Variation in gene expression within and among natural populations

Marjorie F. Oleksiak[1], Gary A. Churchill[2] & Douglas L. Crawford[1]

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Evolution may depend more strongly on variation in gene expression than on differences between variant forms of proteins[1]. Regions of DNA that affect gene expression are highly variable, containing 0.6% polymorphic sites[2]. These naturally occurring polymorphic nucleotides can alter *in vivo* transcription rates[3–7]. Thus, one might expect substantial variation in gene expression between individuals. But the natural variation in mRNA expression for a large number of genes has not been measured. Here we report microarray studies addressing the variation in gene expression within and between natural populations of teleost fish of the genus *Fundulus*. We observed statistically significant differences in expression between individuals within the same population for approximately 18% of 907 genes. Expression typically differed by a factor of 1.5, and often more than 2.0. Differences between populations increased the variation. Much of the variation between populations was a positive function of the variation within populations and thus is most parsimoniously described as random. Some genes showed unexpected patterns of expression—changes unrelated to evolutionary distance. These data suggest that substantial natural variation exists in gene expression and that this quantitative variation is important in evolution.

Microarrays, which measure the levels of mRNAs for thousands of genes, can be used to assess the variation in gene expression between individuals. For example, microarrays have measured differences in gene expression between groups of humans suffering from cancers or behavioral diseases[8–12]. Because these studies ignored the variation in gene expression between individuals within each group, however, differences in expression that appear to be associated with a specific disease may instead represent random genetic variation.

To obtain a more robust analysis of gene expression, we analyzed the variation in expression both within and between populations in two species of *Fundulus*. These fish have large populations (exceeding 10,000 individuals per location) and relatively low migration rates[13,14], and populations adapt readily to local environmental conditions. Northern populations of *Fundulus* species experience much colder temperatures than their southern counterparts, and have evolved adaptations to temperature in cardiac metabolism, glycolytic enzyme expression and molecular mechanisms affecting enzyme expression[3,4,15,16]. If these adaptations are attributable to changes in gene expression, variation in gene expression should exist both within and between these populations.

We examined 15 individuals (5 each from northern and southern populations of *Fundulus heteroclitus* and 5 from the sister taxon *Fundulus grandis*) to determine the variation in gene expression within and between populations. We raised the fish in a common environment to minimize physiological differences. We used a 'loop design' for the microarray study[17,18] (Fig. 1). In this design, we used labeled RNA from each individual to probe four slides, each containing two spatially separated replicate arrays, for a total of eight arrays. We probed each slide with labeled RNA from two individuals, one individual's RNA labeled with Cy3 and the other individual's RNA labeled with Cy5. Each slide contained samples from different combinations of individuals, and we compared each individual to four other individuals, twice when labeled with Cy3 and twice when labeled with Cy5 (Fig. 1). Our raw data consisted of 120 measurements (15 individuals with 8 replicates) for

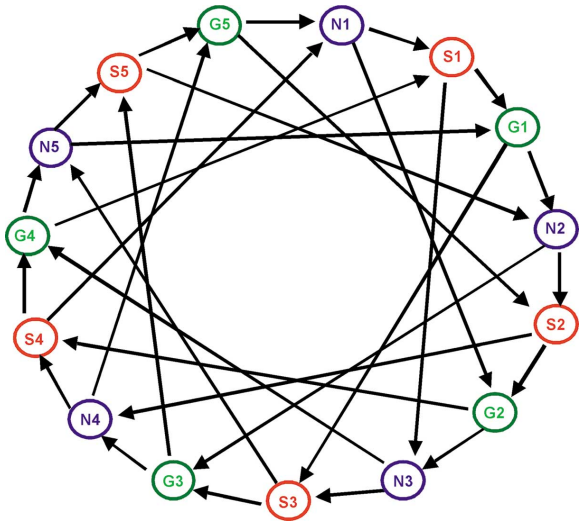

Fig. 1 Loop design used in microarray studies. Each arrow represents a microarray and connects the two individual samples hybridized to it. Each microarray has duplicate arrays of 1,149 genes, of which 907 were used in the analysis. On each microarray, arrows indicate labeling (Cy3 or Cy5): one sample is labeled with Cy3 fluorescent dye (base of the arrow) and the other with Cy5 dye (arrow head). A total of 15 individuals from three populations (northern *F. heteroclitus,* southern *F. heteroclitus,* and sister species *F. grandis*) were used in this study. All 15 individuals were measured in eight replicates. All fish were raised in a common laboratory environment for at least six months to minimize physiologically induced differences. N, northern *F. heteroclitus*; S, southern *F. heteroclitus*; G, *F. grandis.*

