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A Genomewide Assessment of Inbreeding Depression: Gene Number, Function, and Mode of Action

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Abstract: *Although the genetic basis of inbreeding depression is still being debated, most fitness effects are thought to be the result of increased homozygosity for recessive or partially recessive deleterious alleles rather than the loss of overdominant genes. It is unknown how many loci are associated with inbreeding depression, the genes or gene pathways involved, or their mode of action. To uncover genes associated with variation in fitness following inbreeding, we generated a set of inbred lines of* Drosophila melanogaster *for which only the third chromosome varied among lines and measured male competitive reproductive success among these lines to estimate inbreeding depression. Male competitive reproductive success for different lines validated our prediction that equally inbred lines show variation in inbreeding depression. To begin to assess the molecular basis of inbreeding depression for male competitive reproductive success, we detected variation in whole-genome gene expression across these inbred lines with commercially available high-density oligonucleotide microarrays. A total of 567 genes were differentially expressed among these inbred lines, indicating that inbreeding directly or indirectly affects a large number of genes: genes that are disproportionately involved in metabolism, stress and defense responses. Subsequently, we generated a set of outbred lines by crossing the highest inbreeding depression lines to each other and contrasted gene expression between parental inbred lines and F_1 hybrids with transcript abundance as a quantitative phenotype to determine the mode of action of the genes associated with inbreeding depression. Although our results indicated that approximately 75% of all genes involved in inbreeding depression were additive, partially additive, or dominant, about 25% of all genes expressed patterns of overdominance. These results should be viewed with caution given that they may be confounded by issues of statistical inference or associative overdominance.*

Keywords: additivity, inbreeding, inbreeding depression, microarray, overdominance, transcriptional variation

Una Evaluación a Nivel de Genoma de la Depresión por Endogamia: Número, Función y Modo de Acción de Genes

Resumen: *Aunque las bases genéticas de la depresión por endogamia aun están en debate, se piensa que la mayoría de los efectos sobre la adaptabilidad son el resultado del incremento de la homocigosidad para alelos deletéreos recesivos o parcialmente recesivos y no de la pérdida de genes sobredominantes. No se conoce cuantos loci están asociados con la depresión por endogamia, los genes o rutas génicas que están involucrados ni su modo de acción. Para descubrir los genes asociados con la variación en la adaptabilidad después de la*

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endogamia, generamos un conjunto de líneas endogámicas de Drosophila melanogaster *en las que sólo varió el tercer cromosoma y medimos el éxito reproductivo competitivo de machos (ERCM) entre líneas para estimar la depresión por endogamia. El éxito reproductivo competitivo de machos para las diferentes líneas validó nuestra predicción de que líneas igualmente endogámicas muestran variación en la depresión por endogamia. Para iniciar la evaluación de las bases moleculares de la depresión por endogamia para ERCM, detectamos la variación en toda la expresión génica en las líneas endogámicas mediante microseries de oligonucleótidos de alta densidad disponibles comercialmente. Un total de 567 genes se expresaron diferencialmente en estas líneas endogámicas, lo que indica que la endogamia afecta, directa o indirectamente, un gran número de genes: genes que están desproporcionadamente involucrados en respuestas del metabolismo, estrés y defensa. Subsecuentemente, generamos un conjunto de líneas exogámicas mediante cruzas de las líneas con mayor depresión por endogamia y contrastamos la expresión de genes entre líneas endogámicas parentales y los híbridos F_1 utilizando la abundancia de transcripciones como un fenotipo cuantitativo para determinar el modo de acción de los genes asociados con la depresión por endogamia. Aunque nuestros resultados indicaron que aproximadamente 75% de todos los genes involucrados en la depresión endogámica fueron aditivos, parcialmente aditivos o dominantes, cerca de 25% de los genes expresaron patrones de sobredominancia. Estos resultados deben verse con precaución dado que se pueden confundir con temas de inferencia estadística o sobredominancia asociativa.*

Palabras Clave: aditividad, depresión por endogamia, endogamia, microserie, sobredominancia, variación en la transcripción

Introduction

The retention of genetic variation, generally regarded as important in maintaining high levels of fitness and allowing populations to adapt to changing environmental conditions, has been a major concern in the fields of conservation biology, agriculture, and evolutionary biology. As populations become smaller and increasingly isolated, they tend to lose genetic variability owing to genetic drift and inbreeding (Frankel & Soulé 1981; Lande 1988). Inbreeding, a product of mating among related individuals, is characterized by an increase in homozygosity and is usually followed by a decrease in fitness (i.e., inbreeding depression). Two main hypotheses have been formulated to account for the existence of inbreeding depression and heterosis (gain in fitness following outbreeding) (reviewed in Wright 1977; Charlesworth & Charlesworth 1999; Whitlock et al. 2002). The overdominance hypothesis (East 1908) argues that heterozygotes are superior to each homozygote. Loss of heterozygotes through inbreeding will in turn decrease the mean value of traits associated with fitness and lead to inbreeding depression, whereas the (partial) dominance hypothesis (Davenport 1908; Jones 1917) argues that most mutations are neutral or deleterious and generally recessive (Mackay 2001). Increasing the proportion of homozygotes via inbreeding will increase the probability of unmasking these deleterious alleles (Charlesworth & Charlesworth 1999) leading to inbreeding depression (Keller & Waller 2002). Which of the hypotheses explain most of the decline in fitness associated with inbreeding is still being debated (Ritland 1996; Karkkainen et al. 1999; Roff 2002). Although evidence exists to support both models (Crow 1993; Hughes 1995), the dominance hypothesis appears to be the favored one on the basis of available empirical data and theory (Charlesworth & Charlesworth 1999).

Although inbreeding depression has been a central theme of biological research for over a century, little is known about its underlying molecular basis. For instance, how many loci may be involved in causing inbreeding depression, the genes or gene pathways involved, and their mode of action are unknown. Given that most ecologically important traits are polygenic (Falconer & Mackay 1996), one may expect a large proportion of the genome to be involved in inbreeding depression. Several authors argue that inbreeding depression may actually result from the summation of a large number of genes of small effect, whereas others argue that a few genes of large effect lead to the most severe inbreeding depression, without consensus to date (Keller & Waller 2002).

An understanding of the molecular basis of inbreeding depression requires knowledge of variation at the whole-genome level. Most studies investigating the molecular basis of inbreeding depression have focused on single genes or on random neutral makers that offer little or no insight into the functional basis of inbreeding depression. With the generation of vast amounts of DNA sequence information and the advent of microarrays, it is possible to describe biological processes, such as inbreeding depression, from a total genomic perspective (White et al. 1999). Changes in multigene patterns of expression can provide clues about regulatory mechanisms and broader cellular functions and biochemical pathways leading to phenotypic differences. Specifically, microarrays provide a powerful tool for assessing the number of loci involved in inbreeding depression and clues about gene function at these loci.

