The Functional Genomics of Inbreeding Depression: A New Approach to an Old Problem

KEN N. PAIGE

The fitness consequences of inbreeding have attracted the attention of biologists since the time its harmful effects were first recognized by Charles Darwin. Although inbreeding depression has been a central theme in biological research for over a century, little is known about its underlying molecular basis. With the generation of vast amounts of DNA sequence information and the advent of microarrays we are now able to describe biological processes from a total genomic perspective. This article reviews the ways in which microarrays have advanced our understanding of the molecular basis of inbreeding depression, including our first look at the number of genes associated with inbreeding depression, which genes or functional classes of genes are responsible for the decrease in fitness associated with inbreeding, the underlying cause of inbreeding depression—overdominance or partially recessive deleterious alleles—and environmental influences on gene-expression patterns.

Keywords: microarray, Drosophila melanogaster*, inbreeding depression, fitness, genomics*

Inbreeding is defined as the mating of individuals related by ancestry. In normally outbreeding species, inbreeding results in a decline in fitness (survival and reproduction), termed inbreeding depression (Darwin 1876). Beliefs concerning the detrimental effects of inbreeding far antedate scientific observation (Wright 1977). Incest, for example, has long been forbidden in many cultures, with the belief that it leads to the abnormal development of children (Zirkle 1952, Wright 1977). In a letter to Lubbock in 1870, Charles Darwin wrote:

> In England and many parts of Europe the marriages of cousins are objected to from their supposed injurious consequences: but this belief rests on no direct evidence. It is therefore manifestly desirable that the belief should be either proved false, or should be confirmed, so that in this latter case the marriages of cousins might be discouraged…. It is, moreover, much to be wished that the truth of the often repeated assertion that consanguineous marriages lead to deafness and dumbness, blindness, &c, should be ascertained: and all such assertions could be easily tested by the returns from a single census. (cited in Hedrick and Kalinowski 2000, p. 139)

Garrod (1902, 1908) was the first to present compelling evidence for inbreeding depression in humans, showing that an excess prevalence of cousin marriages resulted in abnormal traits such as albinism or alkaptonuria (a blackening of the urine caused by a mutation in the enzyme homogentisate 1,2-dioxygenase, which also leads to cartilage and heart valve damage and the formation of kidney stones; Wright 1977). Of course, Darwin (1876) supplied ample evidence for inbreeding depression from extensive plant breeding experiments. These involved no fewer than 57 species of plants from 52 genera and 30 families. Inbred plants were on average shorter, flowered later, weighed less, and produced fewer seeds than those that were outcrossed. The effects of inbreeding were substantial, showing a 41% reduction in seed production and a 13% decline in height (Darwin 1876, Frankham et al. 2002).

Since Darwin's time, inbreeding depression has been documented in essentially all well-studied populations of outbreeding plants and animals. Despite overwhelming evidence from laboratory and domestic species, there was considerable skepticism regarding the occurrence of inbreeding depression in the wild (see Frankham 1995 for a discussion). Crnokrak and Roff (1999), however, provided irrefutable evidence that inbreeding depression commonly occurs in the wild. They reviewed 34 papers investigating inbreeding depression in the wild for 34 taxa from 157 data sets and showed that in 141 cases (90%) inbred individuals fared worse than their outbred counterparts.

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The importance of inbreeding depression

Inbreeding depression is important in many aspects of evolutionary biology, agriculture, animal husbandry, conservation, and human health (Meghji et al. 1984, Charlesworth and Charlesworth 1987, Abplanalp 1990, Bittles et al. 1991, Lacy 1997, Rudan and Campbell 2004, Rojas et al. 2009). Inbreeding depression, for example, strongly affects the evolution of plant mating systems, selecting against self-fertilization and biparental inbreeding (Darwin 1876, Lande and Schemske 1985, Keller and Waller 2002). Inbreeding is an extremely important issue for agricultural systems, as it not only has direct and dire consequences in terms of decreasing yields but also can enhance susceptibility to a suite of pathogens and pests (Burdon and Marshall 1981, Chahal and Gosal 2002). From a conservation perspective, one of the primary goals is to maintain sufficient genetic variation that populations are able to respond evolutionarily (adapt) to changing environmental conditions (Franklin 1980). As populations become smaller and increasingly isolated as a result of the direct and indirect effects of human activities (including the loss and degradation of habitat, the introduction of alien species, pollution, and the overexploitation of species), they tend to lose genetic variability due to genetic drift. In particular, random sampling of successively smaller subsets from generation to generation can mimic the effects of nonrandom mating, increasing the inbreeding coefficient, which can in turn manifest itself as a loss in individual fitness (Heschel and Paige 1995). Inbreeding is also common in some human populations and can lead to serious health issues. For example, among Saudi Arabian provinces, 52% to 68% of all unions are consanguineous (i.e., members have a recent common ancestor), with first-cousin marriages being the most frequent. The major harmful effects of inbreeding are a higher frequency of autosomal recessive diseases and an increased frequency of morbidity and mortality (El-Hazmi et al. 1995, Rudan and Campbell 2004).