[1]*School of Biological Sciences, 5007 Rockhill Road, University of Missouri–Kansas City, Kansas City, Missouri 64110-2499, USA.* [2]*The Jackson Laboratory, 600 Main Street, Bar Harbor, Maine 04609, USA. Correspondence should be addressed to D.L.C. (e-mail: crawforddo@umkc.edu).*

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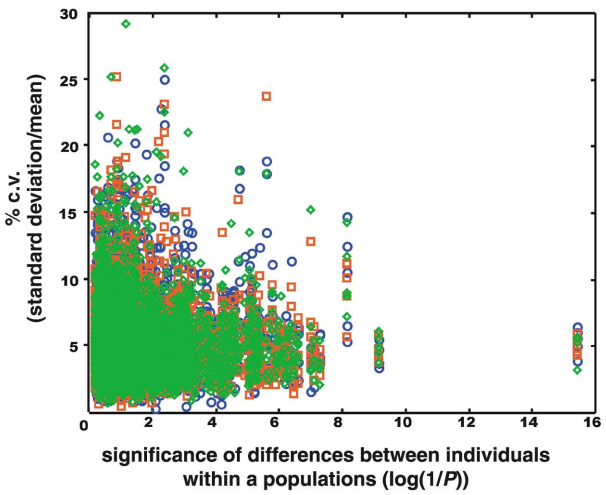

Fig. 2 Error variance versus significance between individuals within populations. Experimental errors for measures of mRNA expression for each individual for each gene are reported as %c.v. (13,605 gene–individuals). Significance between individuals within a population is reported as the inverse log of the *P* value calculated from the ANOVA (for example, '4' is equal to a *P* value of 10^{-4}). Blue circles, individuals from the *F. heteroclitus* northern population; red squares, individuals from the *F. heteroclitus* southern population; green diamonds, *F. grandis* individuals.

each of 907 genes. The loop design is substantially different from the most commonly used 'reference microarray' design, in which each RNA sample of interest is used to probe the same reference sample and all values are expressed as ratios of the sample signal to the reference signal.

We proposed to answer two questions. First, what proportion of genes are differentially expressed between individuals within the same population? Second, how many genes are differentially expressed between populations? To address these questions, we applied ANOVA methods to the $\log_e$ normalized data[18]. Unlike most microarray strategies (but similar to one previous study[19]), ours did not depend on assessing ratios of fluorescent signals, whereby only large differences can be detected. Instead, we investigated which genes showed statistically significant variations in expression.

The expression levels of 161 genes (18%) were significantly different between individuals within the same population at the nominal *P* value of 0.01 (Fig. 2), as determined using standard statistical tables or permutation analyses within each gene. This number of significant genes is 18 times larger than the nine false positives expected under the null hypothesis when $P = 0.01$. To provide tighter control of type I errors (falsely rejecting the null hypothesis), we considered applying a multiple-testing adjustment to these tests[20]. Experiment-wide control of type I error at the 5% level corresponds to an individual test *P* value of 6×10^{-5}. Only 37 of the 161 genes showed significant differences in expression between individuals at this level of stringency, which may be overly conservative. We chose to use the significance level of $P = 0.01$ and accept a greater type I error in our analyses.

The proportion (18%) of loci differing significantly in expression between individuals within the same population is similar to the percentage of loci that differ significantly in expression between different strains of yeast[21] (24%) and the percentage of loci that show non-zero variance in *Drosophila melanogaster*[19] (25%), as determined by previous studies. These studies by necessity used pooled samples, and thus could not measure variation in expression between individuals in natural populations. In humans there is a large variation in gene expression between individuals; in a global comparison of mRNA levels of chimpanzees and humans, there was greater variation within the human population than between human and chimpanzee populations[22]. These results support our finding of large variation in gene expression between individuals and emphasize the importance of examining individual variation.