To assess aspects of the molecular basis of inbreeding depression, we used high-density oligonucleotide microarrays to detect variation in gene expression across inbred lines of *Drosophila melanogaster*. In addition, to assess the mode of action of the genes associated with inbreeding depression, we generated a set of outbred lines by crossing high inbreeding depression lines to each other and contrasting gene expression between these parental inbred lines and their F_1 hybrid offspring showing heterosis. Specifically, we addressed the following questions: How many genes are associated with inbreeding depression? Which genes or functional classes of genes are responsible for the decrease in fitness associated with inbreeding? and What is the underlying cause of inbreeding depression, overdominance or partially recessive deleterious alleles?

Methods

Drosophila Stocks

To assess the molecular basis of inbreeding depression, we initially compared 6 isogenic substitution lines (i.e., all individuals within each line were genetically identical, with the exception of the third chromosome, which was substituted for a different wild-type chromosome in each line) of *D. melanogaster* that varied in their degree of inbreeding depression. Next, we compared inbred with outcrossed lines generated from a subset of these inbred lines. We obtained third-chromosome substitution lines of *D. melanogaster* from J. Leips (University of Maryland–Baltimore County, Baltimore, Maryland) for this study. Within each substitution line, flies were identically homozygous for all genes on the third chromosome and each line contained a different wild-type third chromosome derived from a single wild population collected in Raleigh, North Carolina (DeLuca et al. 2003). All other chromosomes (and hence genes) were identical across lines. Consequently, genetic variation between lines was due solely to variation naturally found in the third chromosome that encodes for approximately 30% of the genome.

Use of these substitution lines presents several advantages over other methods. It controls for epistatic interactions (interactions between genes at different loci) by maintaining a common genetic background among lines. It also allows one to capture the genetic variance present in the third chromosome as it was in the wild at the time of capture (although this does not preclude the possibility that a given third chromosome may vary over differing genetic backgrounds). Breeding flies over several generations in the lab may cause adaptation to laboratory conditions; thus, certain alleles relevant to inbreeding depression could be lost. To ensure that these 6 lines were isogenic, 7 variable microsatellite loci distributed along chromosomes 2 and 3 were used (3 on chromosome 2, and 4 on chromosome 3) (Schug et al. 1998). All were homozygous within each line. Microsatellites on chromosome 3 varied and those on chromosome 2 did not.

Male Competitive Reproductive Success (MCRS)

To assay inbreeding depression we measured MCRS, which provides an assessment of the average reproductive success of each male genotype (Drnevich et al. 2004). To measure variation in MCRS among genotypes, a full diallel (i.e., a crossing design) consisting of all possible crosses between males and females within and between our 6 lines was performed. To account for potential maternal or density effects, we conducted 2 generations of constant-density breeding (i.e., same numbers of individuals for both adults and larvae for both rearing and breeding). Initially, 7 males and 7 females from each isogenic line were placed together in a vial. The virgins collected from these crosses were then used as parental flies in the full diallel. Subsequently, male offspring from the diallel crosses were used in the MCRS assay. These males were reared at constant larval density with 25 larvae per vial and collected as virgins. Two independent replicates were created for each line cross, from the full diallel, with each replicate reared in a separate incubator (blocks). The flies were bred on a standard cornmeal medium at 25 °C, with a fixed illumination cycle of 12-h light and 12-h dark.

Male reproductive success was assayed under "competitive" conditions (MCRS) to mimic the conditions under which normal reproduction takes place, a commonly accepted proxy for male fitness in fruit flies (Drnevich et al. 2004). For each isogenic line, we placed 3 virgin experimental wild-type males (w^+) in a vial with 3 virgin ebony males (e/e) and 3 virgin ebony females, such that the experimental male competed with the ebony male for female access. Ebony is a recessive mutation; only homozygous individuals express the ebony phenotype, allowing one to differentiate offspring sired by the ebony male and the wild-type experimental male. Each competitive assay was replicated 4 times for every cross within each of the 2 replicates (blocks). Males were allowed to mate with females for 7 days, and then experimental male flies were flash frozen and collected for RNA extraction and microarray analysis. Seventeen days after initial contact between male and female flies, we counted the number of wild-type (w^+) and ebony (e/e) adult offspring. The MCRS values for each genotype were calculated as the least-square means from a linear-mixed model that included genotype, block, and replicate within block. Although variation in MCRS could result from differences in offspring viability, results from previous studies showed no significant differences in larval viability among varying genotypes (Drnevich et al. 2004).

Inbreeding Depression and Gene Action

We compared MCRS in the inbred isogenic genotypes with those of the outbred genotypes to measure inbreeding depression (ID). For example, we used isogenic line A as a parental line to produce several different outbred genotypes. We used the following equation to compare the reproductive success of line A with the mean reproductive success of all heterozygous lines for which A was a parent:

$$\mathrm{ID} = \frac{|W_A - W_{Aj}|}{W_{Aj}},$$

where W_A is the MCRS of the inbred line and W_{Aj} is the mean MCRS of the outbred crosses involving line A (Charlesworth & Charlesworth 1999).

To investigate the mode of action (e.g., additive, dominant, or overdominant) of the genes associated with the variation in inbreeding depression between lines, we crossed high inbreeding depression lines to each other to generate outbred lines that showed heterosis. The high inbreeding depression lines, 483, 83, and 33, were crossed in the diallel described earlier to generate lines 483 × 83, 483 × 33, 83 × 33 and their reciprocal crosses (e.g., 483 males × 83 females and 483 females × 83 males). The MCRS and inbreeding depression were assayed and calculated as above.

