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Variation within and among species in gene expression: raw material for evolution

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Abstract

Heritable variation in regulatory or coding regions is the raw material for evolutionary processes. The advent of microarrays has recently promoted examination of the extent of variation in gene expression within and among taxa and examination of the evolutionary processes affecting variation. This review examines these issues. We find: (i) microarray-based measures of gene expression are precise given appropriate experimental design; (ii) there is large inter-individual variation, which is composed of a minor nongenetic component and a large heritable component; (iii) variation among populations and species appears to be affected primarily by neutral drift and stabilizing selection, and to a lesser degree by directional selection; and (iv) neutral evolutionary divergence in gene expression becomes nonlinear with greater divergence times due to functional constraint. Evolutionary analyses of gene expression reviewed here provide unique insights into partitioning of regulatory variation in nature. However, common limitations of these studies include the tendency to assume a linear relationship between expression divergence and species divergence, and failure to test explicit hypotheses that involve the ecological context of evolutionary divergence.

Keywords: comparative biology, evolution, functional genomics, gene expression, genetic drift, microarrays, natural selection

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Introduction

In 1966 extensive protein polymorphism was discerned (Hubby & Lewontin 1966; Johnson *et al*. 1966; Lewontin & Hubby 1966). Since then much attention has focused on determining the relative roles of mutation, drift, and natural selection affecting protein variation (Gillespie 1991). In the decade following the uncovering of extensive protein polymorphism it was proposed that variation in gene control elements, rather than the protein themselves, were likely an important source of adaptive variation (King & Wilson 1975). Until recently, this hypothesis has received relatively little attention (Pierce & Crawford 1997; Ferea *et al*. 1999). However, with the recent advent of microarray technology, genome-wide analysis of regulatory variation has become a common laboratory procedure. The goal of this review is to summarize the extent of variation in gene expression among individuals and taxa uncovered by microarray studies, and to explore the roles of neutral drift and natural selection in accounting for variation. We start with a brief overview of microarrays: their applications and utility. We then proceed in three sections. First, technical sources of variance associated with microarray studies are reviewed. Second, the extent of expression variation among individuals within populations is reviewed. Third, we review the extent of gene expression variation among taxa, and evaluate the evidence for how neutral drift and natural selection interact to govern the evolution of gene expression.

These reviews focus on differences in gene expression measured by microarrays that quantify steady state concentration of a gene's mRNA. It should be noted that mRNA translation, degradation, and protein turnover either expand or contract inter-individual variation emergent at the protein level. It is the active amount of a protein that will effect a phenotypic change, thus microarray measures are proxies for this biochemical parameter.

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Microarrays defined

Microarrays are thousands of 100–250 micron spots of DNA bound to microscope slides in a precise and known pattern

(Ramsay 1998; Schena *et al*. 1998). Each DNA spot quantitatively hybridizes to a specific mRNA so that expression of thousands of individual genes can be measured simultaneously. Importantly, microarray techniques are sensitive: 1.5-fold or less differences in mRNA concentration are typically determined (Gibson & Weir 2005), and each gene/DNA spot has a sensitivity of 15 attomoles (Schena *et al*. 1998) or approximately 1 out of 300 000 transcripts can be measured (Hill *et al*. 2000).

Many studies demonstrate a robust relationship between microarray measures and phenotypic variations, yet one still may question whether microarray studies are worth the investment in time and money. That is, do microarrays provide novel insights not possible through less global approaches? Clearly, for many studies the answer is yes. Microarray studies of gene expression have demonstrated that novel genes define honeybee foraging or nursing behaviour (Whitfield *et al*. 2003), are associated with diseases (Friddle *et al*. 2000; Sorlie *et al*. 2001; Chen *et al*. 2003), and define new insights into well-studied physiological systems such as diabetes (Odom *et al*. 2004) and caloric restriction (Tsuchiya *et al*. 2004). They have elucidated unexpected patterns of gene expression involved in mitochondrial and cytosolic protein synthesis upon physiological stress (DeRisi *et al*. 1997; Eisen *et al*. 1998) and complex interactions among different pathways which explain phenotypic variation (Schadt *et al*. 2003; Oleksiak *et al*. 2005; Segre *et al*. 2005). We would have never guessed that genes involved in the basic assembly of transcriptional complexes would be involved in the physiological response to cyclic temperature change (Podrabsky & Somero 2004). Studies in killifish revealed unanticipated variation in metabolic gene expression and variation in which pathways are 'important' for explaining cardiac metabolism (Oleksiak *et al*. 2005). These novel insights are possible by measuring the expression of many different genes involved in different pathways and biological functions instead of examining the usual characters, traits, or genes. Thus, we would conclude that microarray studies are informative and provide insights not possible by other molecular or genetic means.

Technical variation

With no measurement of error, there can be no basis for statistical inference (Kerr & Churchill 2001). Since differences among mRNA samples are the experimental property of interest, technical replication (or repeated measures within a sample) is necessary. In microarray experiments, technical variance is composed of both random and systematic components.

Random factors contributing to technical variance include variation among replicate spots within a slide hybridization and among slides (Kerr & Churchill 2001; Churchill 2002). Correlation between replicate spots within a hybridization typically exceeds 95%, and decreases to between 60% and 80% with replicate slides (see review in Churchill 2002). Spatially separated replicate spots within a slide increase precision by minimizing variation introduced by scratches or dust or local hybridization effects. However, since correlations are lower among slides than within slides (Jin *et al*. 2001), extensive replication within slides at the expense of replication among slides could underestimate technical variance and artificially inflate differences among individual samples. Random variance can also be increased at the data analysis level by including subtraction of background intensities from spot signal intensities (Qin *et al*. 2004).

Systematic sources of variation include different dyes [correlations between samples labelled with different dyes decrease to below the 60–80% range (Churchill 2002)] and multiple print tips (print group effects). Dye effects are controlled for by including dye swaps in the experimental design (Liang *et al*. 2003). Printing effects are controlled for by data normalization (for example, see Yang *et al*. 2002). Widespread systematic biases can be introduced with different methods for RNA isolation and labelling and with different microarray platforms (cDNAs, oligo, Affymetrix chips), and caution should be exercised when comparing different data sets (Irizarry *et al*. 2005; Larkin *et al*. 2005; Weis & Consortium 2005). However, carefully controlled studies can yield highly concordant results across laboratories (Dobbin *et al*. 2005).

The existence of cell-specific (Bakay *et al*. 2002; Cobb *et al*. 2005) and tissue-specific (Lee *et al*. 2002; Whitehead & Crawford 2005b) expression patterns highlight another source of experimental variation that could artificially inflate differences among individuals in certain studies. If RNA is extracted from whole organisms and there are differences in organ sizes (e.g. one individual has an enlarged liver or larger testes relative to another), differences among individuals may only represent these tissue differences and not specific cellular changes among individuals. The same problem holds if portions of tissues are inconsistently isolated in tissues with cell types heterogeneously distributed across the tissue (e.g. as in liver or kidney).