Genetic basis of inbreeding depression

Following the rediscovery of Mendelian genetics, two main hypotheses were formulated to account for the existence of inbreeding depression and heterosis (gain in fitness following outbreeding; reviewed in Wright 1977, Charlesworth and Charlesworth 1999). The overdominance hypothesis (East 1908) argues that heterozygosity confers properties that are superior to either homozygote. The loss of heterozygosity through inbreeding will thus decrease the mean value of traits associated with fitness and lead to inbreeding depression (Lynch and Walsh 1998). The (partial) dominance hypothesis (Davenport 1908), on the other hand, argues that most mutations are neutral or deleterious and generally recessive (MacKay 2001). Increasing the proportion of homozygotes through inbreeding will raise the probability of unmasking these deleterious alleles (Charlesworth and Charlesworth 1999), leading to inbreeding depression (Keller and Waller 2002). Which of the two hypotheses explains most of the decline in fitness associated with inbreeding is still being debated (Ritland 1996, Kärkkäinen et al. 1999, Roff 2002). Although evidence exists to support both models (Hughes 1995, Li ZK et al. 2001, Carr and Dudash 2003), the dominance hypothesis appears to be favored on the basis of available empirical data and on theoretical grounds (Charlesworth and Charlesworth 1999). Others have suggested that inbreeding depression may also be explained, at least in part, by synergistic or epistatic interactions among genes (Templeton and Read 1994, Charlesworth 1998).

Molecular approaches to inbreeding depression

Although inbreeding depression has been a central theme in biological research for more than a century, little is known about its underlying molecular basis. For example, we have no idea how many loci may be involved in causing inbreeding depression of fitness and its components. The number may well be tens, hundreds, or even thousands (Frankham et al. 2002). The genes or gene pathways associated with inbreeding depression are also unknown. Furthermore, the underlying cause of inbreeding depression, overdominance or partially recessive deleterious alleles, is still debated.

For several decades, molecular genetic approaches to inbreeding depression have relied predominantly on the use of neutral markers (i.e., those that are nonfunctional or those that are not under selection, such as RFLPs [restriction fragment length polymorphisms] or microsatellite markers) for assessing levels of genetic variation (heterozygosity). Using RFLPs, one can detect genetic variation among individuals by cutting each individual's DNA with a specific restriction enzyme, subjecting those DNAs to electrophoresis in agarose gels, and transferring them to charged nylon membranes by use of Southern blots. Nylon membranes are then hybridized to radioactively or biotin-labeled clones (i.e., specific pieces of DNA). Useful clones will be those showing polymorphism (i.e., variation in the size of the restriction fragment) between two or more individuals. Microsatellites are di-, tri-, and tetranucleotide repeats found throughout the genomes of most organisms, varying in size from 10 to 100 repeats, and are present roughly every 1000 base pairs. Once identified, microsatellites are amplified using the polymerase chain reaction and sized using gel electrophoresis or an automated sequencer. Microsatellites owe their variability to an increased rate of mutation compared with other neutral regions of the DNA. High rates of mutation can be explained predominantly by slippage during DNA replication. Each of these techniques allows the detection of homozygous and heterozygous loci for each DNA sample. For example, from a conservation perspective, populations of varying size could be compared for levels of genetic variability. Small populations with low levels of variation very likely have the highest risk of inbreeding depression and have a high risk of experiencing continuing losses of genetic variation resulting from perennial bottlenecks and genetic drift (Wayne and Morin 2004).

More recently, neutral markers have been used for mapping quantitative trait loci (QTL, or stretches of DNA that are

closely linked to the genes that underlie the trait in question) in an effort to understand the molecular basis of inbreeding depression (Carr and Dudash 2003). However, quantitative genetic approaches fall short; they alone have not been useful for directly identifying genes contributing to variation in inbreeding depression, assessing allelic effects, estimating the number of loci involved, or determining whether some loci within the genome exhibit strong overdominance.

With the generation of vast amounts of DNA sequence information and the advent of complementary DNA (cDNA) microarrays (Schena et al. 1995), we can now describe biological processes from a total genetic perspective (White et al. 1999). Microarrays allow the examination of the expression of all genes within the genome at once, permitting analysis of an organism's response to any biological phenomenon of interest. Experimental comparisons enable one to not only better assess the behavior of genes previously implicated in a given process but also to assist in the discovery and identification of genes and gene pathways associated with particular biological phenomena (Harmer and Kay 2000). In the case of inbreeding depression, changes in the multigene patterns of expression can provide clues about the number of genes involved, their modes of action, and the biochemical pathways leading to such effects. This newly founded study of gene-expression patterns is commonly referred to as "functional genomics" (Amundson 2008). Functional genomics can be defined as a field of molecular biology that makes use of the vast wealth of data produced by genome sequencing projects to facilitate our understanding of gene function and gene interaction. In this review, I specifically address the following issues using microarrays: 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? What is the underlying cause of inbreeding depression, overdominance or partially recessive deleterious alleles? How does environment influence gene expression associated with inbreeding and inbreeding depression?

Microarrays and gene expression

Microarray methods are an extension of standard nucleic-acid hybridization procedures (Northern and Southern blots) that enable the simultaneous monitoring of messenger RNA (mRNA) levels for thousands of genes among differing samples. The two most common technologies are cDNA microarrays and oligonucleotide expression arrays. The rationale behind microarray technology is to hybridize a sample of mRNA (that has been extracted, reverse transcribed to cDNA, and fluorescently labeled) to a set of gridded DNAs on a solid support (glass slide or a nylon membrane). The DNAs normally used are short cDNA sequences of expressed genes (called expressed sequence tags, or ESTs), or short (25-mer, Affymetrix chips) or long (70-mer) oligonucleotides representing distinct transcribed protein-coding genes. More recently, researchers developed long-oligonucleotide bead-based arrays (50-mer, Illumina BeadArrays with a 29-mer molecular address; Gunderson et al. 2004), which are gaining popularity. Affymetrix uses multiple probes for each gene, whereas Illumina arrays have approximately 30 copies of the same oligonucleotide on the array, providing an internal technical replication that Affymetrix lacks. Nonetheless, experimental comparison of the performance of Affymetrix and Illumina gene-expression platforms gives comparable and repeatable results (see Barnes et al. 2005). The thousands of simultaneous hybridization reactions occur under stringent conditions that enable preferential binding of each sample cDNA with its homologous DNA sequence on the chip or membrane. The level of hybridization detected is assumed to be proportional to the amount of that mRNA species in the sample assayed (i.e., the level of gene expression; Ranz and Machado 2006).