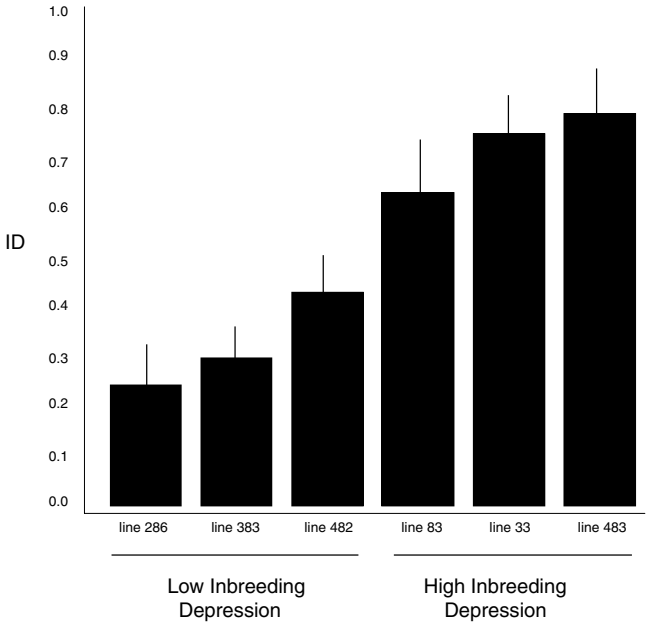

Figure 1. Variation in inbreeding depression (ID) across inbred lines. Inbreeding depression is expressed as the percent decrease in male competitive reproductive success (MCRS, 1 SE shown). The 3 tallest bars are not significantly different from one another and the 3 shortest bars are not significantly different from one another ($p < 0.05$).

Expression Arrays

For the expression analysis, mRNA was extracted from the wild-type experimental males from the competitive assays (from the 6 inbred lines and 3 outcrossed lines). Two sets of 6 males were randomly selected and pooled per replicate (block) and 2 independent arrays were constructed per genotype to produce true biological replicates. Flies were selected from all 4 male competitive assays for each replicate. Two males from each of 2 of the 4 competitive assay vials and 1 male from each of the remaining 2 were chosen. These flies were anesthetized with CO_2 and flash frozen prior to RNA extraction.

Total RNA was extracted with standard TRIzol (Life Technologies, Carlsbad, California) protocols. Messenger RNA was reverse transcribed and labeled with the MessageAmp kit (Ambion, Austin, Texas) and biotin labeled dCTP and dGTP (ENZO Diagnostics, Farmingdale, New York). Affymetrix *Drosophila* GeneChip Arrays (Version 2.0) were hybridized at the University of Illinois Keck Center. Chips contained 18,769 transcripts with 14 probes per transcript. Feature intensities on each chip were quantified with MAS 5.0 software.

Following hybridization, the Perfect Match (PM) probes for all arrays were initially quantile normalized with the Affy package in Bioconductor (Irizarry et al. 2003) to remove nonbiological variation among arrays. Only the PM data were used for the remainder of the analysis, MM probes were ignored because they tend to increase random noise in the data.

Genes Associated with Variation in Inbreeding Depression among Inbred Lines

To detect genes associated with variation in inbreeding depression among inbred lines (from low inbreeding depression to high inbreeding depression, Fig. 1), we analyzed mRNA expression of each gene with hierarchical regression (Sullivan et al. 1999) on a gene-by-gene basis. We used the following mixed model in PROC MIXED, SAS (release 9.13): $\log_2(\mathrm{PM}) = u + \mathrm{ID} + P + P^*\mathrm{ID} + B + e$, where $\log_2$(PM, perfect match) is the $\log_2$ transformed, normalized PM intensity of each feature, u is the intercept; ID is the random intercept (given that ID lines represent randomly chosen samples of flies), P is the probe effect (fixed effects), and B is the block effect (random effects). To remove outliers from the data set, we computed residuals for the model and removed probes with an R student >3 (0.80% of probes were removed or 25,114/3,153,192 probes) (Drnevich et al. 2004). To correct for multiple testing, we adjusted the nominal p value with a step-wise Bonferroni correction at $p < 0.05$, a very conservative correction (for a nominal $p < 0.000019$) (implemented in SAS with PROC MULTEST). This resulted in <1 false positive gene being detected among the 18,769 transcripts.

Gene Clustering and Gene Ontology

We performed gene clustering with Dchip (1.3). The significance level was set at $p < 0.01$ for genotype clustering and $p < 0.001$ for gene clustering (centroid linkage method and ranked correlation were used as distance metrics). The functional categorization of each gene was analyzed with the web-based programs NetAffX (https://www.affymetrix.com/analysis/netaffx/index.affx) and FlyBase (http://flybase.bio.indiana.edu/) (complete list available from J.F.A. on request). To assess whether some GO categories (i.e., categorization of genes based on molecular function) were overrepresented, we imported the list of differentially expressed genes among inbred lines into DAVID (http://david.abcc.ncifcrf.gov/), a program designed to interpret functional information for a variety of model organisms (Dennis et al. 2003). This program uses a hypergeometric test to compare a list of genes for a given GO category with that of a reference gene list and thus provides a null distribution (all genes on the array). DAVID computes a ratio of enrichment and a p value corresponding to the significance of enrichment (Dennis et al. 2003).

Variation among Inbred Parental Lines and F_1 "Hybrids"

For the genes identified as being associated with variation in inbreeding depression (detected by the hierarchical regression above), we used an analysis of variance to assess whether there were expression differences between inbred parental lines (483, 83, and 33) and each of the F_1 "hybrids" from the diallel cross (e.g., 483, 83). We used a likelihood ratio test to determine the optimal linear model to describe these data. We compared the following full and reduced models $\log_2(\mathrm{PM}) = u + G + P + P^*G + B(L)_{\mathrm{random}} + e$ and $\log_2(\mathrm{PM}) = u + G + P + P^*G + B_{\mathrm{random}} + e$, where G is the genotypic cross (parental 1 × parental 2, forming the F_1 hybrid), P is the probe, B is block, and $B(L)$ is the block nested within line. We subtracted the –2 residual log likelihood of the full model from the reduced model and than compared the likelihood ratio with a 1° of freedom chi-square distribution. Most genes showed no significant differences after correction for multiple testing. Genes showing significant differences between the 2 models were removed from the analysis because it is difficult to make biological sense of these genes (i.e., genes for which there was a significant block × line interaction; expression levels were high in one block and low in another).

For the remaining analyses, where genes showed no significant effects between the 2 models above, we used the reduced model implemented in PROC MIXED in SAS 9.13, on a gene-by-gene basis for every cross independently. We used contrast statements to address the difference between midparent and F1 for every cross. We controlled for multiple testing with a false discovery rate (FDR) of $p < 0.01$ (i.e., 1 in every 100 comparisons represented a false positive).

Mode of Action

We used gene expression as a phenotype to calculate the dominance coefficient d (Falconer & Mackay 1996) for each of the 3 crosses (generating outbred lines), focusing on genes showing significant variation for all 3 crosses. The d is the deviation between the phenotype of the heterozygote F_1 hybrid and the mean phenotype of the homozygous parents (d = heterozygote – [homozygote 1+ homozygote 2]/2). The degree of dominance of each gene was calculated using the ratio d/a, where a is half the difference in expression between the 2 homozygotes (a = abs | homozygote 1 – homozygote 2 | / 2). A ratio of $d/a = 0$ indicates exact additivity (the heterozygote is intermediate in expression between the 2 homozygotes), whereas $d/a = >+1$ or < -1 indicates perfect dominance (the heterozygote is equivalent in expression to 1 of the 2 homozygotes) and ratios greater than +1 or less than −1 are indicative of overdominance or underdominance, respectively (heterozygote expression is higher or lower than either of the 2 homozygotes). In addition, we used the contrast for d (difference between midparent and F_1) to determine whether or not d was significantly different from zero, indicating deviation from additivity (see Fig. 4).