Technical replication can never substitute for true biological replication. As succinctly stated by Churchill (2002), one cannot study differences in height between the sexes by repeatedly measuring one man and one woman. To distinguish between populations or experimental treatment requires knowledge about biological variation. Pooling samples does not reduce the need for replication. If individual samples must be pooled, then independent biological replication of separate pools is required for statistical inference among groups (Kendziorski *et al*. 2003, 2005). Unlike earlier microarray studies, most journals will no longer accept manuscripts without adequate sampling.

Replication requires more resources and appropriate experimental design can increase the efficiency of resource utilization and optimize statistical power. Reference and balanced are the two basic designs. In reference designs, all experimental samples are labelled with one dye and each cohybridized with a common reference sample that is labelled with a second dye. In balanced designs (such as loops; Oleksiak *et al*. 2002), experimental samples are labelled with both dyes and hybridized to each other. For the same number of slides, twice the number of experimental samples can be included in a balanced design compared to a reference design, leading to improved precision and increased statistical power (Kerr & Churchill 2001). Furthermore, error due to technical variability is highest for reference designs compared to others such as loop and multiple dye-swap designs when using the same number of arrays (Kerr 2003).

Until recently, much of microarray experimentation, especially in the medical sciences, has been applied to distinguish expression profiles among two or three groups. However, in the emerging field of ecological and evolutionary genomics it may increasingly become desirable to compare expression profiles across many groups, such as among many parental and hybrid species or among metapopulations. It should be clear that in these cases, reference designs will become highly inefficient and other existing designs will be most efficient for optimizing resource utilization and minimizing error variance (for reviews on experimental designs see Kerr 2003; Rosa *et al*. 2005).

Variation among individuals

Inter-individual differences in gene expression are seldom reported in microarray studies, likely due to two general reasons. First, it is technically difficult to sample individuals for many model organisms. Samples from flies (*Drosophila* sp.), worms (*Caenorhabditis elegans*), and yeast (*Saccharomyces cerevisiae*) are simply too small to yield enough RNA to measure expression in individuals and samples are pooled. Second, many experimental designs seek only to test for differences among treatments, and choose to replicate samples of pooled individuals to estimate error variance. We would stress that without within-sample measures of variation (denominator of *F*-ratio), among-sample measures of variation (numerator of *F*-ratio) cannot be statistically verified (Sokal & Rohlf 2001).

Inter-individual differences in gene expression have been measured in humans, mice, and fish, and within-population variation has also been estimated for yeast (Table 1). Variation is expected to be minimal between genetically identical individuals and to increase among more distantly related individuals. Variation in gene expression among individuals is characterized within and across inbred lines, among strains of yeast, and among outbred individuals within populations. It is apparent that within-population variation is widespread and that expression variation has a large heritable genetic component (for example, see Stamatoyannopoulos 2004; Gibson & Weir 2005). Even with large variation among individuals, difference among experimental groups, such as disease state, are detectable when applying appropriate experimental and statistical designs.

Variation within and across inbred strains

Pritchard *et al*. (2001) examined expression variation among normal healthy male mice of the same age from an inbred strain. Surprisingly, results indicated a significant minority of genes were differentially expressed among genetically identical individuals; 3.3%, 1.9%, and 0.8% of genes varied in expression among individuals in kidney, testis, and liver, respectively. The authors found that many of the variable genes encoded components of the immune system and others were stress-inducible, indicating that differences in immune status and in the process of sacrificing subjects may be important sources of variability in gene expression experiments.

Because individuals within an inbred strain are nearly genetically identical (Bailey 1982), an inbred strain can be considered equivalent to a single individual, and variation in expression across strains analogous to inter-individual variation within populations. Crosses between strains of inbred lines have revealed extensive segregating variation in expression and high levels of heritability. Approximately one-quarter of genes differed in expression between inbred strains of *Drosophila melanogaster* (Jin *et al*. 2001). Between one-quarter and one-half of all genes were differentially expressed between a laboratory inbred strain of yeast and a wild vineyard isolate (Brem *et al*. 2002). Crosses between these strains indicated high heritability of parental differences; 84% of observed variation was genetic. Similarly, large proportions of genes were differentially expressed between inbred strains and F_2 individuals of resulting crosses in mice (33% of genes) and maize (76% of genes) (Schadt *et al*. 2003).

Yeast within-population variation

Two notable studies examined expression variation in natural isolates of the yeast *S. cerevisiae*. Expression variation was first characterized between phenotypic variants of progeny from a single parental strain (Cavalieri *et al*. 2000), and another study by the same group (Townsend *et al*. 2003) examined variation in expression among four natural isolates (within a population). The comparison among progeny used a dye-swap experimental design and a twofold difference threshold for determining differentially expressed genes. The authors detected 6% of genes differentially expressed

Table 1 Studies of gene expression variation among individuals within taxa

Organism	Citation	Source of error variance	Statistical criteria	Proportion of genes variable within taxa	Proportion of genes variable among populations
Yeast					
Among progeny within strain	Cavalieri *et al*. (2000)	No error variance	Twofold difference	6%	
Among isolates	Townsend *et al*. (2003)	5 replicate slides (dye swap)	95% credible intervals, Bayesian analysis	7%	
Among inbred lines	Brem *et al*. (2002)	6 replicate cultures	$P < 0.005$, Wilcoxon–Mann-Whitney test	25%	
Fruit flies					
Among inbred strains	Jin *et al*. (2001)	6 replicate slides (dye swap), pooled samples	nonzero contribution to variance	~25%	
Maize					
Among inbred strains	Schadt *et al*. (2003)	dye swap	$P < 0.05$ for at least 10% of samples	77%	
Mice					
Within strain	Pritchard *et al*. (2001)	4 replicate slides (dye swap)	$P < 0.05$ for ANOVA	3.3%	
Among offspring or parents of cross	Schadt *et al*. (2003)	dye swap	$P < 0.05$ for at least 10% of samples	33%	
Among inbred strains	Sandberg *et al*. (2000)	2 replicate mice from each strain	> 1.8-fold change	1%	
Humans					
Among related individuals	Cheung *et al*. (2003)	4 replicate slides	s^2 among individuals > s^2 among replicates	Not determined	
Among unrelated individuals	Cobb *et al*. (2005)	Not reported	s^2 among individuals > s^2 among replicates	Not determined	
	Morley *et al*. (2004)	2 replicate slides	s^2 among individuals > s^2 among replicates	42%	
	Whitney *et al*. (2003)	Not reported	Reference design, >twofold change	Not determined	
Fish					
Among unrelated individuals	Oleksiak *et al*. (2002)	4 replicate slides (dye swap) + 2 spots/slide	$P < 0.05$ for ANOVA	18%	2%
	Oleksiak *et al*. (2005)	2 replicate slides (dye swap) + 8 spots/slide	$P < 0.01$ for ANOVA	94%	10%
	Whitehead & Crawford (2005b)	4 replicate slides (dye swap) + 6 spots/slide	$P < 0.05$ for ANOVA*	82%	3%
	Whitehead & Crawford (2005a)	2 replicate slides (dye swap) + 6 spots/slide	$P < 0.05$ for ANOVA	69%	12%†

