, there are 13 *aa*, 15 *ad*, 17 *da*, and 18 *dd* epistatic values that reach significance at the 0.1% level (Fig. 4). Positive epistatic values predominate over negative values for *ad* and *da*, but there are about equal numbers of positive and negative values for *aa* and *dd*. The means for the significant *aa*, *ad*, *da*, and *dd* values, respectively, are 0.0037, 0.0203, 0.0404, and 0.0197. Three of the largest *dd* values are positive, suggesting overdominance in which the double heterozygotes have greater FA than expected based on a purely additive model. Only three of the 30 instances of epistasis exhibit significance for one pure

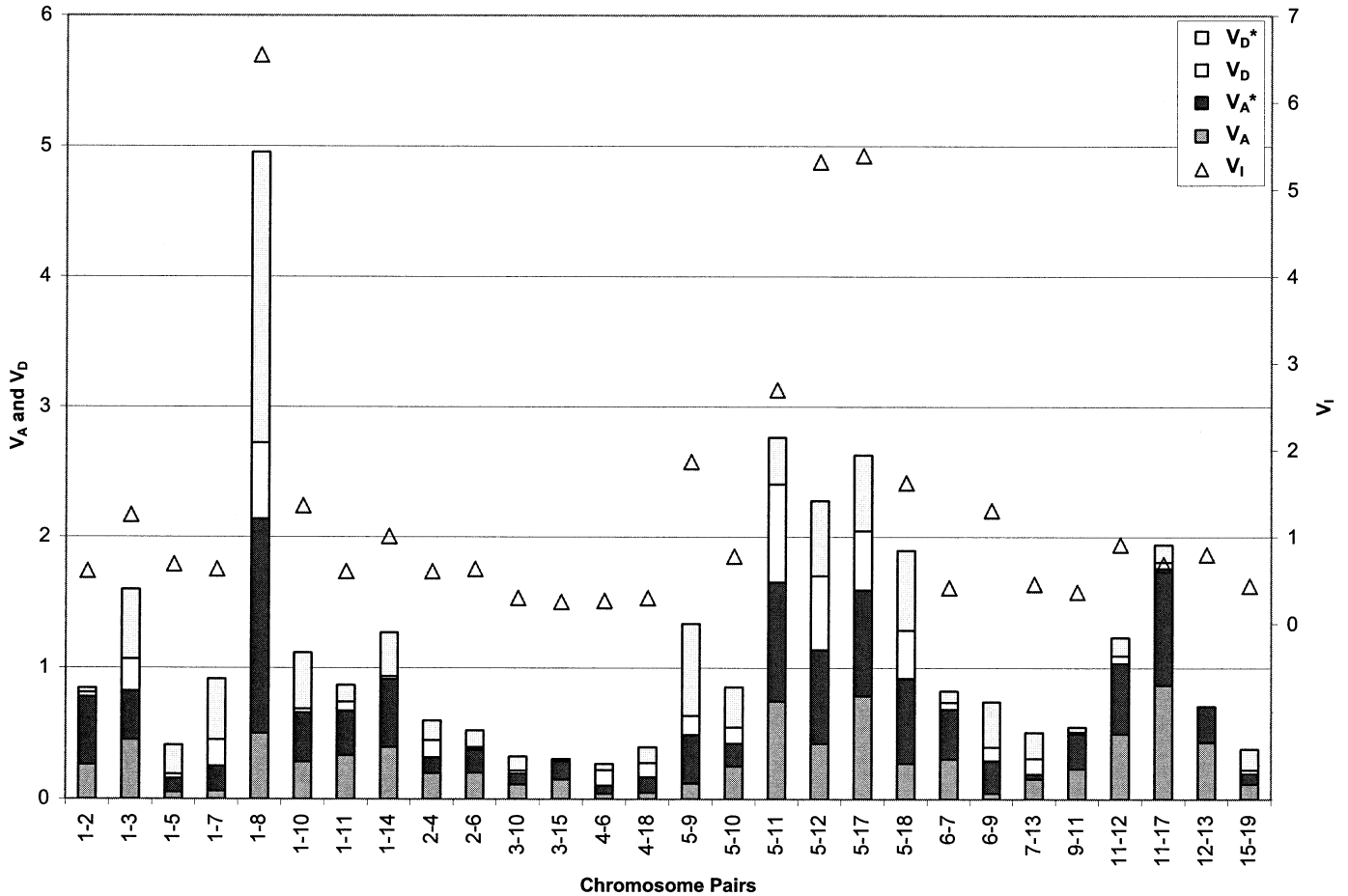


FIG. 3. Additive genetic (V_A) and dominance genetic (V_D) variance estimated with and without (asterisk) epistatic effects, and epistatic genetic variance (V_I) for centroid size generated by each of the 28 epistatic combinations reaching significance at the 0.001 level.

form of epistasis, whereas the remaining 27 cases show more complex patterns. Overall, there is a large amount of epistasis for the unsigned asymmetry of centroid size in these mice.