Results

Inbreeding Depression

Inbred lines of *D. melanogaster* showed considerable variation in the degree of inbreeding depression expressed, with a decrease in MCRS ranging from a low of 24% to a high of 79% among the 6 inbred lines ($p < 0.05$; Fig. 1). From the microarray analysis, we uncovered 567 probe sets that were differentially expressed after Bonferoni adjustment ($p < 0.05$) among these lines. The hierarchical clustering (Fig. 2) showed 2 significant line clusters ($p < 0.01$), separating high inbreeding depression lines from low inbreeding depression lines.

Gene Ontology and Inbreeding Depression

From a functional standpoint, genes associated with variation in inbreeding depression were represented by a large variety of functional categories, from behavioral genes ("response" genes) to those associated with metabolism (Fig. 3). Enrichment analysis (bottom of Fig. 3) revealed significant overrepresentation of genes involved in metabolism (e.g., genes for organic acid, amino acid, lipid, and carbohydrate metabolism), stress (e.g., heat shock proteins) and defense (primarily to bacterial and fungal infections).

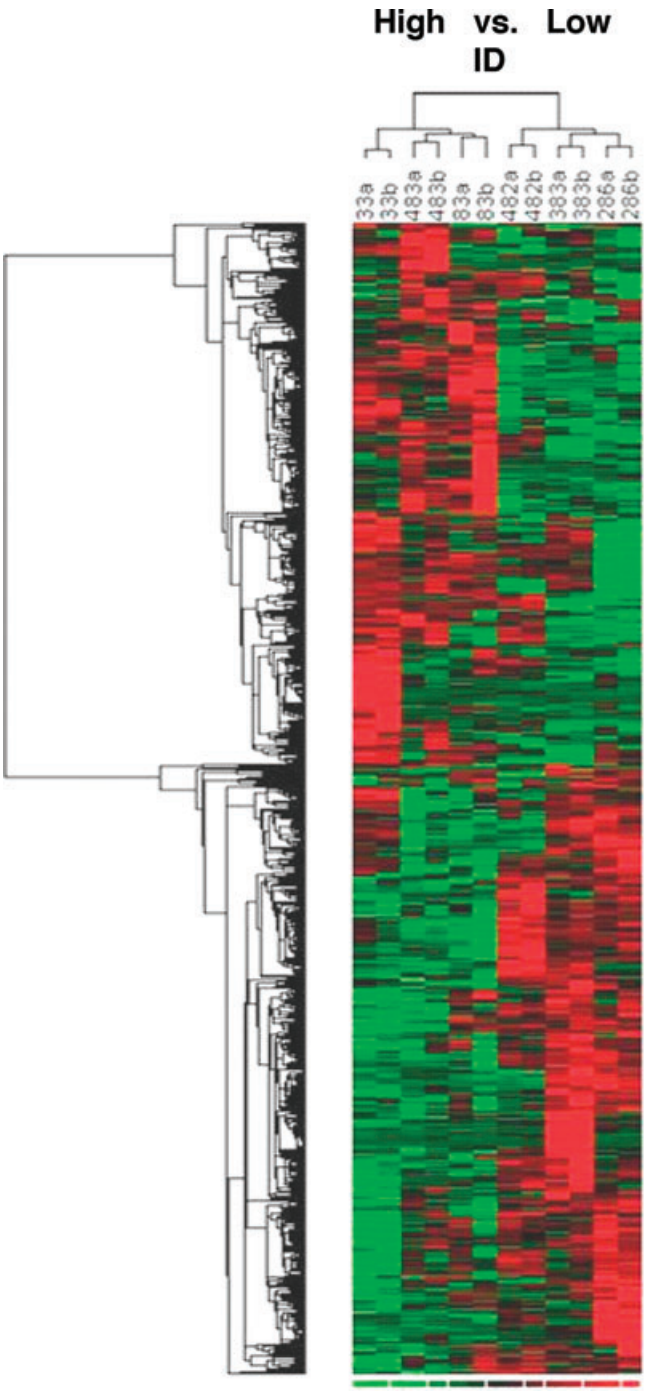

Figure 2. Hierarchical clustering of gene expression by line. Transcript abundance is represented from high (red) to low (green) with lines and array replicates in columns and genes in rows. Clustering distinguishes high inbreeding-depression (ID) lines (83, 33 and 483) from low inbreeding lines (286, 383, and 482).

Gene Action

Gene expression was significantly different for 219, 160, and 241 genes between P1, P2, and F_1 for cross 483 × 33, 483 × 83, and 83 × 33 (FDR $p < 0.05$), respectively. Forty-six genes were common to all 3 crosses and 34 were located on the third chromosome. The GO patterns showed that these genes were associated with metabolism and oxidative stress.

Eighty-eight, 82, and 120 genes behaved in an additive fashion for cross 483 × 33, 483 × 83, and 83 × 33 (*d/a* for these genes was not significantly different from 0). In addition, 101, 30, and 52 genes behaved in a dominant fashion for cross 483 × 33, 483 × 83, and 83 × 33, respectively. Furthermore, we uncovered 61 overdominant genes for cross 483 × 33, 45 for cross 483 × 83, and 59 for cross 83 × 33 (only 5 genes were overdominant and common to all 3 crosses; functions are unknown). Two crosses (483 × 83 and 83 × 33) had significantly more overdominant genes, with about twice as many overdominant as underdominant genes and no difference for overdominant and underdominant genes for cross 483 × 33.

Discussion

Results from the microarray analysis indicated that inbreeding depression for MCRS involves a large number of transcripts (567). Thus, inbreeding depression was either the product of a relatively large number of genes of small effect, rather than a few genes of large affect, or caused by a few key genes that affect the expression of many other genes (e.g., epistatic and pleiotropic effects on gene expression; e.g., Featherstone & Broadie 2002). When we assessed the proportion of genes differentially expressed across inbred lines by chromosomal location, 62% of the differentially expressed genes were on the third chromosome and approximately 38% of differentially expressed genes were on the second, fourth, and X chromosomes (29, 0.4, and 8, respectively). Because these lines are variable only for the third chromosome, any variation on the second, fourth, and X chromosomes had to originate from genes located on the third chromosome and thus are products of transregulation or epistatic interactions. These results suggest a significant amount of inbreeding depression could be due to a few key genes. Gene differences on the third chromosome in combination with these transregulatory effects likely explain much of the variation in genetic architecture among the differing line crosses (Fig. 5). Nevertheless, we have no information about the relative importance of individual genes responsible for the observed variation in inbreeding depression. Thus, listing differences on a gene-by-gene basis alone is uninformative. Instead, we considered genes on the basis of their functional categorization.