An ANOVA analysis calculates significance using an *F* statistic, and significant *F* values require that the variation between samples is significantly larger than the residual variation within samples[20]. Thus, finding significant differences between individuals requires that the variation between individuals be larger than experimental variation (for example, variation due to printing, hybridization, array differences and other factors). One measure of the experimental variation is the coefficient of variation (c.v.) of gene expression for each individual among the eight replicates, which equals the standard deviation divided by the mean, expressed as a percentage. Nearly all (99%) of the genes for each individual had a $\text{c.v.}_{\text{error}}$ of less than 15% (Fig. 2). The statistical significance of the differences in expression of 161 genes depended on this small experimental error. We minimized experimental error by using eight replicate measures per individual for each gene and using normalized data rather than the ratio typically used in a reference design. Ratios of two values, each having its own variation, have larger experimental variation[20]. Not surprisingly, genes for which there was little experimental variation (low $\text{c.v.}_{\text{error}}$ values) showed the greatest significant differences in expression between individuals within the same population, and genes with large experimental variation values did not differ significantly (Fig. 2).

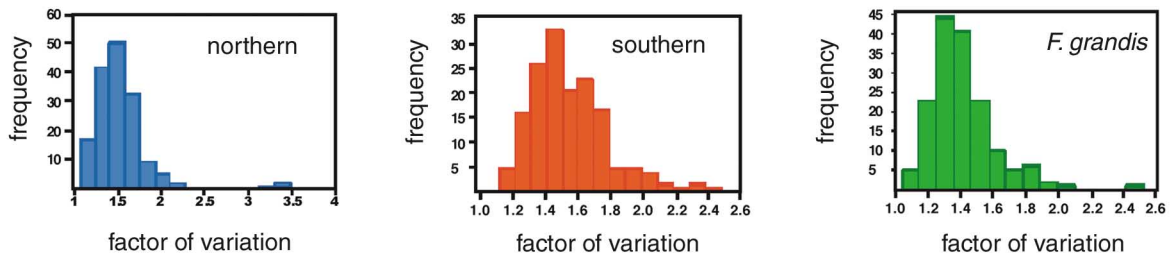

Fig. 3 Frequency distributions of range of gene expression levels among individuals within a population. Frequencies are only for 161 genes whose mRNA expression levels are significantly different among individuals within a population. Factor of variation on the abscissae is the ratio between individuals that have the smallest and largest mean levels of expression within each population (that is, the range). Individual expression is the average of eight replicates. The northern *F. heteroclitus* population is shown in blue, the southern *F. heteroclitus* population in red and the sister species, *F. grandis*, in green.

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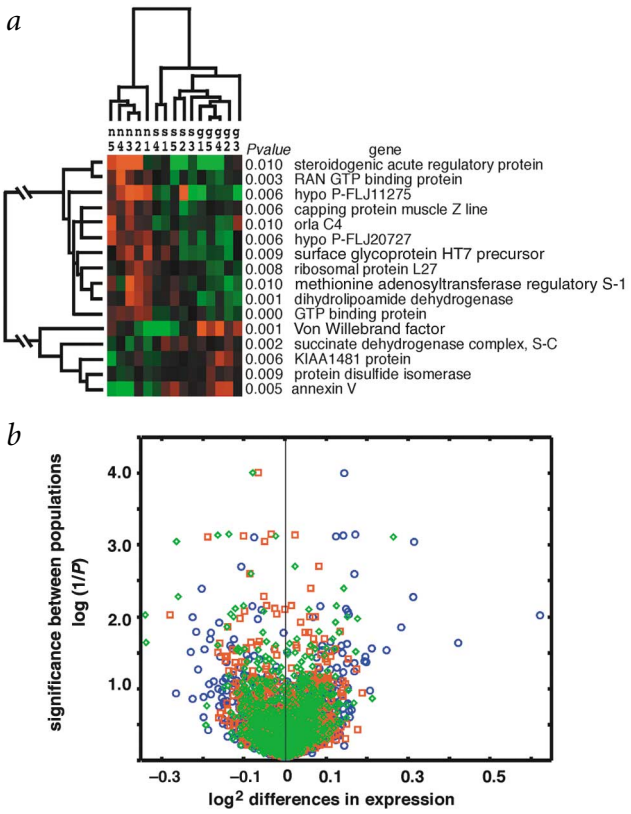