Figure 5 depicts the values for the additive, dominance, and epistatic genetic variance of the unsigned asymmetry of centroid size (all $\times 1000$), calculated with and without epistasis as before, for each of the 30 combinations of loci. The average V_A values calculated with and without epistasis are 0.000122 and 0.000179 (mean ratio of 0.68). Thus, as was the case for centroid size, epistasis for centroid size asymmetry has suppressed its additive genetic variance in the majority (18 of 30) of cases. The average ratio for dominance genetic variance calculated with and without epistasis is 0.93, suggesting that the suppression of V_D for centroid size asymmetry by epistasis is not as great as that seen for centroid size. Epistatic genetic variance for centroid size asymmetry averages 0.00284, considerably larger once again than the average for either V_A or V_D . As a consequence, the enhancement of the total genetic variance for centroid size asymmetry due to epistasis at these 30 combinations of sites is rather massive, the average ratio of genetic variance calculated with and without epistasis being 5.76. Clearly, therefore, epistasis exerts its largest effect (average contribution of 85%) on the

overall genetic variance of centroid size asymmetry through the epistatic variance for this character.

Genomic Distribution of Epistatic Interactions

Figure 6 depicts intervals for each chromosome that exhibited five or more significant ($P < 0.05$ in overall F -test) epistatic interactions for centroid size and centroid size asymmetry. In the study by Klingenberg et al. (2001), there were 12 QTLs (including two on chromosome 11) identified for centroid size itself in these mice, and these are indicated in the figure as well if their position mapped within any of the epistatic intervals for centroid size or asymmetry.

As may be seen in Figure 6, there are 46 intervals on the various chromosomes that were responsible for five or more epistatic interactions affecting centroid size. A small region (6 cM) on chromosome 2 exhibited 10 significant interactions, and a single site on chromosome 1 (at 122 cM from *DIMit3*) showed nine such epistatic interactions with sites on other chromosomes. Chromosome 11 exhibited the greatest number of total epistatic interactions (33), but chromosomes 1, 5, 7, 10, 11, 12, 15, and 17 all show 20 or more significant interactions. Of the 46 intervals shown in Figure

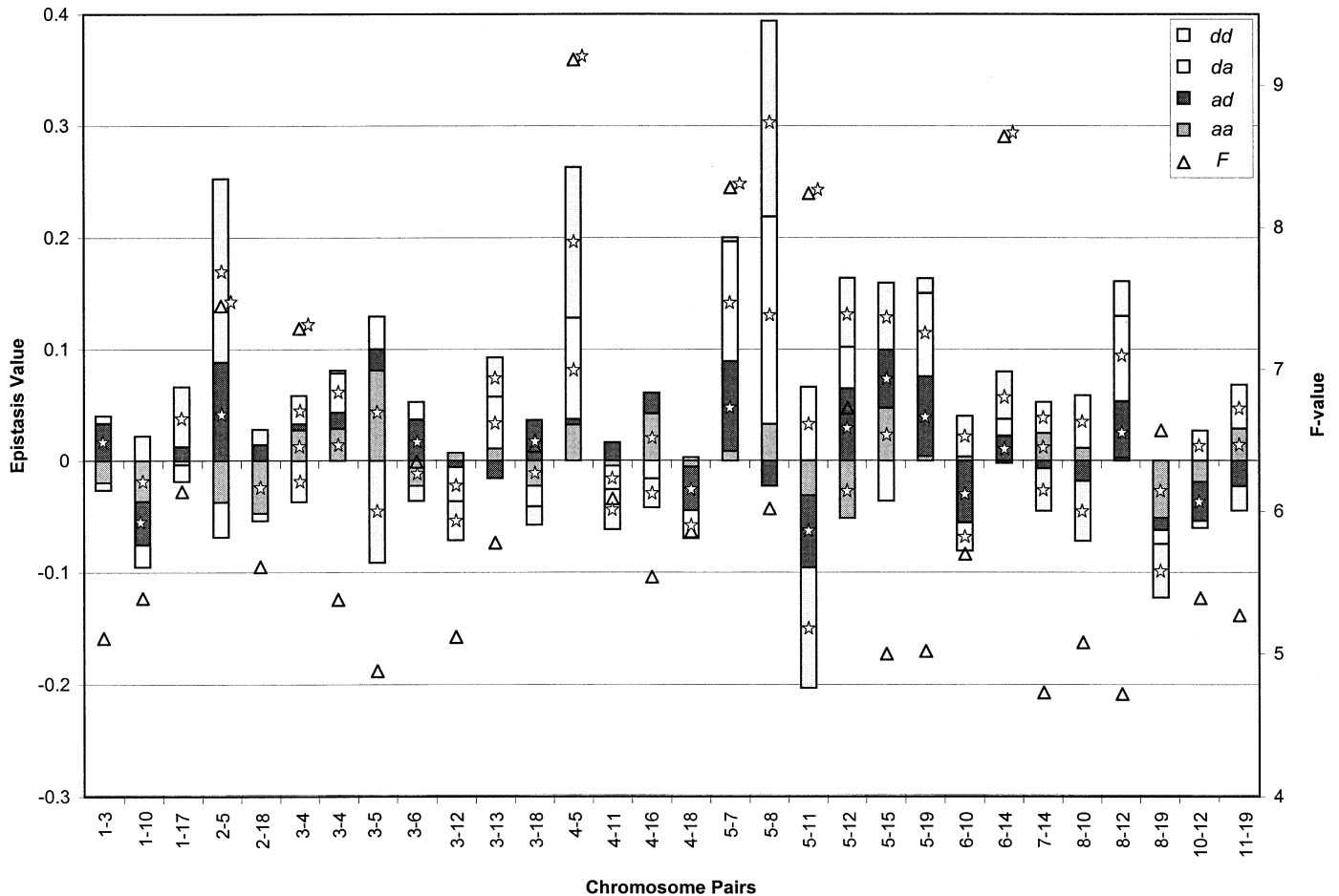


FIG. 4. Epistatic values (*aa*, *ad*, *da*, *dd*) for the 30 pairs of quantitative trait loci affecting centroid size asymmetry significantly at the 0.1% level in the overall *F*-tests. Stars indicate significance at the 5% level for the epistatic values and at the experimentwise level for the *F*-values.