Ultra-high-throughput sequencing (e.g., Illumina sequencing) is also emerging as an attractive alternative to microarrays for studying mRNA expression levels (Marioni et al. 2008, Shendure 2008). This sequencing technology uses massively parallel Sanger sequencing reactions to simultaneously sequence millions of short fragments of DNA (e.g., 32-base-pair [bp] regions). All sequence reads are aligned against the whole genome using the Illumina-supplied algorithm ELAND, which is designed to be particularly efficient for 32-bp reads. The overall expression of each gene is calculated using the number of reads mapping to exons within each gene. Comparisons to the expression patterns of Affymetrix chips using the same RNA samples (human liver and kidney, unrelated to inbreeding or inbreeding depression) showed that these two independent measures are highly correlated (a Spearman correlation of approximately 0.73). Sequencing data, however, allowed greater detection of genes with low levels of expression, and the detection of novel transcripts and alternative splice variants (see Marioni et al. 2008 for details), all of which would be useful in uncovering genes responsible for inbreeding depression.

Inbreeding and inbreeding depression: Gene number and function

To understand the molecular basis of inbreeding depression, we first assessed variation in gene expression across experimentally inbred lines of *Drosophila melanogaster* using commercially available oligonucleotide microarrays of Affymetrix (the *Drosophila* GeneChip Array, *www.osa.sunysb.edu/udmf/ArraySheets/drosophila2_datasheet.pdf*; Kristensen et al. 2005, 2006, Ayroles et al. 2009). Choosing to work with *Drosophila* has many advantages for genome analysis. Most important, the full-genome sequence has been published (Adams et al. 2000). *Drosophila* is also one of the model systems for identifying genes and gene function. Overall, the *Drosophila* genome is relatively small, estimated to contain more than 14,000 protein-coding genes (Lin et al. 2007). In addition, more than two-thirds of the known protein-coding genes have been assigned to functional categories including immunity, gene regulation, development, metabolism, transport, behavior, and DNA replication and

repair (Adams et al. 2000, Lin et al. 2007). Thousands of research papers have examined the physiology, development, biochemistry, and genetics of this organism. Thus, *Drosophila* offers a unique opportunity for conservation biologists, ecologists, and evolutionary biologists to make use of these data to understand the genetic basis of traits under study. Furthermore, the majority of what we currently know about inbreeding and inbreeding depression comes from studies of *Drosophila* (Charlesworth and Charlesworth 1999).

In the first whole-genome study on how inbreeding (but not inbreeding depression per se) affects gene expression, Kristensen and colleagues (2005) compared lines inbred to the same level (F = approximately 0.67) at different rates (fast versus slow) to those of noninbred lines of *D. melanogaster.* Kristensen and colleagues wanted to know which genes and gene pathways were affected by inbreeding, and whether differences in the effects of inbreeding may be determined in part by the rate of inbreeding. If the effective population size is dramatically reduced, then sudden and extreme inbreeding will result, with selection having a minor effect and random fixation predominating. Slower inbreeding may cause less inbreeding depression, given that more generations are available for selection to act, purging deleterious recessives.

The results of this study uncovered a total of 466 genes that were differentially expressed among inbred and noninbred lines. Functional categorization of these genes and an assessment of whether they were overrepresented (using the Gene Ontology database and EASE, expression analysis systematic explorer, in the program DAVID, respectively; Dennis et al. 2003, Hosack et al. 2003) indicated a disproportionate involvement of stress resistance and metabolism in inbreeding. For example, stress-response genes coding for molecular chaperones such as Hsp60 (AFFYID: 15203_at), Hsp83 (AFFYID: 143198_at), and the heat-shock protein cognate 1 (AFFYID: 143191_at) were differentially up-regulated in one or both of the inbred treatments. Heat-shock proteins are part of the cellular stress response and are known to positively affect survival (Feder 1999, Farkas et al. 2000), playing an important role in protein-protein interactions by helping to maintain proper protein conformation. In addition, a number of genes coding for antibacterial peptides were up-regulated with inbreeding. These included Diptericins (AFFYID: 143443_at; AFFYID: 147473_at), Defensin (AFFYID: 143607_at), and Thor (AFFYID: 153432_at), all of which have well-described antibacterial functions (Bulet et al. 1999, Beutler 2003, Ganz 2003). The large representation of genes involved in defensive responses to biotic agents is consistent with what we know of inbreeding and inbreeding depression (Kristensen et al. 2006); inbred organisms are often more susceptible to environmental challenges (e.g., Hedrick and Kalinowski 2000, Keller and Waller 2002). Lower metabolic efficiency (up-regulation of metabolic genes) in inbred individuals may play a key role in explaining inbreeding depression; that is, leaving less energy for reproduction due to higher metabolic costs associated with genetic stress (e.g., the up-regulation of heat-shock proteins, energy metabolism, sucrose metabolism, and amino acid production). In particular, Kristensen and colleagues (2006) showed that inbreeding results in increased adenosine triphosphate and nicotinamide adenine dinucleotide phosphate production, indicative of increased metabolic demand (e.g., 38 genes were significantly differentially up-regulated in the glycolytic pathway). Consistent with this interpretation, Myrand and colleagues (2002) showed that energy metabolism (oxygen consumption) is significantly higher in homozygous individuals when compared with heterozygous individuals.