Genes associated with variation in inbreeding depression revealed an overrepresentation of those involved in metabolism, stress, and defense, which is consistent with a recent study in *D. melanogaster* by Kristensen et al. (2005). Lower metabolic efficiency (upregulation of

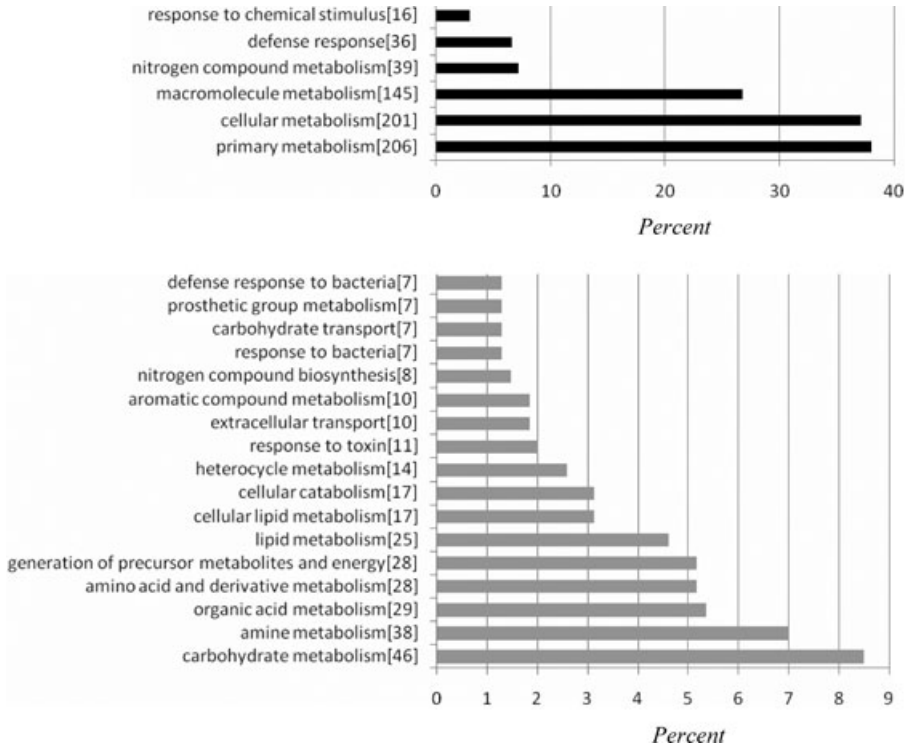

Figure 3. Gene ontology analysis for genes significantly associated with inbreeding depression. Values in brackets represent number of genes involved in a pathway (gene ontology levels shown in black and grey; coloration represents greater gene resolution from general, black, to more specific, grey). Genes within a pathway are expressed as a percentage of the total of all genes. Enrichment analysis (grey bars) revealed significant overrepresentation of genes involved in metabolism, stress and defense.

metabolic genes) in homozygous individuals may play a key role in explaining inbreeding depression (Kristensen et al. 2006), which leaves less energy for reproduction owing to higher metabolic costs associated with genetic stress (e.g., indicative of the upregulation of heat-shock proteins, sucrose metabolism, and production of amino acid). The large representation of genes involved in defensive responses to biotic agents is consistent with what is known about inbreeding and inbreeding depression; inbred organisms are often more susceptible to environmental challenges (e.g., Bijlsma et al. 2000; Hedrick & Kalinowski 2000; Keller & Waller 2002). Several genes uncovered here have been studied intensively relative to inbreeding (e.g., diptericins, attacin-A, Hsp70; Kristensen et al. 2005). In particular, stress response genes, such as Hsp 70B (AFYYI D: 1632841_x_at; a heat-shock protein) were upregulated in the high inbreeding depression lines and are known to positively affect survival (Lis 1998; Feder 1999; Farkas et al. 2000). Several immune response genes, such as cecropin B (AFFYID:1626530_at), which is associated with a humoral response to bacterial infection, were also upregulated in the high inbreeding depression lines in this study (Hoffmann & Reichhart 1997; Govind 1999). The low representation of genes involved with development may reflect the fact that we used adult male flies.

Of particular note are 46 differentially expressed genes held in common across the 3 highly inbred lines. These genes include those associated with metabolism and oxidative stress and appear to represent key genes associated with inbreeding depression. For example, one of these genes held in common is AFYYID: 1638074_at, a glutathione transferase, which is important in resisting lipid peroxidation. This gene is downregulated in the highly inbred lines making them more susceptible to oxidative damage.

Although our results indicated that approximately 75% of all genes involved in inbreeding depression were additive, partially additive, or dominant, about 25% of all genes expressed patterns of overdominance (Fig. 5). This was unexpected, given that fitness effects of inbreeding are thought to result from deleterious recessive alleles that have been unmasked by inbreeding. The role played by overdominance in *Drosophila* is thought to be minimal at best as a mechanism leading to inbreeding depression (Lynch 1991; Hughes 1995; Charlesworth & Charlesworth 1999; Keller & Waller 2002). In an experimentally comparable study, Gibson et al. (2004) found that only 5% of genes in *D. melanogaster* contributed to patterns of overdominance. Although the genetic basis of this difference is unknown, one possibility may be that there are differences in genetic architecture; Gibson et al. crossed disparate populations from Russia and North America. Alternatively, Gibson et al. used a more stringent cutoff, where $|d/a|>1.32 \log_2$ to indicate overdominance as opposed to our cutoff of $|d/a|>1.00 \log_2$. Furthermore, in a recently published paper, Hughes et al. (2006) found fewer genes with significant patterns of overdominance (<2%). The difference is due to the use of a more stringent criterion in Hughes et al., wherein they calculated 80 and 95% confidence intervals for a and d. If upper and lower limits for d and a do not overlap,