6, only six coincide with the positions for QTLs significantly affecting centroid size. Thus, the vast majority of these interactions are between QTLs not necessarily affecting centroid size itself.

There are 42 intervals among the various chromosomes that exhibited five or more significant epistatic interactions affecting centroid size asymmetry. About one-half of these intervals appear to be similar to those for centroid size, but it seems clear that many of them also represent different regions of interactions. Especially noticeable is the fact that 11 different regions (including two on chromosome 7) show nine or more significant interactions. Chromosomes 5 (28), 7 (28), and 11 (25) exhibit the greatest number of total epistatic interactions. Eight of the 12 known QTLs for centroid size map within regions exhibiting significant epistatic interactions for centroid size asymmetry, but the majority of these intervals once again do not contain these QTLs.

DISCUSSION

Our results clearly show that epistasis influences FA of mandible centroid size in this population of mice. The evidence for this seems overwhelming—there were far more epistatic combinations reaching significance at each of three

levels (5%, 1%, and 0.1%) than would be expected by chance alone. Our approach does not make it possible to differentiate true from false positives, and no doubt a number of these cases of apparent epistasis are false positives. At the same time, we are also certain that there were some false negatives here as well that represent true examples of epistasis that have gone undetected. Whatever the true level of epistasis here, its obvious abundance is consistent with previous speculation that epistasis might play an important role in FA (McKenzie and Clarke 1988; Livshits and Kobylansky 1991; Clarke 1994; Leamy 1997).

Although few in number, past studies appear to have been much less successful in detecting epistasis for FA. For example, neither Blows and Sokolowski (1995) nor Polak and Starmer (2001) were able to discover significant epistasis for FA of bristle number in *Drosophila*. Both of these studies used traditional methods of statistical testing of epistasis, which rely on the analysis of means from various line crosses (Cavalli 1952; Mather and Jinks 1982; Lynch and Walsh 1998), however, and they do not include any epistatic effects contributing to additive or dominance genetic variance (Cheverud and Routman 1995; Wade 1996). Perhaps most such studies have been biased against discovering epistasis