Surprisingly, comparisons between fast- and slow-inbred lines showed no significant differences in gene expression, despite the expectation that slow inbreeding should be less deleterious and cause changes in gene expression between the two inbred treatments. However, more gene transcripts were differentially expressed between noninbred and slow-inbred lines (362 genes) than between noninbred and fast-inbred lines (171 genes). One possible explanation for the observed difference in gene number is the higher variance in gene expression within the fast-inbred treatment. Between-line variance is expected to increase with the severity of inbreeding (Lynch and Walsh 1998); this experiment was the first to show this on the level of whole-genome expression (Kristensen et al. 2005). Most genes that were differentially expressed with either fast or slow inbreeding also responded in the same direction. Additionally, the overlap between genes being differentially expressed under both types of inbreeding when compared with noninbred controls was found to be greater than would be expected by chance (67 genes, 50 up-regulated and 17 down-regulated; Kristensen et al. 2005).

In a more recent study, Demontis and colleagues (2009), using single-nucleotide polymorphisms (SNPs) in DNA coding regions of slow- versus fast-inbred lines, demonstrated that slow inbreeding maintains more genetic diversity than fast inbreeding. Demontis and colleagues used 40 SNPs known to be contained within genes that are differentially expressed between inbred and outbred individuals (see Kristensen et al. 2005, 2006). In their study, populations that took 19 generations (slow lines) rather than one generation (fast lines) to reach the same level of inbreeding maintained 10% higher levels of allelic richness and 25% higher levels of heterozygosity (Reed 2009). The increased heterozygosity in the slow-inbred lines is attributed to the favoring of heterozygous individuals over homozygous individuals by natural selection, either by associative overdominance or balancing selection, or a combination of the two (Demontis et al. 2009).

In a study similar to Kristensen and colleagues' (2005) on inbreeding, Ayroles and colleagues (2009) used third-chromosome substitution lines of *D. melanogaster* to assess the genetic basis of inbreeding depression. 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 were identical across

lines. Consequently, genetic variation between lines was due solely to variation naturally found in the third chromosome, encoding approximately 40% of the genome.

Ayroles and colleagues (2009) assessed the degree of inbreeding depression among lines by measuring male competitive reproductive success (MCRS), providing an average assessment of reproductive success of each male genotype. Male reproductive success was assayed under "competitive" conditions wherein inbred experimental males had to compete with ebony males to gain access to ebony females. This approach mimics the conditions under which normal reproduction takes place, and is a commonly accepted proxy for male fitness in fruit flies (Drnevich et al. 2004). Ebony is a recessive mutation; only homozygous individuals express the ebony phenotype, allowing one to differentiate between offspring sired by the ebony males and experimental males. Each competitive assay lasted seven days, during which males were allowed to mate with females; after seven days the experimental males were flash frozen and collected for RNA extraction and microarray analysis. Ayroles and colleagues (2009) assessed inbreeding depression by comparing MCRS in the inbred isogenic genotypes with that of an "outbred" genotype (i.e., they were heterozygous for genes on the third chromosome but identical and homozygous for all other chromosomes, given all lines shared the same genetic background for all but the third chromosome) generated from crosses among all six isogenic lines. For example, isogenic line A was used as a parental line to produce several different outbred genotypes; the reproductive success of line A would be compared with the mean reproductive success of all heterozygous lines for which A was a parent, using the following equation:

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

where ID is the degree of inbreeding depression, W_A is the MCRS of the inbred line, and W_{Aj} is the mean MCRS of the outbred crosses involving line A (Charlesworth and Charlesworth 1999).

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 a 24% reduction in fitness to a high of a 79% reduction in fitness among the six inbred lines. From the microarray analysis, Ayroles and colleagues (2009) uncovered a total of 567 genes that were differentially expressed among these six inbred lines. We summarized gene-expression results in a two-way hierarchical clustering (figure 1) illustrating two significant line clusters, separating high inbreeding depression lines from low inbreeding depression lines. Clustering distinguishes genes that are differentially up- or down-regulated in high inbreeding depression lines from those in low inbreeding depression lines, gene by gene. Interestingly, these results would allow one to predict relative fitness from expression profiles alone. Expression results were similar to those uncovered by Kristensen and colleagues (2005); enrichment