Fig. 4 Pattern and significance of gene expression between populations. ***a***, Patterns of mRNA expression for the 15 genes whose expression was significantly different between populations. Values shown in red are relatively larger than the overall mean and values shown in green are relatively smaller than the overall mean. The *P* values, based on ANOVA, and gene names are listed on the right. Two clusters are displayed. The dendrogram on the left (Gene Cluster) groups genes with similar patterns of expression between individuals. The dendrogram on top (Individual Cluster) groups individuals based on similar patterns of expression for the 15 genes. Individuals are designated by a letter–number combination: n, northern *F. heteroclitus* population; s, southern *F. heteroclitus* population; g, *F. grandis* population. Clustering of genes used average linking correlation values[30]. ***b***, Significance of differences between populations versus magnitudes of differences. Levels of mRNA expression for each gene for each population are reported as $\log_2$ population means minus the $\log_2$ grand mean. The significance between populations is expressed as the $\log_{10}$ of the inverse *P* value from the ANOVA. Symbols are as in Fig. 2.

What is the magnitude of expected difference in mRNA expression between individuals? For the 161 genes that showed significant variation, the difference in expression between two individuals within the same population typically varied by a factor of 1.5, but varied by a factor of 2.0 or more for many genes (Fig. 3). These data indicate that two individuals within the same population can have a large difference in gene expression. For mRNAs encoding enzymes, changes of this magnitude could effect a larger change in reaction rates than the protein polymorphisms that influence enzyme kinetic constants[4,23] (K_m, K_{cat} and others).

We addressed variation between populations by an analysis of estimates of differential expression from an ANOVA model that excluded non-biological variation (for example, dye, spot and slide effects[18]). The expression of 15 genes was significantly different (at $P = 0.01$) in the three *Fundulus* populations (Fig. 4*a*,*b*). The pattern of clustering among individuals (Fig. 4*a*) indicated that most of these differences separate the northern *F. heteroclitus* population from both the southern *F. heteroclitus* population and the *F. grandis* population. This number of significant genes (15) is 2 standard deviations (s.d. = 3) above the 9 false positives expected at $P = 0.01$. No genes differed significantly at the multiple test–corrected threshold (F critical value of 25.1, $P = 6 \times 10^{-5}$). Regardless of which *P* value was used, the genes with more significant *P* values showed only small differences between populations (Fig. 4*b*). For these small differences in mRNA between populations to be significant, the variation within each population would have to be even smaller. The small variation in the expression of these genes within a population could reflect tight regulation or stabilizing selection, suggesting that small changes in these genes may produce biologically important differences. In analyzing significant patterns of gene expression related to disease, the more interesting genes may be those that have little variation between healthy individuals, not only because their small variance may reflect a biologically important change, but also because they are easily distinguished by statistical analysis.

This investigation underscores the importance of obtaining an independent and representative population sample as the basis for inference in gene expression studies. For example, the expression of translocase, NADH dehydrogenase chain 2 and phosphohippolin did not differ between populations, but differed between individuals by factors of 4.0, 3.9 and 3.4, respectively. Forty-eight (5%) genes whose expression was not significantly different between populations differed in expression between individuals by a factor greater than 2.0 (ignoring population structure). These measures of differential gene expression reflect normal biological differences among individuals. We raised all fish in a common environment, they appeared healthy and we used only heart ventricles for RNA extractions; thus, the majority of this variation is not due to different physiological conditions or differences in tissue types. Instead, these differences within and between populations should be considered 'normal' or expected. Some of these differences are due to genetic variation within and among populations, but because we used natural populations, other factors (maternal effects, potential genotype–environment interactions) could also be important. These data suggest that studies seeking to compare individuals must consider the natural variation in gene expression between individuals

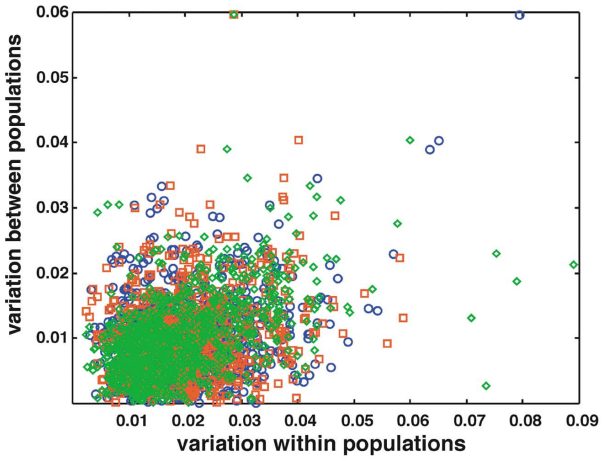

Fig. 5 Variation between and within populations. The variance in mRNA expression for each gene for each individual is plotted against the variance between populations. Variance was standardized by dividing by the mean expression value. Variation was calculated as the mean squares within populations based on individual means or as the mean squares between populations based on the population means. Symbols are the same as in Fig. 2.