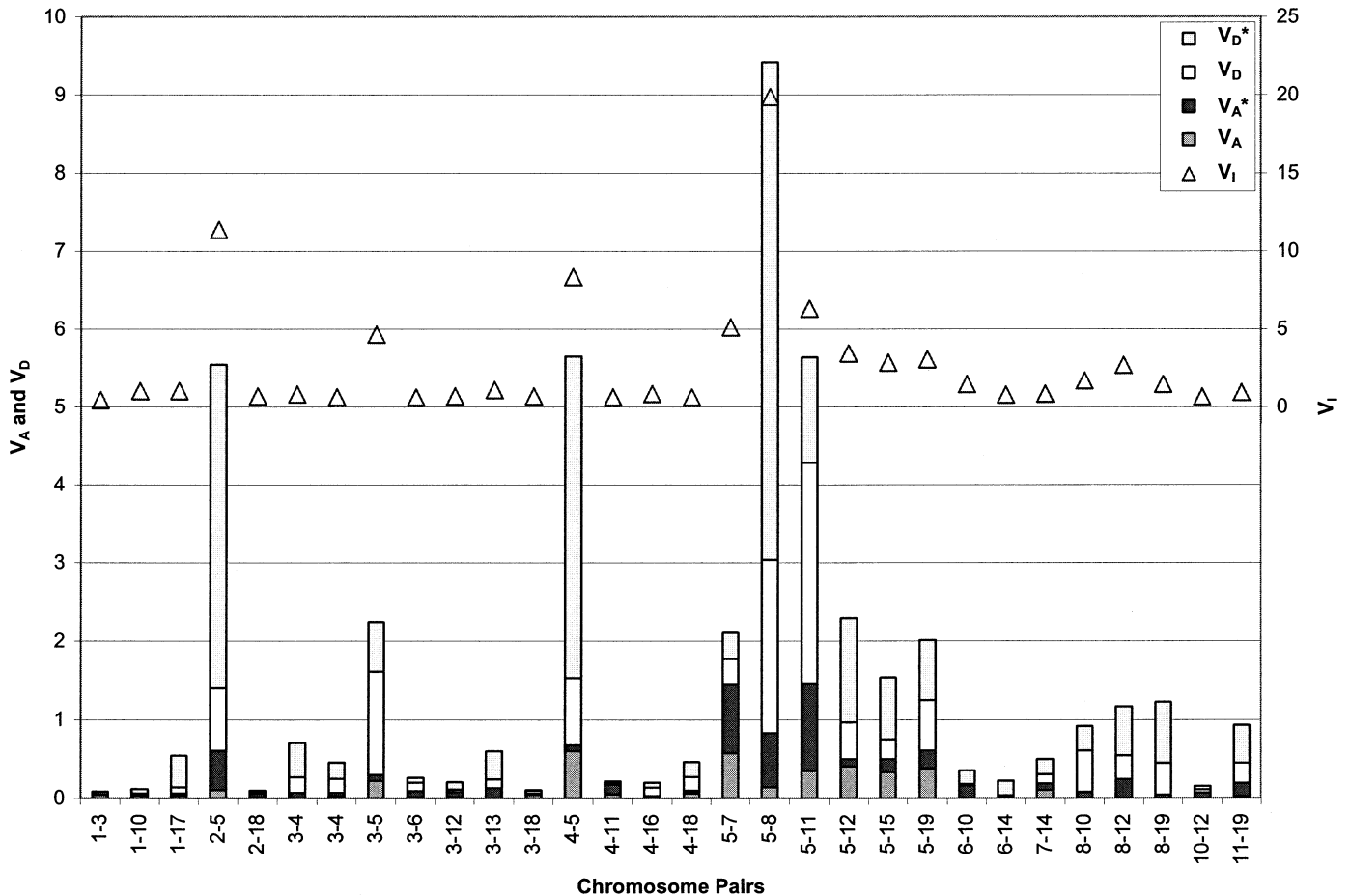


FIG. 5. Additive genetic (V_A) and dominance genetic (V_D) variance estimated with and without (asterisk) epistatic effects, and epistatic genetic variance (V_I) for centroid size asymmetry generated by each of the 30 epistatic combinations reaching significance at the 0.001 level.

because of the statistical inadequacy of these traditional testing approaches. These studies also have generally only used loci showing significant single locus effects, loci that may not be among those most involved in generating epistasis (Lynch and Walsh 1998, p. 487). With the whole-genome-scan approach taken here, therefore, we might expect to find many more instances of significant epistasis for various characters, including the asymmetry in these characters.

We discovered epistasis for FA in mandible centroid size when there was only one QTL (on chromosome 5) that directly affected this character in these same mice (Klingenberg et al. 2001). Actually this QTL was detected using the untransformed FA values for centroid size and was not well supported statistically (Klingenberg et al. 2001); furthermore, a rerun of the QTL analysis using the Box-Cox transformed FA values used here showed no QTLs for FA in centroid size. Thus, although no genes directly affecting mandible centroid size asymmetry in our mice could be detected, this character clearly was significantly influenced by the interactions of loci at a large number of sites. Individual genes affecting FA apparently are quite rare (Leamy et al. 1997, 1998), and even the often-cited example of a locus in Australian blowflies that confers resistance to the insecticide diazinon and alters asymmetry (McKenzie and Clarke 1988)

actually appears to produce antisymmetry rather than elevated FA levels (Palmer 1996). Antisymmetry reverts to FA (at its original level) in these resistant blowflies as a result of selection for modifying loci (McKenzie and Clarke 1988), however, clearly suggesting an epistatic basis for FA. Perhaps we will generally find that genetic variability of FA in various characters is generated primarily by epistatic interactions of genes, rather than by their direct effects.

Klingenberg and Nijhout (1999) might have predicted the large amount of epistasis for FA discovered for mandible size asymmetry, for they found rampant epistasis for FA in a developmental model that simulated random noise in a bilateral character. What is particularly interesting about this model is that this epistasis for FA was generated solely by altering various parameters controlling development of the (left-right side means of the) bilateral character itself. This implies that normal genetic variation inherent in the development of a bilateral character is sufficient to produce genetic variation (including epistasis) in FA in this character, and that no genes uniquely affecting FA need to be hypothesized (Klingenberg and Nijhout 1999). Our results suggest that a number of sites throughout the genome are involved in epistasis for FA in centroid size, however, sites that do not always correspond to those for QTLs that affect centroid size itself.