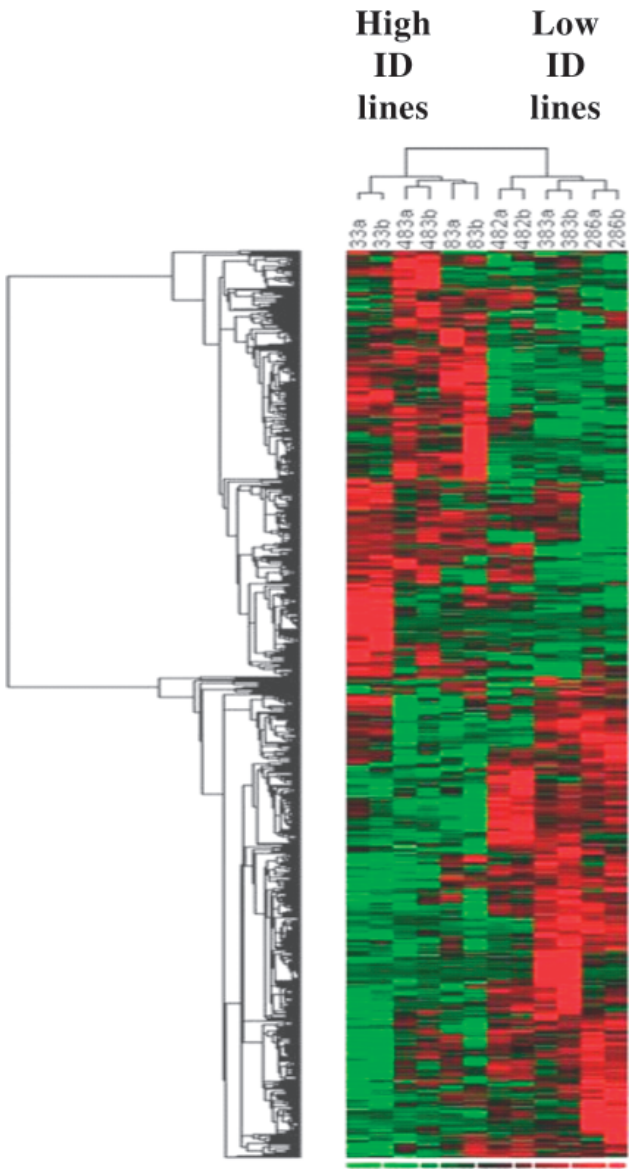

Figure 1. Hierarchical clustering of gene expression by line. Transcript abundance is represented from high (red) to low (green) with lines and array replicates shown in columns and genes shown in rows. Clustering distinguishes high inbreeding depression (ID) lines (83, 33, and 483) from low ID lines (286, 383, and 482). Note the high degree of similarity among low ID lines and among high ID lines, and the dissimilarity between low and high ID lines. Source: Ayroles and colleagues (2009).

analysis revealed significant overrepresentation of genes involved in metabolism (e.g., amino acid and carbohydrate metabolism), stress (e.g., heat-shock proteins), and defense (primarily to bacterial and fungal infections).

The large number of differentially expressed transcripts (567) can be interpreted in three ways: (1) inbreeding depression is the product of a relatively large number of genes of small effect, rather than a few genes of large effect; (2) inbreeding depression is caused by a few key genes that

affect the expression of many other genes (e.g., epistatic effects on gene expression); or (3) inbreeding depression is caused by a relatively large number of genes of small effect that also affect the expression of many other genes (e.g., epistatic effects on gene expression). When Ayroles and colleagues (2009) assessed the proportion of genes differentially expressed across inbred lines by chromosomal location, they found that 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). Since these lines were variable only for the third chromosome (see above), any variations found on the second, fourth, and X chromosomes had to originate from genes located on the third chromosome and were therefore products of *trans*-regulation or epistatic interactions. Such *trans*-regulatory effects suggest that a significant amount of inbreeding depression could be due to a relatively small number of genes (i.e., less than the 567 uncovered) that are causing cascading effects within affected gene pathways (e.g., genes affecting metabolism).

Recent studies by Hughes and colleagues (2006) suggest that *cis*-regulation accounts for approximately 51% of the genetically variable transcripts (on chromosome three in this case), on the basis of the proportion of genes that are *trans*-regulated on chromosomes X and two. *Cis*-effects, however, account for more genetic variation (70%) in gene expression than do *trans*-effects; consequently, *cis*-effects are larger than *trans*-effects. Meiklejohn and colleagues (2003) pointed out that 35% to 80% of QTLs that influence expression of a gene map to the gene itself in studies of yeast, mice, and maize, suggesting *cis*-regulation. Furthermore, the fraction of *cis*-acting genetic factors increases with more stringent statistical cutoffs (Meiklejohn et al. 2003, Schadt et al. 2003), suggesting that large changes in expression may be related to *cis*-effects, whereas *trans*-effects are more often of smaller effect.

Ayroles and colleagues (2009) also assessed the number of genes held in common across the three high inbreeding depression lines and found 46 genes. These genes include those associated with metabolism and oxidative stress and may represent genes associated with inbreeding depression (i.e., genes that are likely indirectly affected by cascading affects on the pathway—it is unlikely that all 46 genes result from the unmasking of deleterious recessives in all three high inbreeding depression lines). For example, one gene held in common is a glutathione transferase important in resisting lipid peroxidation. This gene is down-regulated in the highly inbred lines, making them more susceptible to oxidative (cellular) damage.

Ultimately, studies beyond those of microarray analyses will be required to differentiate between models of gene action (i.e., many genes of small effect, a few genes of large effect, or some combination of the two extremes) and the relative importance of each gene to fitness. Nonetheless, our first look at whole-genome interactions upon inbreeding and its consequent fitness effects is a step in the right direction.

Inbreeding depression: Mode of action

Ayroles and colleagues (2009) also assessed the mode of gene action associated with inbreeding depression (whether gene expression is additive, dominant, or overdominant; see figure 2). They generated a set of three "outbred" lines (recall that the lines differ with respect to allelic variation for genes on the third chromosome; all other chromosomes were genetically identical) by crossing the three homozygous high inbreeding depression lines to each other and contrasting gene expression between these parental inbred lines and their F1 hybrid offspring showing heterosis for the third chromosome.