*Data from Whitehead & Crawford (2005b) re-analysed using nested ANOVA design for purposes of comparison.
†Unpublished data from the study reported in Whitehead & Crawford (2005a).

between progeny with different phenotypes. The comparison of expression patterns among natural isolates (Townsend *et al*. 2003) included five replicate hybridizations per sample and Bayesian analyses that estimated 95% credible intervals. Their analysis detected 7.2% of genes differentially expressed among isolates.

Although both studies (Cavalieri *et al*. 2000; Townsend *et al*. 2003) detected very similar proportions of differentially expressed genes, one would expect a greater proportion of genes differentially expressed among isolates than among progeny of a single strain. One potential explanation is that measures in the progeny comparison experiment were relatively imprecise owing to little technical replication. Lower precision and inclusion of arbitrary thresholds (twofold difference in expression) could lead to a higher false-positive rate than in the among-isolates analysis.

Variation within outbred populations of other species

Variation among individuals within outbred populations has typically been measured in humans and fish, and is consistently high. Cheung *et al*. (2003) measured expression variation among 35 human individuals in lymphoblastoid cells. Although the authors did not perform formal *F*-tests, variation among individuals exceeded variance within individuals for the majority genes (see Fig. 1 in Cheung *et al*. 2003) indicating extensive inter-individual differences in expression. Two other studies examined expression variation in human blood sampled multiple times, but experimental and statistical designs allow for limited scope for inference. Considerably more variation was observed between 17 healthy patients than observed within patients (replicate sampling over a 24-h period) (Cobb *et al*. 2005). Whitney *et al*. (2003) also measured expression variation in leucocytes among individuals and their finding are similar to Cobb *et al*. (2005). From these studies, it appears as if expression variation varies with the following factors: temporal variation within an individual introduces a small amount of variation, variation is intermediate among individuals different in age and relatedness, and high variation among individuals is introduced with trauma and among genders. Application of more sophisticated experimental designs and statistical tests are necessary to quantify the relative influence of these factors.

Several studies have examined variation within natural populations of the teleost fish *Fundulus*. These studies have included extensive technical replication and applied nested analysis of variance (ANOVA) to uncover widespread variation in gene expression among individuals within populations (Oleksiak *et al*. 2002, 2005; Whitehead & Crawford 2005a, b). Technical replication included replicate spots within an array, replicate arrays, and dye swaps. Samples were typically hybridized to each other in a loop design, eliminating the need for a common reference sample. The earliest experiment in *Fundulus* indicated 18% of genes differentially expressed among individuals within populations (Oleksiak *et al*. 2002). More recent studies focusing only on metabolic genes have detected higher variation among individuals within populations; 94% of metabolic genes were different among individuals in Oleksiak *et al*. (2005), 82% in Whitehead & Crawford (2005b) (when data are re-analysed in a nested two-way ANOVA), and 69% in Whitehead & Crawford (2005a). Differences were not due to physiological acclimatization to native habitat (Hochachka & Somero 1984; Prosser 1986) because fish for all studies were raised in a common laboratory setting. Difference in body weight or sex were unlikely sources of significant variation because only postreproductive males were used and body weight does not appear to significantly affect gene expression in *Fundulus* (Oleksiak *et al*. 2005). Instead the authors suggest

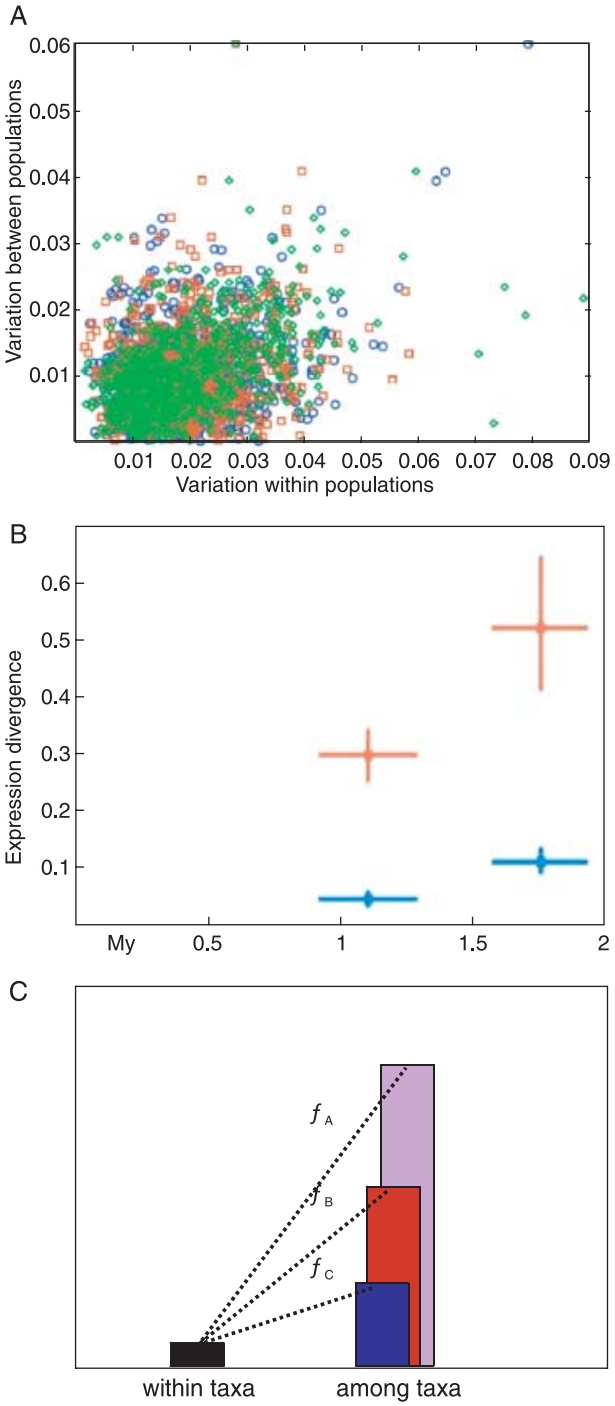

Fig. 1 Gene expression variation with and among taxa. (A) Variation among populations of the fish *Fundulus heteroclitus* is a positive function of within-population variation (Fig. 5 from Oleksiak *et al*. 2002). (B) Genes with high within-species variation in mice (red) tend to diverge faster between species than genes with lower within-species variance (blue) (Fig. 3b from Khaitovich *et al*. 2004). (C) Although variation among taxa should be a function of variation within taxa, the definition of the function (f_A, f_A, or f_C) will depend on gene-specific constraints and phylogenetic distance among taxa (strong, moderate, and weak constraints or small, intermediate, and large phylogenetic distances represented by blue, red, and purple bars respectively).

that much of the variation is more likely due to genetic sources, although this hypothesis needs to be further investigated.