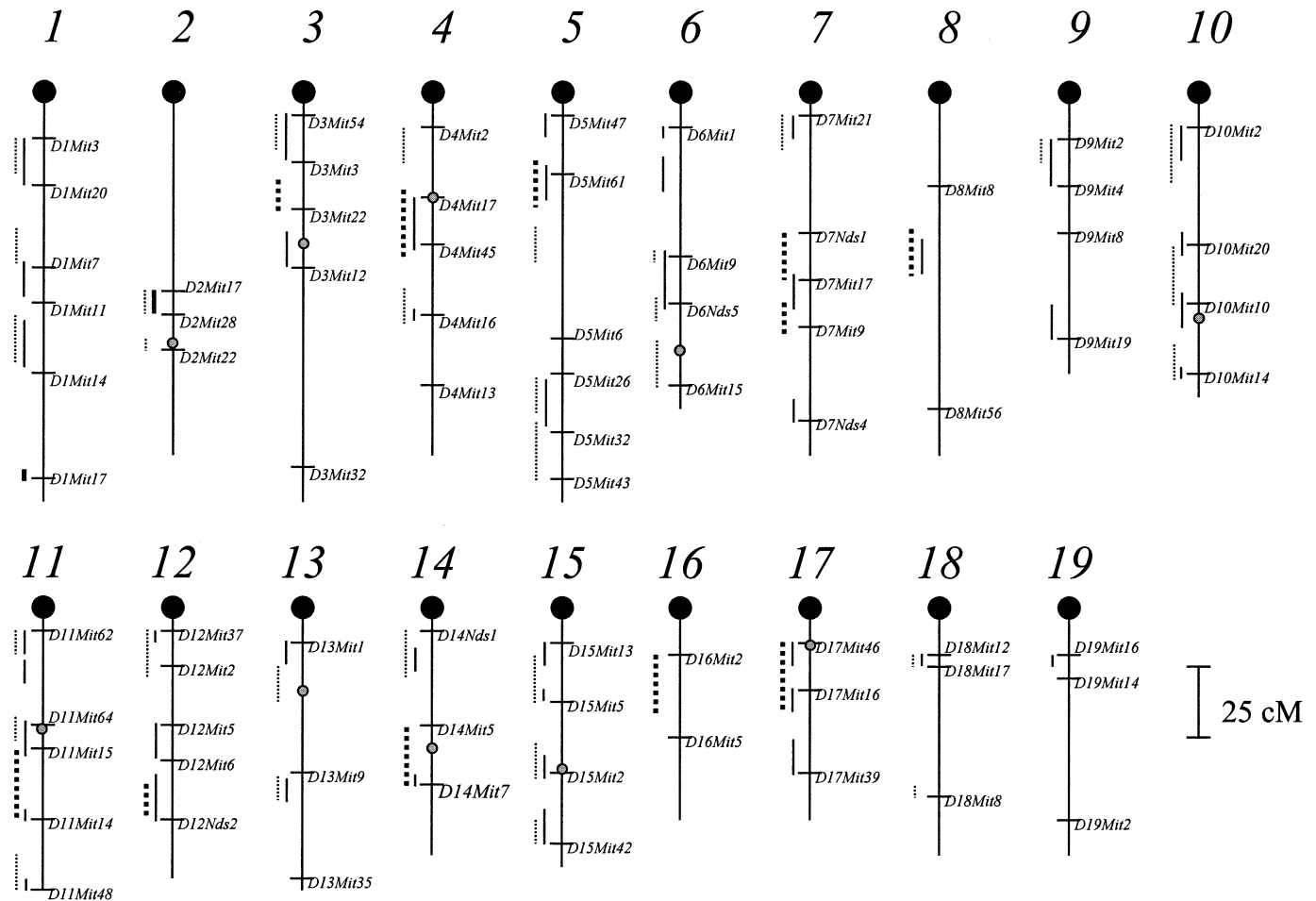


FIG. 6. Chromosomal regions exhibiting repeated (five or more) epistatic interactions for centroid size (solid lines) and centroid size asymmetry (dashed lines). The light solid or dashed lines indicate intervals showing five to eight significant interactions, whereas intervals exhibiting nine to 12 significant interactions are shown as bolder lines. Positions of quantitative trait loci for centroid size mapping within any of the epistatic intervals for centroid size or asymmetry also are shown as gray circles on the chromosomes.

Thus, for this model and its associated predictions (Klingenberg and Nijhout 1999) to be fully tested, we would need to analyze epistasis at all pairwise sites of the QTLs for mandible centroid size.

Most of the epistatic interactions affecting FA in mandible centroid size were complex (e.g., involved more than one form of epistasis); as a result, their influence on V_A and V_D varied considerably among the combinations of loci. In general, however, the 30 epistatic interactions significant at the 0.1% level tended to decrease V_A for FA in centroid size by about one-third, even though the contribution of epistasis to V_A at intermediate gene frequencies is minimal (Cheverud and Routman 1996). Entirely different results might be produced with other allelic frequencies, however, and many more studies are needed to discover what effects epistasis might have on V_A for FA in different characters. It would be interesting if our results prove to be general, for if so, this might help to account for the very low levels of heritabilities typically discovered for FA in various characters (Leamy 1997; Møller and Thornhill 1997; Whitlock and Fowler 1997).

Epistasis also generally suppressed the level of V_D for FA

in mandible centroid size, although to a much smaller extent than for V_A . This does not mean that FA is not influenced by single-locus dominance effects, but rather only that the contributions of any such effects to the total dominance genetic variance would be compromised by epistatic interactions. In fact, 25 of the 60 dominance genotypic values among the 30 combinations of QTLs shown in Figure 5 for centroid size asymmetry reached significance ($P < 0.01$). Of these 25 significant d -values, 17 are negative, indicating that FA values for heterozygotes are generally less than the midhomozygote values for these loci. There also is a significant difference ($P < 0.01$) between the mean values of 0.4328 and 0.4056, respectively, for the four homozygous and four heterozygous genotypes (double heterozygotes excluded) at each of the two loci for all 30 epistatic interactions given in Figure 5. Thus, the overall FA level is higher for the homozygotes than the heterozygotes, as has often been previously found (Leamy 1984; Palmer and Strobeck 1986; Livshits and Smouse 1994).

As already detailed, the greatest contribution of the epistatic interactions identified as significantly affecting centroid size asymmetry was not due to V_A or V_D , but rather to V_I .

The V_I -values averaged far larger than either the V_A - or V_D -values (Fig. 5), and thus were the largest contributors to the total genetic variances. This was expected because we calculated these V_I -values for the 30 cases showing the greatest significance (0.1%) for epistasis and epistasis contributes the maximum to V_I at intermediate gene frequencies (Cheverud and Routman 1996). Different results would result from different gene frequency assumptions (Cheverud and Routman 1996); nonetheless, it seems quite clear that FA in the mandible centroid size in this population is greatly influenced by epistasis. For this particular population, the primary contribution of epistasis to the overall genetic variance is through V_I .