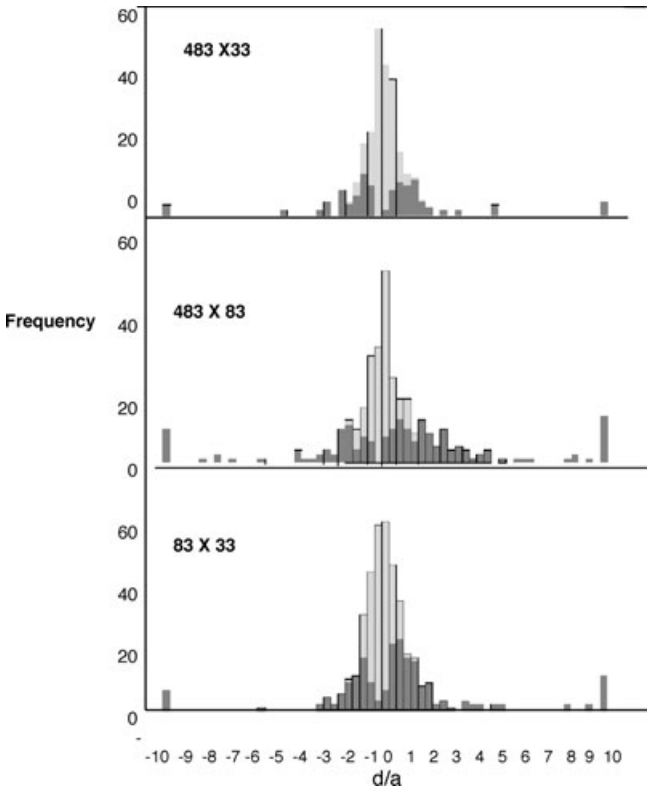

Figure 4. Distribution of d/a *(dominance to additivity ratio) for each of the 3 outbred crosses among high inbreeding depression lines. The dark grey area represents genes for which* d *was significantly different from zero, indicating non-additivity. Genes with* d/a *values* ≥ 10 *and* ≤ -10 *were pooled with values of 10 and* -10*.* d/a $= 0$ *refers to exact additivity,* d/a $= +1$ *or* -1 *indicates perfect dominance and ratios* d/a $= \geq +1$ *or* < -1 *are indicative of overdomiance or underdominance, respectively.*

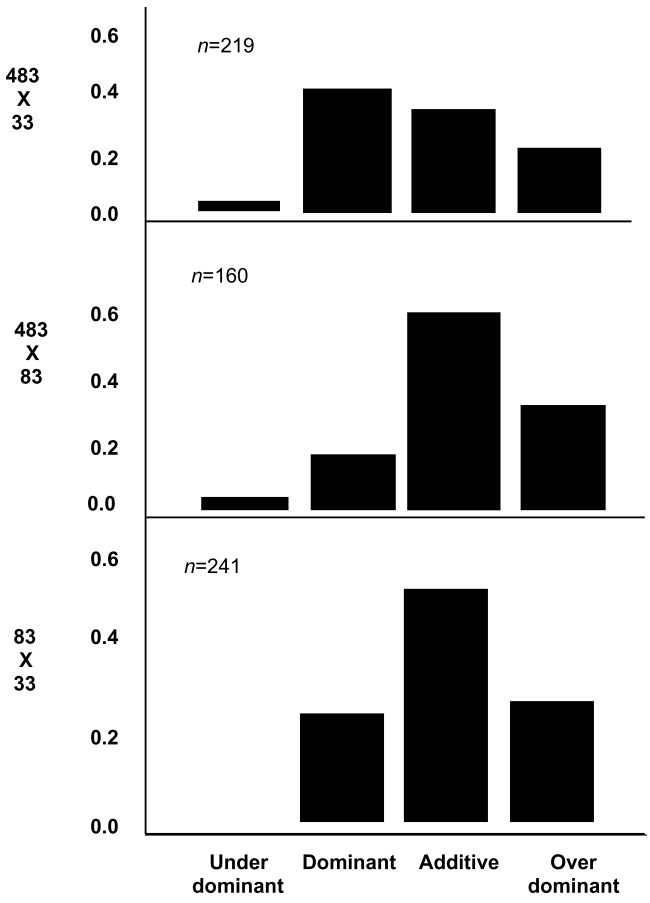

*Figure 5. Percentage of genes categorized as under- or overdominant, additive, and dominant for each of the 3 outbred crosses among lines with high inbreeding depression (*n *is the number of genes showing variation between the 2 parental lines and the "F_1 hybrid").*

there is statistical support for overdominance. Our results were similar to those of Hughes et al. (2006) when we used their criteria. Whether these differences among studies and analyses are of biological significance or not is open to question.

One confounding problem that has plagued studies of inbreeding depression and the role of overdominance is the issue of associative overdominance. Associative overdominance is the apparent heterozygote advantage that results from 2 closely linked loci (A and B) in repulsion disequilibrium for deleterious alleles (i.e., the dominant allele on one homologue complements the deleterious allele on the other). Individuals heterozygous for this linkage group will appear to have the highest fitness because they bear a dominant allele at both locus A and B (Carr & Dudash 2003). Thus, it is not straightforward to infer true overdominance from associative overdominance from our data. Associative overdominance may be the explanation for the unusually high number of overdominant loci we uncovered because the third chromosome was made completely homozygous within each line, leading to complete gametic phase disequilibrium in the F_1 lines, a situation that leads to more widespread associative overdominance. This may explain the extreme values (<-10 and >10) observed for *d/a* ratios (Fig. 4).

Most of what is known to date about the molecular basis of inbreeding depression at the genome level comes from agricultural studies, where quantitative trait loci for maize and rice have been mapped for a variety of traits, including those associated with heterosis. It appears that overdominance plays a major role in inbreeding depression in these crop species (Luo et al. 2001; Birchler et al. 2003). These organisms have mixed mating strategies of both selfing and outbreeding; thus, they evolved to tolerate high levels of inbreeding, which minimizes inbreeding depression and the role deleterious recessives play in contributing to patterns of inbreeding depression (Berg & Redbo-Torstensson 1999).

Although strong selection applied to crop species could result in stronger gametic phase disequilibrium, compelling arguments have been made against associative overdominance in studies of both maize and rice (e.g., see Stuber et al. 1992; Luo et al. 2001; Carr & Dudash 2003). In other species, it appears that inbreeding depression results primarily from the accumulation of deleterious recessives, as in *Mimulus guttatus*, *M. micranthus* (Dudash & Carr 1998), and *Mus musculus* (Caballero & Keightley 1998). In light of these seemingly contradictory results, it is important to note that in the majority of studies only a miniscule fraction of the genome has been sampled, making any general mechanistic interpretation premature.