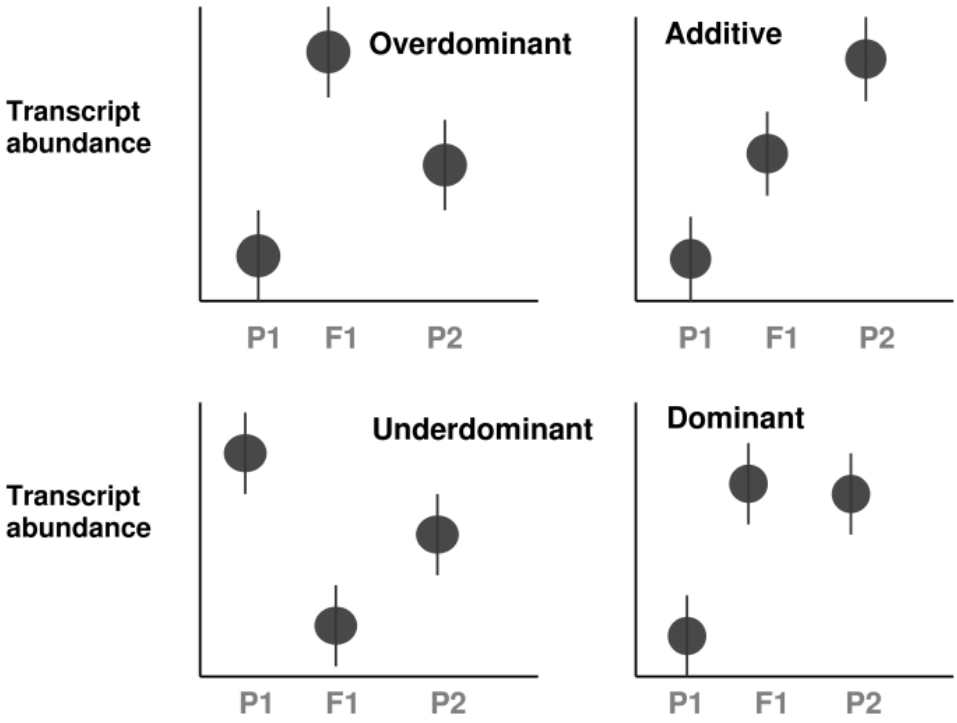

Figure 2. Mode of action for additive, dominant, underdominant, and overdominant gene expression. Comparisons are for inbred homozygous parental lines (P1 and P2) versus an F1 heterozygous cross between the two parental lines. Expression is additive if the F1 is intermediate between the two parents, dominant if one of the parents has the same expression as the F1, and over- or underdominant if the expression is greater than or less than the two parental lines. Bars represent ±1 standard error of the mean.

Results indicated that the expression of approximately 75% of all differentially expressed genes associated with inbreeding depression were additive or dominant, and about 25% of all of these genes expressed patterns of overdominance. This was unexpected, given that overdominance for expression is thought to be rare (Gibson et al. 2004). Experimentally comparable studies by Gibson and colleagues (2004) and Hughes and colleagues (2006) found fewer genes showing significant patterns of overdominance (< 5% of genes) in *Drosophila*. The difference appears to be a result of more stringent statistical criteria and cutoffs; whether these differences among studies are of biological significance is open to question. Both genetic mechanisms may contribute to inbreeding depression, although expression studies are correlational and do not translate directly to cause and effect. Furthermore, it is important to point out that only a miniscule fraction of the genome has been sampled in the majority of studies on inbreeding depression to date, making any general mechanistic interpretation premature (Ayroles et al. 2009).

Inbreeding by environmental interactions

Kristensen and colleagues (2006) conducted the first whole-genome study to assess the independent and interactive effects of inbreeding, inbreeding depression, and environmental stress (high temperature) in male *D. melanogaster*. They compared inbred lines (F approximately 0.67) with those of outbred lines of *D. melanogaster* after exposure to nonstressful and stressful (25 degrees Celsius [°C] versus 36°C) environmental conditions. With a conservative false discovery rate of 5% used in this study, 12 genes were differentially expressed between inbred and outbred lines of *Drosophila* under nonstressful conditions (temperatures of 25°C); all 12 genes were involved in metabolism. Following exposure to a high temperature (36°C), 176 individual genes were differentially expressed between inbred and outbred lines, including those associated with metabolism and heat shock (i.e., heat-shock proteins). The fact that more genes are differentially expressed between inbred and outbred lines under stressful conditions is clear evidence for genotype by environmental interactions at the transcription level. These genes represent potentially important candidates for explaining conditionally dependent inbreeding depression (Kristensen et al. 2006).

Kristensen and colleagues (2006) also found that the majority of differentially expressed genes were up-regulated in inbred lines compared with noninbred lines under temperature stress. This finding supports the hypothesis that homozygous individuals exposed to heat stress require more energy, and that the better performance of heterozygous individuals derives from reduced energy expenditure in the maintenance of metabolism. Further support comes from the fact that these inbred lines are less heat resistant and have lowered fitness compared with noninbred lines (Pedersen et al. 2005, Kristensen et al. 2006).

In both environments the within-gene variance in expression levels in the inbred lines was higher than within both the noninbred lines and in the heat stressed lines (both inbred and noninbred) than the nonstressed lines. Increased genetic variation in expression is expected given the randomness associated with drift (unmasking of deleterious alleles and the breakdown of heterosis) among different lines (Falconer and Mackay 1996, Kristensen et al. 2006). Assuming a high correlation between gene expression levels and phenotype, from an evolutionary perspective, population differentiation may occur at a faster rate under harsh environmental conditions independent of inbreeding and drift (Kristensen et al. 2006). Overall, results show that inbreeding and heat stress greatly affect the genes associated with metabolism, and that these two distinct types of stresses synergistically affect gene-expression patterns.

Generalized stress response

Interestingly, results similar to Kristensen and colleagues (2005) and Ayroles and colleagues (2009) were uncovered in a study by Landis and colleagues (2004) on the effects of aging and oxidative stress in outbred lines of *D. melanogaster*; enrichment analysis revealed significant overrepresentation of genes involved in metabolism (e.g., amino acid and carbohydrate metabolism), stress (e.g., heat-shock proteins), and defense (primarily to bacterial and fungal infections). Landis and colleagues (2004) observed 913 genes that were differentially expressed with aging and 593 genes that were differentially expressed with oxidative stress. Kristensen and colleagues (2005) tested whether observed overlap was higher than expected among inbreeding, aging, and oxidative stress treatments and showed that the overlap was significantly higher in all comparisons. Overall, 34 genes were differentially expressed across all three treatments; these included metabolic genes (e.g., adenosine [AFFYID: 143062_at] and NAD-dependent methylenetetrahydrofolate dehydrogenase [AFFYID: 151767_at]) and stress and defense response genes (e.g., Hsp83 [AFFYID: 143198_at] and Diptericin [AFFYID: 147473_at]).