From the results of this analysis, we cannot say what genes might be responsible for the extent and magnitude of epistatic effects on centroid size asymmetry. It is clear, however, that there are specific areas on several of the chromosomes that were repeatedly involved in such interactions (Fig. 6). Several sites on chromosomes 5 and 7, in particular, showed the most significant epistatic interactions for centroid size asymmetry, and these areas would be particularly good choices for finer-scale mapping that could eventually narrow down the precise sites of the genes involved. We might expect many of these genes to be involved in some way with the formation of the mandible, for it is difficult to see how they might affect asymmetry in this structure otherwise. This would certainly be the expectation if the Klingenberg and Nijhout (1999) developmental model for FA is correct. Some of the sites involved in epistasis affecting FA in centroid size do not correspond to those affecting centroid size itself (Klingenberg et al. 2001), although more QTLs were uncovered when individual dimensions on the mandible (rather than centroid size alone) were analyzed (Leamy et al. 1997). Many of the epistatically important regions for centroid size FA (Fig. 6) also are similar to those identified by Cheverud (2000) for body size in these mice, so perhaps some of these genes also have effects that are not restricted to the mandible. Beyond this, the precise nature of the genes involved in epistasis for centroid size FA will have to await the results of further analyses.

ACKNOWLEDGMENTS

We thank S. Beyene, M. Butler, E. Cheverud, D. Irschick, and N. Vasey for help with laboratory work; G. Conroy for the use of digitizing equipment; S. Cropp for computer assistance; C. P. Klingenberg for comments on an earlier draft of this paper; S. Clark for assistance with the figures; and S. Tonsor and two anonymous reviewers for useful revision suggestions. This research was supported in part by funds provided by the University of North Carolina at Charlotte, and in part by National Science Foundation grant DEB-9726433 and National Institutes of Health grant DK52514.

LITERATURE CITED

- Blows, M. W., and M. B. Sokolowski. 1995. The expression of additive and nonadditive genetic variation under stress. *Genetics* 140:1149–1159.
- Cavalli, L. L. 1952. An analysis of linkage in quantitative inheritance. Pp. 135–144 in E. C. R. Reeve and C. H. Waddington, eds. *Quantitative inheritance*. Her Majesty's Stationery Office, London.
- Cheverud, J. M. 1996. Developmental integration and the evolution of pleiotropy. *Am. Zool.* 36:44–50.
- . 2000. Detecting epistasis among quantitative trait loci. Pp. 58–81 in J. Wolf, E. Brodie II, and M. Wade, eds. *Epistasis and the evolutionary process*. Oxford Univ. Press, New York.
- . 2001. A simple correction for multiple comparisons in interval mapping genome scans. *Heredity* 87:52–58.
- Cheverud, J. M., and E. J. Routman. 1995. Epistasis and its contribution to genetic variance components. *Genetics* 139:1455–1461.
- . 1996. Epistasis as a source of increased additive genetic variance at population bottlenecks. *Evolution* 50:1042–1051.
- Cheverud, J. M., J. Rutledge, and W. Atchley. 1983. Quantitative genetics of development: genetic correlations among age-specific trait values and the evolution of ontogeny. *Evolution* 37:895–905.
- Cheverud, J. M., E. J. Routman, F. A. M. Duarte, B. V. Swinderen, K. Cothran, and C. Perel. 1996. Quantitative trait loci for murine growth. *Genetics* 142:1305–1319.
- Cheverud, J. M., E. J. Routman, and D. K. Irschick. 1997. Pleiotropic effects of individual gene loci on mandibular morphology. *Evolution* 51:2004–2014.
- Clarke, G. M. 1992. Fluctuating asymmetry: a technique for measuring developmental stress of genetic and environmental origin. *Acta Zool. Fenn.* 191:31–35.
- . 1993. The genetic basis of developmental stability. I. Relationships between stability, heterozygosity and genomic coadaptation. *Genetica* 89:15–23.
- . 1994. The genetic basis of developmental stability. I. Relationships between stability, heterozygosity and genomic coadaptation. Pp. 17–25 in T. A. Markow, ed. *Developmental instability: its origins and evolutionary implications*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Dryden, I. L., and K. V. Mardia. 1998. *Statistical analysis of shape*. Wiley, Chichester, U.K.
- Falconer, D. S., and T. F. C. Mackay. 1996. *Introduction to quantitative genetics*. Longman, New York.
- Graham, J. H. 1992. Genomic coadaptation and developmental stability in hybrid zones. *Acta Zool. Fenn.* 191:121–131.
- Haley, C. S., and S. A. Knott. 1992. A simple regression method for mapping quantitative trait loci in line crosses using flanking markers. *Heredity* 69:315–324.
- Hutchison, D. W., and J. M. Cheverud. 1995. Fluctuating asymmetry in tamarin (*Saquinus*) cranial morphology: intra- and interspecific comparisons between taxa with varying levels of genetic heterozygosity. *J. Hered.* 86:280–288.
- Klingenberg, C. P., and H. F. Nijhout. 1999. Genetics of fluctuating asymmetry: a developmental model of developmental instability. *Evolution* 53:358–375.
- Klingenberg, C. P., L. J. Leamy, E. J. Routman, and J. M. Cheverud. 2001. Genetic architecture of mandible shape in mice: effects of quantitative trait loci analyzed by geometric morphometrics. *Genetics* 157:785–802.
- Lander, E. S., P. Green, J. Abrahamson, A. Barlow, M. Daley, S. Lincoln, and L. Newburg. 1987. Mapmaker: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics* 1:174–181.
- Leamy, L. 1984. Morphometric studies in inbred and hybrid house mice. V. Directional and fluctuating asymmetry. *Am. Nat.* 123:579–593.
- . 1997. Is developmental stability heritable? *J. Evol. Biol.* 10:21–29.
- . 1999. Heritability of directional and fluctuating asymmetry for mandibular characters in randombred mice. *J. Evol. Biol.* 12:146–155.
- Leamy, L. J., E. J. Routman, and J. M. Cheverud. 1997. A search for quantitative trait loci affecting asymmetry of mandibular characters in mice. *Evolution* 51:957–969.
- . 1998. Quantitative trait loci for fluctuating asymmetry of discrete skeletal characters in mice. *Heredity* 80:509–518.
- . 1999. Quantitative trait loci for early- and late-developing