The difference in inter-individual variation detected between the earlier and more recent *Fundulus* studies could reflect differences in the sets of genes assayed or differences in the precision of measures. In Oleksiak *et al*. (2002), 1200 random genes were used, whereas in the more recent *Fundulus* microarray studies (Oleksiak *et al*. 2005; Whitehead, Crawford 2005a, b) only metabolic genes were quantified. A more parsimonious reason for the differences reported would be differences in the precision of gene expression measures. One measure of this precision is the CV_{error}: the standard deviation for the replicate measures divided by the mean. For example, in Oleksiak *et al*. (2002) although 95% of genes had CV_{error} less than 10%, many genes had CV_{error} greater than 5%. Whereas, in Whitehead & Crawford (2005b), 95% of genes had CV_{error} less than 5%. It is important to note that the precision of measurements contributes to determining whether differences among samples can be resolved. As technical sources of variance continue to be minimized, more studies may detect more genes having individual-specific patterns of gene expression.

Sources of inter-individual variation

It is clear that extensive variation in gene expression exists within populations, and variation even exists among individuals within inbred lines. Experimental design and statistical approaches will strongly influence whether or not these differences are detected and accounted for when testing for differences between experimental groups. What factors are responsible for the variation among outbred individuals? It is generally agreed that much of the variation in gene expression for a particular environmental condition is heritable (Stamatoyannopoulos 2004; Gibson, Weir 2005). The genetic basis for differences in microarray measures are most readily shown in studies using different lines or strains of yeast (Ferea *et al*. 1999; Cavalieri *et al*. 2000; Brem *et al*. 2002; Townsend *et al*. 2003), *Drosophila* (Jin *et al*. 2001; Rifkin *et al*. 2003; Nuzhdin *et al*. 2004; Ranz *et al*. 2004), or mice (Pritchard *et al*. 2001). Among humans, studies have demonstrated a greater similarity in microarray measures of mRNA between monozygotic twins, than among dizygotic twins, siblings or unrelated individuals (Cheung *et al*. 2003; Sharma *et al*. 2005; Tan *et al*. 2005). Additionally, microarray and quantitative trait loci (QTL) studies have been combined (gene expression QTLs: eQTLs) to identify both *cis*- and *trans*-acting loci that are related to differences in gene expression in *Drosophila* (Wayne & McIntyre 2002; Wang *et al*. 2004), yeast (Brem *et al*. 2002; Yvert *et al*. 2003; Brem & Kruglyak 2005; Ronald *et al*. 2005), mice (Schadt *et al*. 2003; Chesler *et al*. 2005; Doss *et al*. 2005; Ghazalpour *et al*. 2005), humans (Monks *et al*. 2004; Morley *et al*. 2004; Schadt *et al*. 2005) and trees (Kirst *et al*. 2005).

The eQTL studies in yeast, mice and humans suggest that both *cis*- and *trans*-acting regulators affect patterns of gene expression. In general between 20% and 30% of differential expressions are due to a *cis*-acting eQTL (Doss *et al*. 2005; Ronald *et al*. 2005). However, in mice, 70% of the eQTLs with high LOD scores (> 7.0) were *cis*-acting (Schadt *et al*. 2003). For yeast and mice, *cis*-acting eQTLs were confirmed by allelic specific quantification (Doss *et al*. 2005; Ronald *et al*. 2005). These studies often find a single eQTL near the expression locus of interest. Yet with more powerful analyses, gene expression becomes more complex involving many loci and often a few loci that affect the expression of many genes (Schadt *et al*. 2003, 2005; Monks *et al*. 2004; Stamatoyannopoulos 2004; Brem & Kruglyak 2005; Gibson, Weir 2005). For mice, gene expression was affected by more than one QTL for 40% of the genes with a significant eQTL (Schadt *et al*. 2003; Chesler *et al*. 2005). Similar results are seen in yeast, humans and maize (Schadt *et al*. 2003; Monks *et al*. 2004; Brem & Kruglyak 2005). In yeast, more than five QTLs were mapped to more than 50% of genes with a significant eQTL (Brem & Kruglyak 2005) and multiple eQTL are found in other organisms (Schadt *et al*. 2003; Monks *et al*. 2004). These data suggest a complex regulation of gene expression in which polymorphism among many loci affects the variation in gene expression.

A quantitative measure of the genetic basis for phenotypic variation is h^2 (narrow sense heritability Va/Vp; additive genetic variation divided by phenotypic variation). Significant heritable variation in gene expression is common in the three model systems (yeast, mice and men) even though most of these studies lack sufficient power for detecting QTL because of small sample sizes (de Koning & Haley 2005). In 10 lines of *Drosophila*, 663 of 7886 measured genes (8%) had significant heritability with a median $h^2 = 0.47$ (quartile range 0.39–0.60) (Nuzhdin *et al*. 2004). Among 112 *S. cerevisiae* segregants, 3546 out of 5727 measured genes (62%) had $h^2 > 0.69$ (Brem & Kruglyak 2005). Using lymphoblast human cell lines, among 15 families 762 out of 2430 (31%) of differentially expressed genes had a significant h^2 with a median of 0.34 (Monks *et al*. 2004). Notice that, except for yeast, sample sizes were 15 or less.

Much of gene expression variation measured by microarrays is genetic; expression differs between inbred lines, is associated with QTLs, and has $h^2 > 30\%$. Additionally, it appears that much of the genetic variation is due to many loci (Schadt *et al*. 2003; Brem & Kruglyak 2005) with *cis*-acting polymorphisms affecting approximately 25% of loci (Doss *et al*. 2005; Ronald *et al*. 2005). These data, combined with measures of natural variation in gene expression, suggest that polymorphism in mRNA expression should provide ample material for evolution.