Similar results were also recently uncovered in Antarctic fishes; many up-regulated genes coded for proteins that respond to environmental stress such as heat-shock proteins, antioxidants (that alleviate oxidative damage to cells), lipid metabolism, and innate immunity (Zuozhou et al. 2008). Thus, these results suggest that these genes may be candidates for stress resistance in general, acting to maintain homeostasis in organisms exposed to diverse stresses (Kristensen et al. 2005).

Summary and implications

It is clear that inbreeding depression is associated with a large number of gene transcripts, primarily genes associated with metabolism, stress, and defense. Genetic drift alone is an unlikely explanation for these patterns given the reasonably large number of replicates within and across several studies that have repeatedly uncovered the same genes and gene pathways. Whether there is a large number of genes of small effect or a few key genes that affect the expression of many other genes (i.e., epistatic effects) is unclear.

However, when the proportion of genes differentially expressed across inbred lines by chromosomal location were assessed, approximately two-thirds of the differentially expressed genes were on the third chromosome, and approximately one-third of the differentially expressed genes were on the second, fourth, and X chromosomes. Since these lines were variable only for the third chromosome, variation found on the second, fourth, and X chromosomes had to originate from genes located on the third chromosome, and are therefore products of *trans*-regulation or epistatic interactions. Thus, results suggest that a significant amount of inbreeding depression may be due to a smaller number (i.e., less than 567) of genes that in turn affect other genes.

Overall, these studies have focused primarily on male attributes of fitness; it remains to be seen whether patterns uncovered here will hold true for traits associated with other components of fitness (e.g., female side of fitness). Interestingly, a comparison of male and female fitness components in six inbred lines of *D. melanogaster* showed that males had markedly reduced mating ability while female viability and fecundity were less affected (Miller and Hedrick 1993). Furthermore, a number of studies have shown gender-related differences in gene expression in genes shared by both sexes (Connallon and Knowles 2005, Ellegren and Parsch 2007).

Microarray studies have also enabled us to address issues concerning the interactive effects of multiple stressors. The combinatorial stress effects of inbreeding and inbreeding depression and temperature led to the differential expression of a larger number of genes than either stressor alone; clear evidence for genotype by environment interactions at the level of transcription. Furthermore, there was greater variance in gene expression for inbred and temperature-stressed lines—from an evolutionary perspective, population differentiation may occur at a faster rate under harsh environmental conditions independent of inbreeding and drift. Additionally, genes were up-regulated in inbred lines compared with noninbred lines under temperature stress. The fact that these genes are involved primarily in metabolism may explain why fitness is lower in inbred lines, which shunt energy to maintenance rather than reproduction. Moreover, genes and gene pathways uncovered in the studies discussed above respond to inbreeding and inbreeding depression at the molecular level in much the same way as they do to other environmental or physiological stressors such as oxidative stress, aging, and temperature, demonstrating a generalized stress response that affects fitness.

Results from these studies may also shed light on specific issues important to evolution and conservation. One of the central questions concerning inbreeding depression in conservation and evolutionary biology relates to the interplay between selection and drift: Can deleterious alleles causing inbreeding depression be removed (purged) efficiently enough by natural selection to reduce the impact of increased inbreeding on population viability? The operation of purging through natural selection depends in part on the magnitude of allele effects; purging is highly effective for alleles with large effects (e.g., lethals) but less so for alleles of small effect (Frankham et al. 2002). Of course, although purging cannot reduce the fraction of the mutational load that is due to true overdominance, it can purge the fraction that is due to dominance (unmasking of deleterious recessives). If, however, there is a reasonably large number of genes contributing to inbreeding depression, then purging might not be very efficient given that so many loci are involved. This could explain, in part, why deleterious recessive genes are maintained in nature and purging is not as common as one might theoretically predict.

Although most genetic models do not currently predict the effect of heterozygous advantage on the rate of loss of genetic variation in small populations, Lesica and Allendorf (1992) estimated expected rates of loss of heterozygosity under different selective intensities and population sizes using computer simulations. Overall, their results indicate that heterozygous advantage would maintain larger levels of genetic variance within populations of small size (< 100). Thus, if overdominance were an important mechanism (having a fitness advantage at the loci examined), heterozygous advantage would slow the loss of genetic variation caused by drift more than 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.

Limitations of microarrays

The "list of genes" resulting from microarray data should not be viewed as an end in itself; it is a first step toward uncovering genes responsible for the traits or patterns of interest. As Feder and Mitchell-Olds (2003) pointed out, "Transcription profiling should be regarded as exploratory data analysis in advance of the manipulative experiments that are needed to provide rigorous verification" (p. 650). Certainly, the results of the microarray studies described above lead to a number of hypotheses ripe for testing. It will also be important to broaden our perspective. To date, microarray studies of inbreeding and inbreeding depression have focused on solely *Drosophila*. It will be important to expand these studies not only to other model organisms but more importantly to nonmodel organisms that we can study under natural conditions in the field. Recently developed next-generation sequencing technologies such as 454 pyrosequencing certainly hold promise for making genome and transcriptome sequences available for more projects and more nonmodel organisms (Hudson 2008). The impact of next-generation sequencing is already being felt in taxa distantly related to the classic molecular model organisms (see e.g., Toth et al. 2007).

Where to from here?