- skull characters in mice: a test of the genetic independence model of morphological integration. *Am. Nat.* 153:201–214.
- Lincoln, S., M. Daly, and E. Lander. 1992. Constructing genetic maps with MAPMAKER/EXP 3.0. Whitehead Institute, Cambridge, MA.
- Livshits, G., and E. Kobyliansky. 1991. Fluctuating asymmetry as a possible measure of developmental homeostasis in humans. *Hum Biol.* 63:441–466.
- Livshits, G., and P. E. Smouse. 1994. Relationship between fluctuating asymmetry, morphological modality and heterozygosity in an elderly Israeli population. Pp. 157–168 in T. A. Markow, ed. *Developmental instability: its origins and evolutionary implications*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Lynch, M., and B. Walsh. 1998. *Genetics and analysis of quantitative traits*. Sinauer Associates, Sunderland, MA.
- Markow, T. A. 1995. Evolutionary ecology and developmental instability. *Annu. Rev. Entomol.* 40:105–120.
- Mather, K., and J. L. Jinks. 1982. *Biometrical genetics: the study of continuous variation*. Chapman and Hall, New York.
- McKenzie, J. A., and G. M. Clarke. 1988. Diazinon resistance, fluctuating asymmetry and fitness in the Australian sheep blowfly, *Lucilia cuprina*. *Genetics* 120:213–220.
- Møller, A. P., and A. Pomiankowski. 1993. Fluctuating asymmetry and sexual selection. *Genetica* 89:267–279.
- Møller, A. P., and R. Thornhill. 1997. A meta-analysis of the heritability of developmental stability. *J. Evol. Biol.* 10:1–16.
- Mouse Genome Database. 2000. Mouse genome informatics project. The Jackson Laboratory, Bar Harbor, ME. Available via <http://www.infomatics.jax.org>.
- Palmer, A. R. 1994. Fluctuating asymmetry analyses: a primer. Pp. 335 in T. A. Markow, ed. *Developmental instability: its origins and evolutionary implications*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- . 1996. Waltzing with asymmetry. *BioScience* 46:518–532.
- Palmer, A. R., and C. Strobeck. 1986. Fluctuating asymmetry: measurement, analysis, patterns. *Annu. Rev. Ecol. Syst.* 17:391–421.
- . 1992. Fluctuating asymmetry as a measure of developmental stability: implications of non-normal distributions and power of statistical tests. *Acta Zool. Fenn.* 191:57–72.
- Parsons, P. A. 1990. Fluctuating asymmetry: an epigenetic measure of stress. *Biol. Rev.* 65:131–145.
- Polak, M., and W. T. Starmer. 2001. The quantitative genetics of fluctuating asymmetry. *Evolution* 55:498–511.
- Routman, E. J., and J. M. Cheverud. 1994. Individual genes underlying quantitative traits: molecular and analytical methods. Pp. 593–606 in B. Schierwater, B. Streit, G. P. Wagner, and R. Desalle, eds. *Molecular ecology and evolution: approaches and applications*. Birkhauser Verlag, Basel, Switzerland.
- . 1995. Polymorphism for PCR-analyzed microsatellites: data for two additional mouse strains and the utility of agarose gel electrophoresis. *Mammal. Genome* 6:401–404.
- . 1997. Gene effects on a quantitative trait: two-locus epistatic effects measured at microsatellite markers and at estimated QTL. *Evolution* 51:1654–1662.
- SAS Institute. 1989. *SAS/STAT user's guide*. Ver. 6. SAS Institute, Cary, NC.
- Sokal, R. R., and J. F. Rohlf. 1995. *Biometry*. 3rd ed. Freeman, San Francisco, CA.
- Swaddle, J. P., I. C. Cuthill, and M. S. Witter. 1994. The analysis of fluctuating asymmetry. *Anim. Behav.* 48:986–989.
- Thoday, J. M. 1958. Homeostasis in a selection experiment. *Heredity* 12:401–415.
- Van Valen, L. 1962. A study of fluctuating asymmetry. *Evolution* 16:125–142.
- Wade, M. 1996. Sewall Wright: gene interaction and the shifting balance theory. Pp. 35–62 in D. Futuyma and J. Antonovics, eds. *Oxford surveys in evolutionary biology*. Vol. 8. Oxford Univ. Press, New York.
- Wagner, G. 1984. On the eigenvalue distribution of genetic and phenotypic dispersion matrices: evidence for a nonrandom organization of quantitative character variation. *J. Math. Biol.* 21:77–95.
- Whitlock, M. C., and K. Fowler. 1997. The instability of studies of instability. *J. Evol. Biol.* 10:63–67.
- Zakharov, V. M. 1989. Future prospects for population phenogenetics. *Sov. Sci. Rev. F. Physiol. Gen. Biol.* 4:1–79.
- . 1994. Appearance, fixation and stabilisation of environmentally induced phenotypic changes as a microevolutionary event. Pp. 229–236 in T. A. Markow, ed. *Developmental instability: its origins and evolutionary implications*. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Corresponding Editor: S. Tonsor