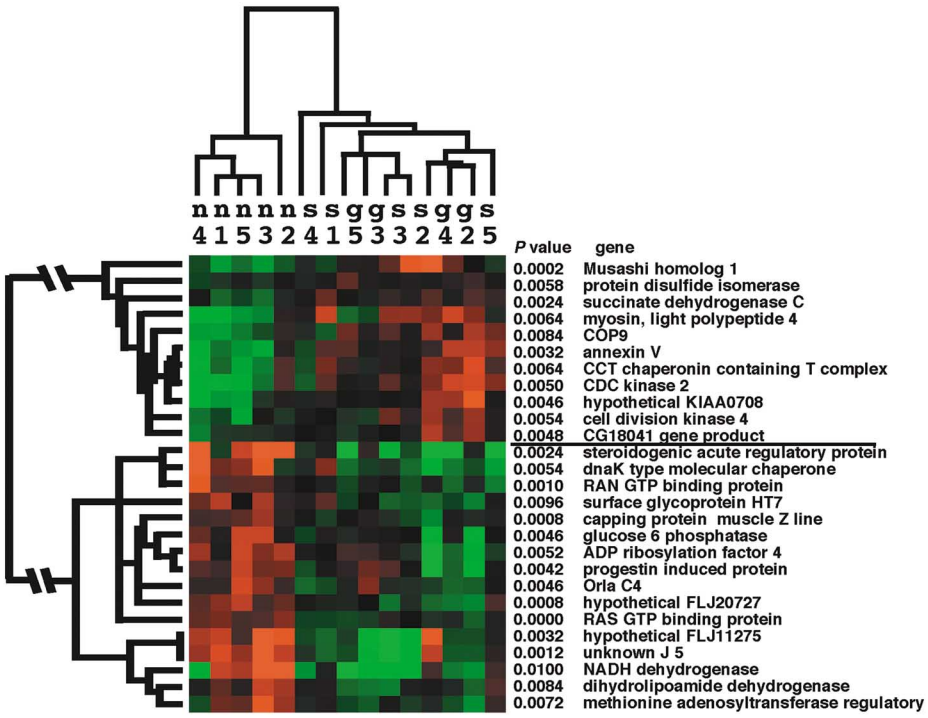

Fig. 6 Patterns of gene expression for northern *F. heteroclitus* population versus combined southern *F. heteroclitus* and *F. grandis* populations. Patterns of mRNA expression were based on the 27 genes whose expression was significantly different in the northern *F. heteroclitus* population than in both the southern *F. heteroclitus* and the *F. grandis* populations. The *P* values, based on permutation of *t* statistics, and gene names are listed on the right. Values shown in red are relatively larger than the overall mean and values shown in green are relatively smaller than the overall mean. Two clusters are displayed. The dendrogram on the left (Gene Cluster) groups genes with similar patterns of expression between individuals. The dendrogram on top (Individual Cluster) groups individuals based on similar patterns of expression for the 27 genes. Individuals are designated by a letter–number combination: n, northern *F. heteroclitus* population; s, southern *F. heteroclitus* population; g, *F. grandis* population. Clustering of genes used average linking correlation values[30].

and the effect of population structure. Normal individual variation in gene expression represents a potential confounding source of variation that cannot be accounted for without replication at the level of the individual. This is especially important in studies of natural, outbred populations (for example, humans), where one can expect to observe substantial variation in expression.