We also found that inbreeding among inbred lines led to high levels of variation in inbreeding depression; range in decline of MCRS was 24–79%. These levels of variation in inbreeding depression are particularly prevalent with fast inbreeding, in which selection is much less efficient at removing deleterious recessives and drift predominates (Kristensen et al. 2005). Such variation was not unexpected given that the third chromosome varied among the inbred lines, and these genes represented a suite of diverse genes that could differentially affect fitness or randomly unmask deleterious recessives among the differing lines. The variation in inbreeding depression uncovered here is similar to that uncovered in previous studies of *Drosophila*. For example, chromosome-2 homozygous males in competition with chromosome-2 heterozygous males have a fitness reduction of about 60%, on average (approximately 52% in this study), with lines ranging from 0% to 93% inbreeding depression (Sved & Ayala 1970). The effects of full-sib mating on competitive male-mating ability in *D. melanogaster* also exhibit considerable variation in inbreeding depression. For example, Fowler and Whitlock (1999) found that inbreeding depression ranged from approximately 10 to 60% among inbred lines after 3 generations of inbreeding. Our lines only varied for the third chromosome, which accounted for approximately 30% of the *D. melanogaster* genome. This result suggests that we would have detected an even higher proportion of variable transcripts had our lines differed for all chromosomes, and this would have resulted in greater variance in inbreeding depression. Nevertheless, the variance in inbreeding depression was as great as those from lines in which all chromosomes varied. This is likely due to the random nature associated with unmasking deleterious recessives and cis- and transregulatory effects influencing the degree of inbreeding depression manifested.

Conservation Implications

The large number of genes significantly affected by inbreeding depression that we found underscores the importance of preserving diversity at the level of the whole genome. Breeding individuals to maintain allelic diversity at a subset of genes, as has been occasionally suggested (e.g., see Hughes 1991), would accelerate inbreeding and a concomitant loss of diversity at the level of the whole genome in small populations (Haig et al. 1990). To maintain viability and evolutionary flexibility, the best strategy is to attempt to retain maximal allelic diversity at all loci within populations (Vrijenhoek & Leberg 1991) while preserving genetic differences occurring among captive and among native populations of the species.

One of the central questions concerning inbreeding depression in conservation biology relates to the interplay of selection and drift: can purging remove deleterious alleles that cause inbreeding depression efficiently enough to reduce the impact of increasing inbreeding on population viability? The fraction of the load that is due to true overdominance cannot be reduced by purging, but the fraction of the load that is due to dominance can be purged. If there are a large number of genes contributing to inbreeding depression, each with a relatively small effect, as is possible, then purging may not be very efficient given that so many loci are involved.

Although genetic theory does not predict the effect of heterozygous advantage on the rate of loss of genetic variation in small populations, Lesica and Allendorf (1992) used computer simulations to estimate expected rates of loss of heterozygosity under different selective intensities and population sizes. Overall, their results indicate that heterozygous advantage maintains larger levels of genetic variance within small populations (<100). Thus, if overdominance was an important mechanism (with a fitness advantage at the loci examined), heterozygous advantage would slow the loss of genetic variation due to drift over what neutral models would predict. In addition, in the case of overdominance both allelic variants would have a higher probability of being maintained in the population, which would not be the case where deleterious alleles are being unmasked by inbreeding.

The genes and pathways we uncovered in this study are consistent with those of other array studies in which inbreeding, oxidative stress, or aging was included as a treatment (Landis et al. 2004; Kristensen et al. 2005, 2006). These genes and pathways include those associated with metabolism, stress, and defense, the identification of which provides a first step toward identifying and understanding the specific roles of genes associated with inbreeding depression.

Overall, microarray technology is a powerful tool for investigating variation in gene expression at the genome level. Although this approach neglects variation in protein structure, variation in gene expression accounts for a large portion of total phenotypic variation and provides the widest possible estimate of genomewide variation. In combination with high-density genotyping and association or QTL mapping (Mackay 2001), the tools for

discovering the genetic determinants of inbreeding depression are available.

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Literature Cited

Berg, H., and P. Redbo-Torstensson. 1999. Offspring performance in three cleistogamous *Viola* species. Plant Ecology **145:**49–58.

Bijlsma, R., J. Bundgaard, and A. C. Boerema. 2000. Does inbreeding affect the extinction risk of small populations: predictions from *Drosophila*. Journal of Evolutionary Biology **13:**502–514.

Birchler, J. A., D. L. Auger, and N. C. Riddle. 2003. In search of the molecular basis of heterosis. The Plant Cell **15:**2236–2239.

Caballero, A., and P. D. Keightley. 1998. Inferences on genome-wide deleterious mutation rates in inbred populations of *Drosophila* and mice. Genetica **102:**229–239.

Carr, D. E., and M. R. Dudash. 2003. Recent approaches into the genetic basis of inbreeding depression in plants. Philosophical Transactions of the Royal Society of London B **358:**1071–1084.

Charlesworth, B., and D. Charlesworth. 1999. The genetic basis of inbreeding depression. Genetical Research **74:**329–340.

Crow, J. F. 1993. Mutation, mean fitness, and genetic load. Pages 3–42 in D. Futuyma and J. Antonovics, editors. Oxford surveys in evolutionary biology. Oxford University Press, Oxford, United Kingdom.

Davenport, C. B. 1908. Degeneration, albinism and inbreeding. Science **28:**454–455.

DeLuca, M., N. V. Roshina, G. L. Geiger-Thornsberry, R. F. Lyman, E. G. Pasyukova, and T. F. C. Mackay. 2003. Dopa decarboxylase (Ddc) affects variation in *Drosophila* longevity. Nature genetics **34:**429–433.

Dennis, G., B. T. Sherman, D. A. Hosack, J. Yang, W. Gao, H. C. Lane, and R. A. Lempicki. 2003. DAVID: Database for annotation, visualization, and integrated discovery. Genome Biology **4:**R60.

Drnevich, J. M., M. M. Murray, E. A. Ruedi, S. Rodriguez-Zas, and K. A. Hughes. 2004. Quantitative evolutionary genomics: differential gene expression and male reproductive success in *Drosophila melanogaster*. Proceedings of the Royal Society of London **271:**2267–2273.

Dudash, M. R., and D. E. Carr. 1998. Genetics underlying inbreeding depression in *Mimulus* with contrasting mating systems. Nature **393:**682–684.

East, E. M. 1908. Inbreeding in corn. Report of the Connecticut Agriculture Experimental Station **1907:**419–429.

Falconer, D. S., and T. F. C. Mackay. 1996. Introduction to quantitative genetics. 4th edition. Longman, Essex, United Kingdom.

Farkas, G., B. A. Leibovitch, and S. C. Elgin. 2000. Chromatin organization and transcriptional control of gene expression in *Drosophila*. Gene **253:**117–136.