APPENDIX

The 75 microsatellite loci used on all 19 autosomes in the F₂ mice. Interval lengths (in cM) are given for each marker as distances from the previous mapped marker. *Cx* indicates the distance from the first scored marker and the most centromeric mapped locus in the Mouse Genome Database (2000). For the last microsatellite markers on each chromosome, interval lengths are based on distances to the most telomeric mapped locus in the Mouse Genome Database (2000). NA, recombination approached 50%.

Locus	Interval	Locus	Interval	Locus	Interval	Locus	Interval
<i>C1</i>	11.0	<i>D5Mit47</i>	24.6	<i>D9Mit19</i>	8.0	<i>D14Nds1</i>	42.0
<i>D1Mit3</i>	6.3	<i>D5Mit61</i>	64.5			<i>D14Mit5</i>	19.2
<i>D1Mit20</i>	35.3	<i>D5Mit6</i>	8.1	<i>C10</i>	16.0	<i>D14Mit7</i>	24.0
<i>D1Mit7</i>	10.9	<i>D5Mit26</i>	30.2	<i>D10Mit2</i>	43.7		
<i>D1Mit11</i>	25.1	<i>D5Mit32</i>	10.5	<i>D10Mit20</i>	19.7	<i>C15</i>	6.7
<i>D1Mit14</i>	41.6	<i>D5Mit43</i>	9.0	<i>D10Mit10</i>	19.4	<i>D15Mit13</i>	23.9
<i>D1Mit17</i>	6.0			<i>D10Mit14</i>	12.0	<i>D15Mit5</i>	28.0
		<i>C6</i>	2.8			<i>D15Mit2</i>	27.2
<i>C2</i>	NA	<i>D6Mit1</i>	51.9	<i>C11</i>	1.5	<i>D15Mit42</i>	15.6
<i>D2Mit17</i>	5.2	<i>D6Mit9</i>	15.1	<i>D11Mit62</i>	44.3		
<i>D2Mit28</i>	12.7	<i>D6Nds5</i>	28.0	<i>D11Mit64</i>	9.1	<i>C16</i>	14.0
<i>D2Mit22</i>	6.0	<i>D6Mit15</i>	1.0	<i>D11Mit15</i>	21.1	<i>D16Mit2</i>	30.0
				<i>D11Mit14</i>	31.4	<i>D16Mit5</i>	34.0
<i>C3</i>	4.6	<i>C7</i>	0.5	<i>D11Mit48</i>	3.0		
<i>D3Mit54</i>	20.0	<i>D7Mit21</i>	51.9			<i>C17</i>	10.0
<i>D3Mit3</i>	14.4	<i>D7Nds1</i>	12.5	<i>C12</i>	1.0	<i>D17Mit46</i>	9.9
<i>D3Mit22</i>	20.2	<i>D7Mit17</i>	12.8	<i>D12Mit37</i>	18.0	<i>D17Mit16</i>	37.2
<i>D3Mit12</i>	70.7	<i>D7Mit9</i>	35.7	<i>D12Mit2</i>	19.9	<i>D17Mit39</i>	13.0
<i>D3Mit32</i>	15.0	<i>D7Nds4</i>	1.6	<i>D12Mit5</i>	8.9		
				<i>D12Mit6</i>	21.9	<i>C18</i>	17.0
<i>C4</i>	6.5	<i>C8</i>	32.0	<i>D12Nds2</i>	7.0	<i>D18Mit12</i>	2.5
<i>D4Mit2</i>	29.5	<i>D8Mit8</i>	83.9			<i>D18Mit17</i>	44.9
<i>D4Mit17</i>	11.2	<i>D8Mit56</i>	9.0	<i>C13</i>	1.0	<i>D18Mit8</i>	12.0
<i>D4Mit45</i>	19.0			<i>D13Mit1</i>	55.3		
<i>D4Mit16</i>	22.1	<i>C9</i>	17.0	<i>D13Mit9</i>	34.8	<i>C19</i>	15.0
<i>D4Mit13</i>	13.0	<i>D9Mit2</i>	12.5	<i>D13Mit35</i>	5.0	<i>D19Mit16</i>	4.6
		<i>D9Mit4</i>	13.7			<i>D19Mit14</i>	50.1
<i>C5</i>	1.0	<i>D9Mit8</i>	44.8	<i>C14</i>	2.5	<i>D19Mit2</i>	5.0