Accepting that the nominal *F* statistic with a *P* value of 0.01 is statistically meaningful, it is difficult to suggest that the differences in expression of 15 genes between populations are biologically important. Most of the variation between populations reflects the variation within a population (Fig. 5). According to the neutral theory of evolution, the variation between populations is a positive function of the variation within populations[24]. Thus, much of the significant variation between populations may represent random genetic drift. There is, however, an alternative hypothesis that is not supported by neutral theory: gene expression in the northern *F. heteroclitus* population differs from the expression in both southern *F. heteroclitus* and *F. grandis* populations. This is unexpected because the northern and southern *F. heteroclitus* populations are genetically more similar to each other than either is to *F. grandis*[25–27]. In order to test this hypothesis, we computed a *t* statistic (with 12 degrees of freedom) contrasting the mean of the northern *F. heteroclitus* population to the joint mean of the southern *F. heteroclitus* and *F. grandis* populations. Twenty-seven genes showed a significant difference in expression at the $P = 0.01$ level, and two exceeded the multiple test–corrected threshold[28]. The cluster diagram (Fig. 6) illustrates the pattern of expression for each of these 27 genes. The northern *F. heteroclitus* population forms one cluster and the southern *F. heteroclitus* and *F. grandis* populations form another (Fig. 6). The gene encoding steroidogenic acute regulatory protein (intracellular cholesterol transport) showed significantly greater expression in northern *F. heteroclitus* populations than in southern *F. heteroclitus* or *F. grandis* populations, yet showed one of the largest variations within a population, and thus must have even greater variation between populations. Other genes with greater expression in the northern *F. heteroclitus* population included those encoding dihydrolimoamide dehydrogenase and two GTP-binding proteins (Ras and Ran). Genes with lower expression in the northern *F. heteroclitus* population included those encoding CDC-like kinase, Musashi (a transcription factor) and succinate dehydrogenase-C. Assuming that much of the variation in expression is heritable, these patterns of expression may be the result of these fish evolving in different environments: cold water for the northern *F. heteroclitus* population and warmer waters for its southern counterpart and *F. grandis*. This is similar to measures of glycolytic enzyme expression[16] or the molecular mechanisms affecting *Ldh-B* expression[3,4] that have evolved by natural selection. The patterns of gene expression that we observed support previous results by suggesting that variation in gene expression may be an important mechanism for evolution by natural selection.

Observations concerning qualitative differences in proteins have had a marked impact on how we view protein biochemistry, physiology and evolution. Knowledge of variation in the quantitative differences in gene expression could have a similar impact. King and Wilson[1] stressed the importance of variation in gene expression in 1975. Referring to protein variation, they suggested that "all the biochemical methods agree that the genetic distance between humans and the chimpanzees is probably too small to account for their substantial organismal differences" and that instead "evolutionary changes in anatomy and way of life are more often based on changes in the mechanisms controlling the expression of genes than on the sequence changes in proteins." Our data, showing substantial variation in the levels of expression for many genes, supports their surmise. These data demonstrate that the variation required for evolution by natural selection is evident in measures of gene expression.

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Methods

Organisms and organs. We captured individual fish used in this study from wild populations in Wiscasset, Maine, USA (northern *F. heteroclitus* population), Sapelo Island, Georgia, USA (southern *F. heteroclitus* population) and Pensacola, Florida, USA (*F. grandis* population). We maintained the fish at 20 °C for more than 6 months before RNA extraction. Light cycle was 14:10 light/dark. We fed the fish frozen brine shrimp and Tetramin Food Flakes *ad libitum* once daily in the late afternoon. During this time, all species came into reproductive condition and spawned. The reproductive tissues were in regression in all species when assayed.

We isolated heart ventricles and allowed them to expel blood by placing them in a cardiac Ringer's solution with heparin for 1–2 min before isolating RNA. Hearts continued to beat and maintain a constant metabolic rate for more than 30 min[15]. Thus, this treatment should not have adversely affected RNA. We isolated total RNA using guanidine isothiocyanate and digested samples with DNase before use.

RNA labeling. We labeled RNA by incorporation of amine-dUTP (5-(3-aminoallyl)-2′-deoxyuridine 5′-triphosphate sodium salt, Sigma) using reverse transcriptase (Promega) and covalently attached fluorescent labels by treating amine-labeled cDNAs with NHS-ester Cy3 or Cy5 (Amersham) in bicarbonate buffer. We quantified fluorescently labeled RNAs bound to microarrays with a laser confocal scanner (ScanArray 4000, Packard Instruments) at a low power setting (70%:60% power/PMT). We analyzed the images using Imagene 4.1 (Biodiscovery). For a gene to be included in this analysis, the fluorescent signal from all replicates for all individuals had to be greater than the negative controls (a plant-specific cDNA). Of the 1,149 *Fundulus* genes, 907 met this requirement. We did not include the other 242 genes because of failed printing in one or more arrays or low signal.