Variation among taxa

In addition to differences among individuals, extensive differences in gene expression can also be detected across demographically distinct groups of individuals such as populations and species (here collectively termed 'taxa'). As is true for other characters that are variable and heritable, gene expression is likely affected by both neutral drift and selection. If variation in gene expression is subjected to neutral drift, then with greater divergence among taxa there will be greater divergence in gene expression (Kimura 1983). In contrast, if variants are subject to natural selection then divergence among taxa tends to increase or decrease depending on native ecological conditions. Here we review studies that quantify variation in expression among populations or species, and the evolutionary forces acting on this variation (Table 2).

Variation among populations

Population comparisons may hold some advantages over species comparisons for examining evolutionary influences on gene expression. One reason is that as phylogenetic distance increases among taxa, divergence in gene expression among groups could become nonlinear and difficult to interpret (see discussion in following section). Another is that as phylogenetic divergence increases, sequence divergence in hybridized probes will confound differences in mRNA concentration when interpreting differential spot signal intensities (Hsieh *et al*. 2003; Karaman *et al*. 2003; Gilad *et al*. 2005). The only vertebrate organism in which among-population within-species variation in gene expression has been examined is in the marine teleost fish *Fundulus*. Oleksiak *et al*. (2002) examined variation in gene expression among three taxa: two populations of *Fundulus heteroclitus*, one from cold northern waters and the other from warm southern waters, and one population of the closely related species *Fundulus grandis* from warm southern waters. Under a neutral drift model, patterns of expression should be most similar among populations within species and least similar between species (Fig. 1). Most genes fit these neutral expectations (Fig. 1A), yet for some genes expression was most similar between southern populations of different species, and most divergent between northern and southern populations within a species. This pattern of expression is indicative of evolution by natural selection.

Although tissue-specific differences in gene expression reflect different metabolic demands, these tissue-specific patterns vary among populations. In *Fundulus*, 76% of metabolic genes were differentially expressed among tissues (Whitehead & Crawford 2005b). Differences among tissues reflected well-established metabolic requirements, suggesting that these measures of gene expression accurately reflect changes in proteins and their phenotypic effects. However, only a small subset (31%) of tissue-specific differences was consistent in all three populations, suggesting that different taxa may achieve the same metabolic ends through regulation of different sets of genes owing to the complex network of genes that can affect metabolism (Cornish-Bowden & Cardenas 1990). These data indicate that expression differences should be verified in more than one population in order to identify patterns that are fundamental to, for example, disease states or mechanisms of toxicity.

To specifically address the adaptive importance of gene expression Whitehead & Crawford (2005a) examined the covariation between gene expression among five populations and an ecologically important parameter: native habitat temperature. *Fundulus* populations are distributed along a steep temperature gradient (Crawford & Powers 1989; Powers *et al*. 1993; Pierce *et al*. 1997; Crawford *et al*. 1999) such that northern populations are approximately 12 °C colder than the extreme southern populations. However, these populations also share a common phylogeny and closely related populations tend to share more similar environments. Thus, clinal variation in gene expression could be due to neutral drift where genetically similar populations have similar patterns of expression or it could be due to adaptive divergence. In order to correct for phylogenetic covariation, genetic distance was accounted for as initially suggested by Felsenstein (1985), then measures of gene expression, now independent of genetic relatedness, were examined for correlation with an environmental gradient. Not surprisingly, phylogeny accounted for much of the variance in expression among populations (Fig. 2A) supporting neutral divergence in gene expression. However, phylogenetically independent variance for some of these genes regressed significantly with the habitat temperature gradient, suggesting adaptive differences. To examine what, if any, other evolutionary forces affected the variation in gene expression along the habitat cline, Whitehead & Crawford (2005a) suggested objective statistical tests to identify genes with expression patterns reflecting balancing and purifying selection. Genes under balancing selection should have higher variation within populations than expected based on among-population variance, and genes under purifying selection should have little variation both within and among taxa. Although many genes were subject primarily to drift, data suggested that natural selection was acting on the expression of 44 out of the 329 metabolic genes (directional selection acting on 13 genes, stabilizing selection acting on 24 genes, and balancing selection acting on 7 genes). These data suggest that divergence among populations is affected by neutral and adaptive differences.

Variation among species

Comparisons among species generally indicate that gene expression variation is a function of phylogeny (Fig. 2),

Table 2 Studies of gene expression variation among taxa

Organism	Citation	Error variance	Proportion of genes variable among taxa	Statistical model	Relative influence of various evolutionary forces			
					Neutral drift	Directional selection	Stabilizing selection	Balancing selection
Fruit flies (*Drosophila* species)	Rifkin *et al.* (2003)	replicate measures for each lineage	up to 42%*	rejection of mutation–drift model; *F*-test	7% of genes	25%	67% of genes	N/T
	Nuzhdin *et al.* (2004)	replicate lines within species	34%	variance among taxa > variance within; *F*-test	majority of genes (N/Q)	many genes (N/Q)	N/Q	N/Q
	Lemos *et al.* (2005)	N/A	N/Q	rejection of mutation–drift model; *F*-test	1% of genes	0% of genes	99% of genes	N/T
Primates (Humans, chimpanzees, macaques)	Enard *et al.* (2002)	N/A	N/Q	correlation b/t variation and phylogenetic distance	dominates for variation in liver and blood	accelerated evolution in brain, but not other tissues	N/T	N/T
	Hsieh *et al.* (2003)	among individuals within species	25% (brain), 35% (liver)	rejection of mutation–drift model; *F*-test	N/T	4% of genes	N/T	N/T
	Khaitovich *et al.* (2004)	N/A	N/Q	correlation b/t within and among-taxon variation, and b/t variation and divergence time; permutation test	N/Q; majority of genes	N/T	N/T	N/T
	Lemos *et al.* (2005)	N/A	N/Q	rejection of mutation–drift model; *F*-test	1–4% of genes	0% of genes	96–99% of genes	N/T
Mammals (Humans and mice)	Yanai *et al.* (2004)	duplicate mouse and human samples	N/Q	cluster analysis	widespread	N/T	N/T	N/T
Mouse species	Lemos *et al.* (2005)	N/A	N/Q	rejection of mutation-drift model; *F*-test	< 1% of genes	0% of genes	100% of genes	N/T
Fish (*Fundulus* populations and species)	Oleksiak *et al.* (2002)	replicate individuals within populations	2%	correlation b/t within and among-taxon variation, correlation b/t variation and phylogenetic distance	N/Q; majority of genes	3% of genes	N/T	N/T
	Whitehead & Crawford (2005a)	replicate individuals within populations	12%	phylogenetic generalized least squares; permutation test	15% of genes	4% of genes	7% of genes	2% of genes

*of genes that vary with development, for comparison between *Drosophila yakuba* and *Drosophila melanogaster* Samarkand strain.
N/A, not applicable; N/Q, not quantified; N/T, not tested, N_e, effective population size.