In combination with high-density genotyping and QTL or association mapping, we now have the tools for discovering the specific genetic determinants of inbreeding depression

(Ayroles et al. 2009). One potential approach is to identify candidate genes by combining microarray data for variation in inbreeding depression with QTL mapping studies using recombinant inbred lines of *Drosophila*. A suite of recombinant inbred lines would be developed from a cross between a high inbreeding depression line and a low inbreeding depression line to ultimately segregate the genetic variation associated with inbreeding depression. QTL mapping allows the discovery of stretches of DNA that are closely linked to the genes that underlie inbreeding depression in these recombinant inbred lines. Microarray data from inbreeding depression studies, in combination with deficiency mutants (DNA deletions), could then be used to uncover candidate genes found within each QTL region (Wayne and McIntyre 2002). To confirm the role of candidate genes in inbreeding depression validation studies such as RNA interference or transformations through gene targeting by homologous recombination will be necessary (see, e.g., Rong and Golic 2000).

Instead of QTL mapping, genomewide association mapping (also known as linkage disequilibrium mapping) has emerged as a tool for uncovering the genetic basis of phenotypic variation such as inbreeding depression. Rather than looking for marker-trait (QTL) associations in a population with known relationships (e.g., offspring of an experimental cross), we look for associations in the population of "unrelated" individuals. Because unrelated individuals are always related at some distance, phenotypically similar individuals may be similar because they share identical alleles inherited through descent—alleles that will be surrounded by short ancestral marker haplotypes that can be identified in genomewide scans. Association mapping has two main advantages over traditional linkage mapping methods. First, no crosses are required, and second, association mapping has greater resolution than linkage mapping because the extent of haplotype sharing between unrelated individuals reflects the action of recombination over many generations. The drawbacks of association mapping stem from the fact that it is an uncontrolled experiment and it is difficult to predict the number of false positives as a result of spurious correlation (Aranzana et al. 2005).

Another approach would be to gain an understanding of the physiological and biochemical effects of inbreeding depression. Metabolomic studies would enable one to assess which fundamental metabolic processes are modified by inbreeding depression, given that the underlying effects of inbreeding depression at the genomic level relate to metabolism. The metabolome is the complete set of small-molecule metabolites (such as metabolic intermediates, secondary metabolites, hormones, and other signaling molecules) within an organism. Pedersen and colleagues (2008) recently conducted just such a study using nuclear magnetic resonance (NMR) spectroscopy. This technique allows the detection of metabolites without any initial separation step (such as gas chromatography or high-performance liquid chromatography). Thus, all kinds of small-molecule metabolites can be measured simultaneously; in this sense, NMR spectroscopy is close to being a universal detector. Pedersen and colleagues (2008) found significant differences in the metabolome between inbred and outbred lines of *D. melanogaster*; in particular, they found inbred lines to have increased levels of maltose and decreased levels of 3-hydroxykynurenine and a galactoside specific to male *Drosophila*. The galactoside, which is transferred from the male accessory glands in the seminal fluid to the female, is thought to be related to mating success and suppression of female receptivity following mating. Thus, lower concentrations may have negative effects on reproduction.

Similarly, a proteomic approach would allow an assessment of the proteins that are differentially affected by inbreeding depression. The proteome is larger than the genome, especially in eukaryotes, in the sense that there are more proteins than genes. This is due to alternative splicing of genes and posttranslational modifications such as glycosylation or phosphorylation. Proteomics will most likely give a much better understanding of the molecular biology of inbreeding depression than genomics, given that the level of transcription of a gene gives only a rough estimate of its level of expression into a protein. An mRNA produced in abundance may be degraded rapidly or translated inefficiently, resulting in a small amount of protein. Expression differences can be detected by two-dimensional gel electrophoresis (e.g., Li H-M et al. 2007), which can also be used to identify posttranslational protein modifications. Small differences in a protein can be visualized by separating a modified protein from its unmodified form.

Only one study has carried out a proteomic approach on the causal effects of inbreeding depression. Pedersen and colleagues (2009) presented a proteomic characterization of a conditional lethal found in an inbred line of *D. melanogaster*. The lethal effect is apparent as a substantial increase in early mortality at high temperatures (29°C) as opposed to normal survival at lower temperatures (20°C), most likely caused by a single recessive major locus. Overall, 45 proteins were found to be significantly differentially regulated in response to high temperatures in this conditional lethal inbred line when compared with both a nonlethal inbred and outbred control line. No proteins were significantly differentially regulated between the inbred and outbred control lines, verifying that differential protein regulation was specific to the genetic defect in the conditional-lethal line. Proteins associated with oxidative phosphorylation and mitochondria were significantly overrepresented within the list of differentially expressed proteins. Dysfunction of mitochondrial function may be a major factor in the high mortality observed in the conditional-lethal line, directly affecting activity and stability of proteins and consequently energy metabolism. This study is of particular significance because the deleterious effects of inbreeding are generally accepted to be more severe in suboptimal or stressful environments, but knowledge of the environmentally induced causative molecular basis is rare.

The technological advances in genotyping, expression profiling, proteomics, and metabolomics have led and

will continue to lead to advances in our understanding of the molecular basis of inbreeding depression and perhaps of more generalized stress responses as well. Our ultimate goal is to devise means for minimizing and reversing the detrimental effects of inbreeding depression. Knowing the details of how genetic variation affects molecular, cellular, and organismal function is essential to understanding inbreeding depression, its evolutionary impact, the conservation of genetic diversity, the probability of extinction, and the susceptibility to pathogen attack and autosomal disease. Lastly, these studies represent a major step toward understanding the relationship between genotype and phenotype, the primary goal of biology in the 21st century.

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Ken N. Paige (k-paige@illinois.edu) is with the School of Integrative Biology at the University of Illinois in Urbana.