Microarrays. We printed *Fundulus* microarrays using sequenced cDNAs isolated from a heart ventricle library[29]. We amplified these cDNAs with amine-linked primers. Amine-tagged cDNAs were covalently bound to Motorola Activated Slides. We verified the identities of all cDNAs used in the microarray by re-analyzing the DNA sequences used for printing.

We printed the second array on each slide after printing the first array (that is, for 50 slides, the replicate array on the first slide would be the 51st printing). Printing pens re-sampled the cDNAs before printing replicate arrays, so replicate arrays can be considered a separate printing. We labeled each individual's mRNA once and hybridized it to different slides. These procedures may explain why the correlations within a slide are similar to the correlations between slides for the same printing (62% versus 64%). Thus, slides versus replicates within a slide did not contribute substantial additional variance. The different dyes also did not seem to add variance: correlations between arrays labeled with the different dyes were 69%. This probably reflects the labeling procedure, in which dyes were bound to amine groups incorporated in the sample cDNAs during reverse transcription. This is different from the microarray study on *D. melanogaster*[19], where there was a small yet consistent dye effect.

Statistics. To identify non-linear fluorescence, we examined signal intensity for each array by plotting the log ratio against the log average intensity for paired Cy3 and Cy5 measurements from each spot[18]. We normalized the raw data using a log-shift transform as previously described[18].

We carried out two separate analyses to test the hypotheses of individual variation within groups and variation between groups. In principle, a single nested analysis could be carried out, but we found this approach to be computationally prohibitive when permutation analysis and multiple-test correction was applied. Our test of variation among groups is an approximation of the full nested model analysis.

To test for individual variation within groups, we employed the ANOVA method previously described[18]. The general model was $y_{ijkg} = m \pm A_i \pm D_j \pm (AD)_{ij} \pm G_g \pm (AG)_{ig} \pm (DG)_{jg} \pm (VG)_{kg} \pm e_{ijkg}$. In this model, y_{ijkgr} is the signal from the i^{th} array with dye j and treatment k for gene g in spot r. The variable m represents the mean signal of all the genes across the entire experiment. The overall variation in arrays and dyes is represented by the terms for array (A_i), dye (D_j) and array–dye interaction ($AD)_{ij}$. The term G_g is the average signal for gene g across arrays, dyes and populations. The term $(AG)_{ig}$ represents the spot effects, or the effects of the replicated DNA spots not being exactly the same (for example, because of morphology or DNA concentration). The term $(DG)_{jg}$ represents gene-specific dye effects due to a gene that labels or binds better with one dye than with another. The term e_{ijkg} or residual represents measurements of experimental error. Finally $(VG)_{kg}$ represents the differences across samples[18]. To construct our F statistics we considered two representations of $(VG)_{kg}$. Under the null hypothesis of no individual variation, treatment k can take on one of three values corresponding to the populations (northern *F. heteroclitus*, southern *F. heteroclitus* or *F. grandis*). Under the alternative hypothesis, treatment k takes on 15 distinct values, one for each individual. We calculated the F statistics separately for each gene and compared them to the tabulated F distribution with d.f. = 12,45 at the $P = 0.01$ significance level. These F statistics were computed on a per-gene basis, and thus there is no need to assume a common error variance across genes.

We addressed the differences between groups using a derived data set that consisted of 15 estimated relative expression values for each gene ($(VG)_{kg}$ from the ANOVA model). In this analysis, 'error' represents the sum of the between-individual variation and the technical (measurement) error. It seemed plausible that there would be variance heterogeneity, and we performed testing on a per-gene basis in order to avoid the assumption of common error variance. The F statistic is the standard test for differences in a one-way classification of 15 individuals into three equal-size groups. We determined significance levels by comparison to the tabulated F distribution with d.f. = 2,12.

For both sets of test statistics we carried out permutation analysis by randomizing the residuals (within each gene) from the fitted null model approximately 10,000 times and re-computing the F statistics. The results did not differ from those obtained using standard statistical tables. To further control for type I errors among all 907 genes, we computed a multiple test–corrected threshold for the F statistic (F_{max}). We computed multiple test–corrected critical values for the F or t statistics by the one-step adjustment method[28]. We computed 10,000 permutations of the original data across all genes and identified the largest F value across all genes in each permuted set. We used the distribution of these F values to determine the critical F value that occurs less than 5% of the time among all permuted sets.

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Competing interests statement

The authors declare that they have no competing financial interests.

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