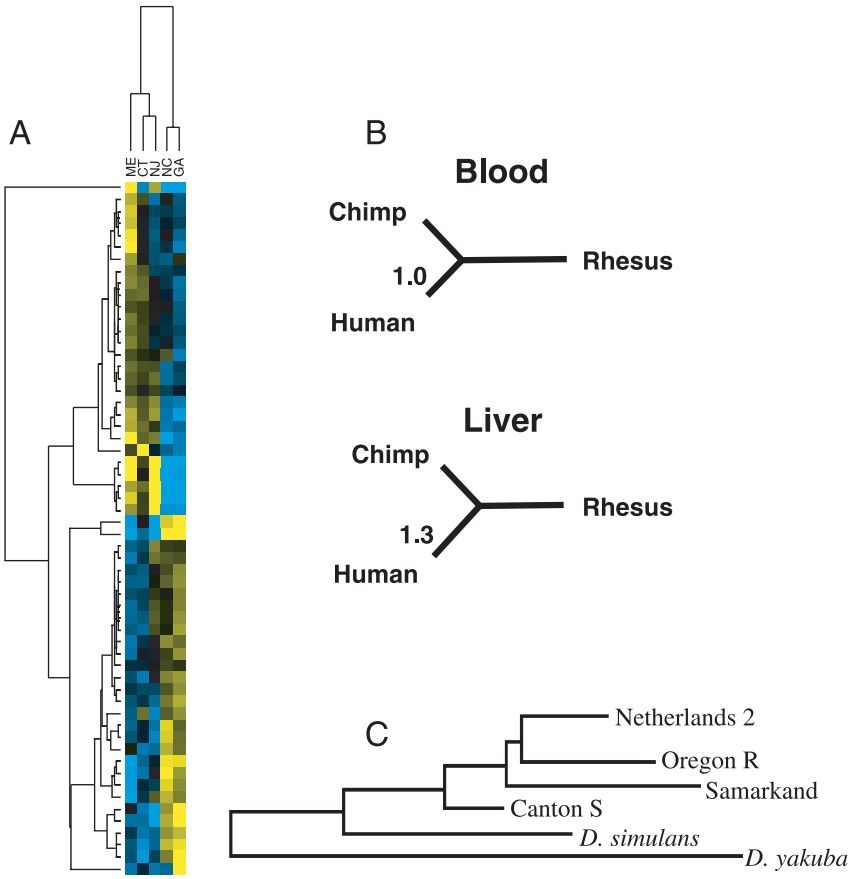

Fig. 2 Gene expression patterns tend to reflect phylogenetic relatedness. Distance trees based on gene expression differences among (A) populations of the marine teleost fish *Fundulus heteroclitus* [Populations are from Maine, ME; Connecticut, CT; New Jersey, NJ; North Carolina, NC; Georgia, GA (Whitehead, Crawford 2005a)], (B) primate species (Enard *et al*. 2002), and (C) species of *Drosophila* and *D. melanogaster* strains Netherlands 2, Oregon R, Samarkand, and Canton S (Rifkin *et al*. 2003).

leading to the conclusion that the majority of variation in gene expression is governed by neutral drift (Khaitovich *et al*. 2004; Yanai *et al*. 2004). Different yardsticks of neutral divergence have been used to accept or reject neutral expectations for character differences. The simplest model follows the neutral expectation that variation among taxa should be a positive function of variation within taxa (variation among = f * variation within, where f is a function; Fig. 1C). This has been analysed by testing whether the variation among taxa is significantly higher than variation within taxa (an F-test). When significant, the neutral expectation is rejected providing evidence for directional selection. For a comparison of expression differences among *Drosophila melanogaster* and *Drosophila simulans*, Nuzhdin *et al*. (2004) applied this approach to identify adaptive divergence in a large proportion of genes. Many of these genes are involved in reproduction (Nuzhdin *et al*. 2004), supporting the finding that most of the interspecific differences in gene expression between *D. melanogaster* and *D. simulans* show sex-biased patterns of expression (Ranz *et al*. 2003). However, statistically significant differences in expression among taxa are not evidence for directional selection per se. This is because the function relating neutral variance among vs. within taxa is unknown and varies for different genes (Fig. 1C). Larger variance ratios should be expected for some genes with fewer constraints on expression compared to genes with higher constraints. That is, the function f is not the same for all genes and thus not easily modelled by standard ANOVA. Additionally, larger ratios are expected for more divergent taxa (this issue is explored in more detail later). *D. melanogaster* and *D. simulans* are not closely related (they diverged on the order of 10^7 generations ago) compared to species of cichlid fish (diverged on the order of 10^5 generations ago) or primates (humans and chimpanzees diverged on the order of 10^5 generations ago). Thus many of the large differences between *D. melanogaster* and *D. simulans* may be due to drift acting over the long time since divergence.

In a second approach, observed variance within and among taxa is compared to expected variance that is scaled by time since divergence and effective population size. Expression divergence that exceeds expected divergence under this neutral model is considered evidence for directional selection. Using this method a significant minority of genes were detected to be subject to diversifying selection among primates (4% of genes) (Hsieh *et al*. 2003) and among fruit flies (1% of genes) (Rifkin *et al*. 2003). A modified version of the above approach sets upper and lower limits on the range of expected trait divergence among taxa due to genetic drift, where expected divergence is estimated

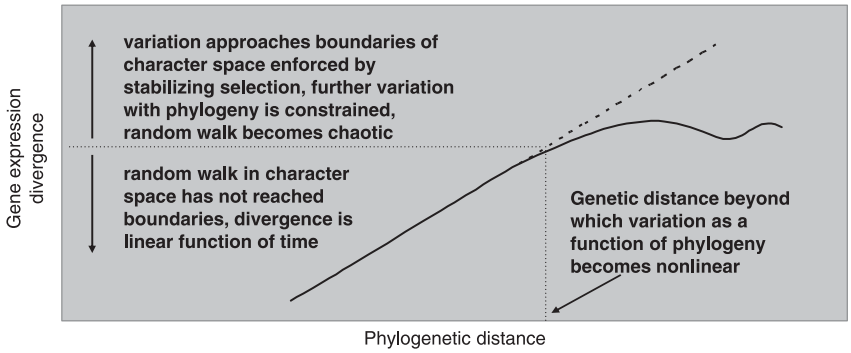

Fig. 3 Relationship between gene expression divergence among taxa and phylogenetic distance between comparisons. Genetic drift interacts with stabilizing selection to diverge or constrain variation among taxa. Among closely related taxa divergence will be primarily governed by genetic drift, and a random walk through character space drives divergence among taxa as a linear function of time. The slope of this line is a function of gene-specific constraints. Among more distantly related taxa, functional constraints on expression divergence will eventually apply as the random walk through character space reaches boundaries set by stabilizing selection. This boundary is also gene-specific as some genes will have wider boundaries than others. Relationship between character divergence and time departs from linearity because there are boundaries to the amount of acceptable (neutral) variation. Beyond this phylogenetic distance, divergence becomes chaotic as drift randomly walks back through character spaces previously traversed because this is the only space available. Volume of character space and the time to nonlinearity will differ among genes.