Featherstone, D. E., and K. Broadie. 2002. Wrestling with pleiotropy: genomic and topological analysis of the yeast gene expression network. BioEssays **24:**267–274.

Feder, H. 1999. Engineering candidate genes in studies of adaptation: the heat-shock protein Hsp70 in *Drosophila melanogaster*. The American Naturalist **154**(supplement):S55–S66.

Fowler, K., and M. C. Whitlock. 1999. The variance in inbreeding depression and the recovery of fitness in bottlenecked populations. Proceedings of the Royal Society of London B **266:**2061–2066.

Frankel, O., and M. Soulé. 1981. Conservation and evolution. Cambridge University Press, Cambridge, United Kingdom.

Gibson, G., R. Riley, L. Harshman, A. Kopp, S. Vacha, S. Nuzhdin, and M. Wayne. 2004. Extensive sex-specific non-additivity of gene expression in *Drosophila melanogaster*. Genetics **167:**1791–1799.

Govind, L. 1999. Control of development and immunity by rel transcription factors in *Drosophila*. Oncogene **18:**6875–6887.

Haig, S. M., J. D. Ballou, and S. R. Derrickson. 1990. Management options for preserving genetic diversity: reintroduction of Guam rails to the wild. Conservation Biology **4:**290–300, 464.

Hedrick, P. W., and S. T. Kalinowski. 2000. Inbreeding depression in conservation biology. Annual Review of Ecology and Systematics **31:**139–162.

Hoffmann, J. A., and J. M. Reichhart. 1997. *Drosophila* immunity. Trends in Cell Biology **7:**309–316.

Hughes, A. L. 1991. MHC polymorphism and the design of captive breeding programs. Conservation Biology **5:**249–251.

Hughes, K. A. 1995. The inbreeding decline and average dominance of genes acting male life-history characters in *Drosophila melanogaster*. Genetical Research **65:**41–52.

Hughes, K. A., J. F. Ayroles, M. M. Reedy, J. M. Drnevich, K. C. Rowe, E. A. Ruedi, C. E. Caceres, and K. N. Paige. 2006. Segregating variation in the transcriptome: cis regulation and additivity of effects. Genetics **173:**1347–1355.

Irizarry, R. A., B. Hobbs, F. Collin, Y. D. Beazer-Barclay, K. J. Antonellis, U. Scherf, and T. P. Speed. 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics **4:**249–264.

Jones, D. F. 1917. Dominance of linked factors as a means of accounting for heterosis. Genetics **466:**465–479.

Karkkainen, K., H. Kuittinen, R. van Treuren, C. Vogl, S. Oikarinen, and O. Savolainen. 1999. Genetic basis of inbreeding depression in *Arabis petraea*. Evolution **53:**1354–1365.

Keller, L., and D. M. Waller. 2002. Inbreeding effects in wild populations. Trends in Ecology & Evolution **68:**252–258.

Kristensen, T. N., P. Sørensen, M. Kruhøffer, K. S. Pedersen, and V. Loeschcke. 2005. Genome wide analysis of inbreeding effects on gene expression in *Drosophila melanogaster*. Genetics **171:**157–167.

Kristensen, T. N., P. Sørensen, K. S. Pedersen, M. Kruhøffer, and V. Loeschcke. 2006. Inbreeding by environmental interactions affect gene expression in *Drosophila melanogaster*. Genetics **173:**1329–1336.

Lande, R. 1988. Genetics and demography in biological conservation. Science **241:**1455–1460.

Landis, G. N., D. Abdueva, D. Skvortsov, J. Yang, B. E. Rabin, J. Carrick, S. Tavare, and J. Tower. 2004. Similar gene expression patterns characterize aging and oxidative stress in *Drosophila melanogaster*. Proceedings of the National Academy of Sciences of the United States of America **101:**7663–7668.

Lesica, P., and F. W. Allendorf. 1992. Are small populations of plants worth preserving? Conservation Biology **6:**135–139.

Lis, J. 1998. Promoter-associated pausing in promoter architecture and postinitiation transcriptional regulation. Cold Spring Harbor Symposium on Quantitative Biology **63:**347–356.

Luo, L. J., Z.-K. Li, H. W. Mei, Q. Y. Shu, R. Tabien, D. B. Zhong, C. S. Ying, J. W. Stansel, G. S. Khush, and A. H. Paterson. 2001. Overdominant epistatic loci are the primary genetic basis of inbreeding depression and heterosis in rice. Genetics **158:**1755–1771.

Lynch, M. 1991. The genetic interpretation of inbreeding depression and outbreeding depression. Evolution **45:**622–629.

Mackay, T. F. C. 2001. The genetic architecture of quantitative traits. Annual Review of Genetics **35:**303–339.

Ritland, K. 1996. Inferring the genetic basis of inbreeding depression in plants. Genome **39:**1–8.

Roff, D. A. 2002. Inbreeding depression: tests of the overdominance and partial dominance hypotheses. Evolution **56:**768–775.

Schug, M. D., C. M. Hutter, K. A. Wetterstrand, M. S. Gaudette, T. F. C. Mackay, and C. F. Aquadro. 1998. The mutation rates of di-, tri- and tetranucleotide repeats in *Drosophila melanogaster*. Molecular Biology and Evolution **15:**1751–1760.

Stuber, C. W., S. E. Lincoln, D. W. Wolff, T. Helentjaris, and E. S. Lander. 1992. Identification of genetic factors contributing to heterosis in a hybrid from two elite maize inbred lines using molecular markers. Genetics **132:**823–839.

Sullivan, L. M., K. A. Dukes, and E. Losina. 1999. Tutorial in biostatistics. An introduction to hierarchical linear modelling. Statistics in Medicine **18:**855–888.

Sved, J. A., and F. J. Ayala. 1970. A population cage test for heterosis in *Drosophila pseudoobscura*. Genetics **66:**97–113.

Vrijenhoek, R. C., and P. L. Leberg. 1991. Let's not throw the baby out with the bathwater: a comment on management for MHC diversity in captive populations. Conservation Biology **5:**252–254.

White, K. P., S. A. Rifkin, P. Hurban, and D. S. Hogness. 1999. Microarray analysis of *Drosophila* development during metamorphosis. Science **286:**2179–2184.

Whitlock, M. C., P. C. Phillips, and K. Fowler. 2002. Persistence of changes in the genetic covariance matrix after a bottleneck. Evolution **56:**1968–1975.

Wright, S. 1977. Evolution and the genetics of populations, Volume 3. Experimental results and evolutionary deductions. University of Chicago Press, Chicago, Illinois.