according to mutation rate and time since divergence. Among-taxon trait variance that exceeds upper or lower limits is considered evidence for directional or stabilizing selection, respectively. Using this approach, Lemos *et al*. (2005) detected a majority of genes (61% to 100%) subject to stabilizing selection among mouse strains, among primates, and among fruit fly species. In contrast, a minority of genes were classified as subject to neutral genetic drift (0% to 24%), and few genes were considered subject to diversifying selection (0% to 12%). What is notable of these data is that the proportion of genes detected to be affected by drift deceases with increasing divergence. That is, drift accounted for variance of a greater proportion of genes among closely related mouse strains than among distantly related *Drosophila* species. At first this appears counter-intuitive, as the effects of drift should increase with increasing divergence, but as divergence increases the relationship between within- and among-taxon variations is likely to break down (Fig. 3, see discussion later).

A third approach has examined asymmetry in gene expression variation along branches of the primate phylogeny to identify patterns that reject neutral expectations (Fig. 2). Proceeding from the early predictions of King & Wilson (1975), much recent attention has focused on comparison of expression variation among primates to identify evolutionary patterns that distinguish human brains from those of our relatives (Enard *et al*. 2002; Caceres *et al*. 2003) (see review in Preuss *et al*. 2004). Expression patterns within a species tended to cluster individuals together and into mutually exclusive species groups (Enard *et al*. 2002) demonstrating that the variation among species exceeds variation within species. Also, patterns among species tended to reflect evolutionary relationships. Humans and chimpanzees are more closely related to each other than either is to the rhesus macaque, and human and chimpanzee expression patterns from blood and liver samples were more similar to each other than either was to the macaque (Fig. 2). However, gene expression patterns in brains of chimpanzees were more similar to the macaque than to humans, indicating accelerated evolution of brain gene expression in the human lineage. In a statistically rigorous re-analysis of the primate data from Enard *et al*. (2002), Hsieh *et al*. (2003) detected more differentially expressed genes across species, with larger differences in expression, in liver tissue than in the brain (35% and 25% of genes differentially expressed across species in livers and brains, respectively). Indeed, both diet and cognitive functions are divergent between primates. What remains unusual about the primate data is the relatively long and asymmetrical branch length in human brain evolution that does not exist for other tissues (Enard *et al*. 2002; Hsieh *et al*. 2003). Another interesting finding of studies among primates is that in brains, differences in expression are biased towards up-regulation in human (Enard *et al*. 2002; Caceres *et al*. 2003). This highlights the confounding influence of probe sequence divergence across species leading to hybridization inefficiencies and false-positive differences in gene expression (Hsieh *et al*. 2003; Gilad *et al*. 2005). However, this appears to be a brain-specific phenomenon as no such bias towards up-regulation in humans is observed in other tissues (Enard *et al*. 2002; Gu & Gu 2003; Hsieh *et al*. 2003).

A fourth approach has been to use neutral markers, such as microsatellites, to quantify genetic distances and to

use genetic distance matrices to correct among-taxon trait variation for nonindependence due to phylogeny (the phylogenetic comparative approach: see Felsenstein 1985; Harvey & Pagel 1991; Garland *et al*. 1992). Genetic distance among taxa is considered a covariable, and maximum trait variation among taxa is allocated to genetic distance. This is a gene-specific approach; the covariance between gene expression and genetic distance is determined for each locus separately. The expectation is that different loci have different relationships with genetic distance; those genes subject to greater expression constraint will be influenced less by genetic drift. The residual variation that remains after taking phylogeny into consideration can then be tested for correlation with ecological parameters of hypothesized evolutionary importance. This approach has been applied to detect the influence of directional selection on glycolytic enzyme expression (Pierce *et al.* 1997) and on expression of central metabolic genes (Whitehead & Crawford 2005a) in populations of the fish *Fundulus* distributed along a strong thermal habitat gradient. This approach holds advantage because the expression of each gene is considered separately and the statistical underpinnings are well established. The weakness is that if there is strong correlation between genetic distance and the ecological parameter, then most of the variation is only attributed to neutral evolution leaving little residual variation to investigate adaptive correlations.

Phylogenetic distance and the influence of drift

An important conflict in evolutionary analysis of gene expression is the relative influence of neutral drift vs. stabilizing selection; many studies find that drift tends to dominate among-taxon variation (Oleksiak *et al*. 2002; Khaitovich *et al*. 2004; Yanai *et al*. 2004; Whitehead & Crawford 2005a), whereas others argue for the dominance of stabilizing selection (Rifkin *et al*. 2003; Lemos *et al*. 2005). We propose that the crux of this conflict hinges on the question: 'What timescale is most appropriate for examining the relative influences of different evolutionary forces?'

Drift and stabilizing selection interact to diverge or constrain variation, and the interaction becomes more complex as phylogenetic distance increases (Fig. 3). Thus, we suggest that binning of gene expression patterns into groups governed by drift or by stabilizing selection is unnecessarily arbitrary since the relative influence of each of these forces will depend on the phylogenetic distance between taxa and gene-specific constraints (Fig. 1C). Genetic drift randomly traverses the character space over which the fitness phenotype is not affected, and the boundaries of that character space are defined by functional constraints set by stabilizing selection. Among closely related taxa, neutral divergence should appear to be a linear function of time. However, there are limits to the amount of mRNA a cell can produce and thus ever-increasing divergence in gene expression is unreasonable. Consequently, expression divergence should become a nonlinear function of phylogenetic distance for pairs of more distantly related taxa since drift is more likely to have reached boundaries set by stabilizing selection. The neutral range of variation will depend on the gene and its function, so expression patterns for some genes will appear to be governed primarily by drift (neutral character space is large or time since divergence is small) whereas at the other end of the continuum (where character space is small or time since divergence is large) constraints on variation by stabilizing selection are more likely to have been imposed (Fig. 3). Identifying the threshold of genetic distance beyond which character variation as a function of phylogeny becomes nonlinear would contribute to resolving some of the apparent contradictions in the literature.

Another apparent conflict relates to the relative influence of directional selection and stabilizing selection. For example, stabilizing selection has been invoked to explain almost all expression variation among *Drosophila* species (Lemos *et al*. 2005), whereas others find widespread evidence for directional selection (Rifkin *et al*. 2003; Nuzhdin *et al*. 2004). We propose that the crux of this conflict hinges on the classical debate over whether among-taxon patterns of variation alone can be used to distinguish the influence of directional selection from the influence of drift interacting with stabilizing selection. The neutral expectation is that variation among taxa (S^2_{among}) be a function of variation within taxa (S^2_{within}) (Khaitovich *et al*. 2004). In order to reject the neutral expectation, the ratio of S^2_{among} to S^2_{within} (an *F*-ratio in ANOVA) must exceed some statistical threshold (a critical *F*-value) (Nuzhdin *et al*. 2004). What is unclear, however, is what this threshold should be. Must S^2_{among} exceed S^2_{within} by a factor of 4, 10, or 20, for neutrality to be rejected in favour of directional selection (Fig. 1C)? The ratio expected under neutrality should be higher for genes with fewer constraints or among more divergent species. One proposed solution has been to scale expected neutral divergence according to time since common ancestor (Hsieh *et al*. 2003; Rifkin *et al*. 2003; Lemos *et al*. 2005). Two applications of the scaling approach suggest that a majority of genes that vary among *Drosophila* species are affected by stabilizing selection (Rifkin *et al*. 2003; Lemos *et al*. 2005). One could imagine that stabilizing selection could serve to maintain low variance within taxa and neutral drift drive divergence between taxa, especially for traits that are important for reproduction. Indeed, many of the genes differentially expressed between *D. melanogaster* and *D. simulans* are sex biased (Ranz *et al*. 2003). This argument comes full circle to a classical debate over whether patterns of trait variation within and among taxa can distinguish the influences of directional selection from neutral drift interacting with stabilizing selection.

Distinguishing selection from drift

Whitehead & Crawford (2005a) argue that since natural selection operates within an ecological context, identifying the effects of selection should involve testing for correlations between trait variation and ecological variation while including phylogenetic comparisons [the 'comparative approach' (Harvey *et al.* 1991)]. Application of this principle to the comparisons of mice and men would be difficult, because ecological factors of influence during evolutionary divergence would be challenging to identify and quantify. In contrast, analyses among closely related species may be more powerful because for shorter phylogenetic distances drift should drive linear divergence over time, and the relative influences of drift and directional selection may be more readily distinguishable. Candidate systems for this approach would be those with well-characterized ecological contexts for relatively recent adaptive radiations; examples could include morphological variants of Caribbean anoles (Losos *et al*. 1998), flight variants among populations of butterflies (Watt *et al*. 1986), populations of killifish from different thermal environments (Powers *et al*. 1993), or morphological and behavioural variants of cichlid fishes (Meyer 1993).

Studying conserved patterns of gene expression

Interspecies comparisons have typically sought to examine how evolution has driven divergence between taxa. Alternatively, identification of conserved transcriptional profiles across taxa could serve to confirm or uncover molecular mechanisms that are fundamental to biological processes. McCarroll *et al*. (2004) examined gene expression patterns of orthologous gene pairs across highly divergent taxa: nematode worms, the fruit fly, and yeast. Although most expression profiles were species specific, they detected conserved patterns associated with such fundamental biological processes as ageing, development, and meiosis. Although data confirmed known molecular mechanisms associated with these processes, genes with unexpected associations were also uncovered.

The study by McCarroll *et al.* (2004) supports the paradigm that mechanisms for important biological processes should be conserved across taxa. Conservation for mechanisms evolved early in the history of life and fundamental to biological processes may be common, and advances in comparative approaches may be powerful for identifying underlying genetic bases. This is not to say, however, that one should expect the same molecular mechanisms to be responsible for derived characters that are shared among independently evolving lineages. When standing variation is present in an ancestral population then independent evolution of the same derived characters via shared genetic mechanisms can occur. For example, freshwater populations of stickleback fishes worldwide have independently evolved reduced armour plating by the same genetic mechanism (Colosimo *et al*. 2005). However, independent evolution of derived characters may not be expected if relevant standing variation in ancestral populations did not exist and new mutations had to arise independently in evolving lineages. For example, different developmental mechanisms give rise to horn appendages among closely related lineages of beetles (Moczek & Nagy 2005). Indeed, Moczek & Nagy (2005) emphasize that little or no phylogenetic divergence is necessary for the evolutionary derivation of different developmental mechanisms. Independently evolving groups may arrive at different evolutionary solutions to common biochemical or physiological challenges, in which case the power of comparative analyses for detecting molecular mechanisms would be weak.

Concluding remarks

Gene expression variation is widespread among individuals and taxa, has a large heritable component, and is subject to influence by natural selection and genetic drift. Experimental sources of variation can decrease the precision of gene expression measures, but can be controlled through careful experimental design including appropriate replication, and through application of robust statistical tests that can accommodate variation. As is true for protein variation, much of gene expression variation appears selectively neutral, although genome-wide analyses indicate that a significant minority of variants appear to be evolving by directional selection. Future studies of the role of gene expression in adaptive variation may benefit from examining variation within ecological contexts.

Neutral drift and stabilizing selection interact to constrain trait variation. Accordingly, it is arbitrary to bin traits into groups affected by drift or by stabilizing selection. It may be more useful to think of traits varying along a continuum with stabilizing selection predominating for traits that vary less than expected, and neutral drift predominating for traits that vary linearly with time, across taxa. Note that when phylogenetic divergence exceeds some threshold, gene expression divergence may become nonlinear and among-taxon comparisons difficult to interpret. As such, insights into the influence of drift and directional selection on gene expression may be best achieved with comparisons among taxa that are relatively closely related.

Appropriate evolutionary studies of gene expression should provide insights into which genes are 'important'. Studies among recently divergent species could begin to address whether common pathways or sets of genes are often involved in adaptive differences, in speciation, or in phenotypic variation. Simply supporting or rejecting this idea would greatly increase our understanding of the nature of biological processes and evolution. Importantly, continued studies of how gene expression varies and how

it affects phenotype will involve more than just microarrays, as the relationships between mRNAs and proteins have to be established. Additionally, to provide functional information to microarray studies will require coupling with physiological, ecological and behavioural studies. By studying hundreds to thousands of genes and experimentally determining how these affect phenotype should provide a broad understanding of how cells work, how organs function, and which genes are likely to effect a phenotypic change. We would suggest that many of the changes in expression are likely to be subtle but important and the interactions among genes may affect the phenotypic outcome. If true, it will be difficult to identify these important changes by just examining a few inbred lines, or only screening for genes of large effect. Instead, we suggest that much more effort needs to be invested in examining the causes and consequences of natural expression variation among outbred species with enough statistical power to define important subtle differences.

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