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A test of the neutral model of expression change in natural populations of house mouse subspecies

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Summary

Changes in expression of genes are thought to contribute significantly to evolutionary divergence. To study the relative role of selection and neutrality in shaping expression changes, we analysed 24 genes in three different tissues of the house mouse (*Mus musculus*). Samples from two natural populations of the subspecies *M. m. domesticus* and *M. m. musculus* were investigated using quantitative PCR assays and sequencing of the upstream region. We have developed an approach to quantify expression polymorphism within such populations and to disentangle technical from biological variation in the data. We found a correlation between expression polymorphism within populations and divergence between populations. Furthermore, we found a correlation between expression polymorphism and sequence polymorphism of the respective genes. These data are most easily interpreted within a framework of a predominantly neutral model of gene expression change, where only a fraction of up to 10% of the changes may have been driven by positive selection. Although most genes investigated were expressed in all three tissues analyzed, significant changes of expression levels occurred predominantly in a single tissue only. This adds to the notion that enhancer-specific effects or transregulatory effects can modulate the evolution of gene expression in a tissue-specific way.

Introduction

The relative role of expression changes versus protein coding changes in shaping evolutionary divergence is still a matter of vivid debate (Tautz 2000; Lemos et al. 2005a; Hoekstra and Coyne 2007; Wray 2007; Carroll 2008; Fay and Wittkopp 2008). Although our knowledge on intra- and interspecific variation and change in gene regulation is growing fast, it is still not clear which general role regulatory variation plays in adaptations. Existing estimates of the proportion of genes under directional or stabilizing selection show wide ranges (Rifkin et al. 2003; Lemos et al. 2005a; Whitehead and Crawford 2006a). Khaitovich et al. (2004, 2005) proposed a predominantly neutral model of transcriptome evolution based on microarray data in primates. They argue that an approximately linear accumulation of expression differences over time, as well as a correlation between expression variance within a

species and expression difference between species are signs for evolutionary divergence processes that are best explained in the context of predominantly neutral changes. However, their conclusions have been critizised on the grounds that their calibration was based on the assumption of neutrality of the pseudogene expression, which may not hold (Svensson et al. 2006). On the other hand, this problem would only affect the question of how much purifying selection needs to be taken into account, but does not change the conclusions with respect to the role of positive selection.

Blekhman et al. (2008) have taken a comparative approach along the same lines and report an excess of regulatory changes in the human lineage compared to chimpanzee and rhesus macaques. This would suggest that there is, in addition to neutral and purifying selection patterns, evidence for positive selection in the human lineage. In a different type of study on expression differences within and between *Drosophila* species, Wittkopp et al. (2008) found a predominance of *cis*-regulatory changes in comparisons between species, which would also suggest an involvement of positive selection. However, both of these studies do not directly provide data on the frequency of positive selection versus neutral change.

To infer microevolutionary mechanisms underlying expression evolution, gene expression patterns should be analysed together with sequence polymorphisms and changes. A first study integrating such data was conducted by Brown and Feder (2005). They used microarray data to choose gene sets with gene expression differences between *Drosophila* strains and sequenced the upstream regions of such genes. They did not find a correlation between sequence change and expression change, which would argue for a decoupling of these parameters. However, their data basis was small and Holloway et al. (2007) and Lawniczak et al. (2008) found in more extensive data sets from *Drosophila simulans* strains that sequence polymorphism in *cis*-acting elements may indeed be an important determinant of expression variation and could be subject to adaptive change.

In our study we aimed to test the relative role of positive selection versus a neutral model (including purifying selection) for expression changes at a microevolutionary scale. This includes analysis of gene expression polymorphism and divergence between populations of two subspecies, combined with an analysis of sequence polymorphisms for the same genes and populations. Our study system are house mouse populations that were sampled in the wild. One represents the western subspecies *M. m. domesticus* and the other represents the eastern subspecies *M. m. musculus*. We used quantitative PCR

analysis on a set of genes previously identified in microarray experiments as being differentially expressed between subspecies (Voolstra et al. 2007). We included three different tissues (brain, testis, and liver/kidney) in the study and compared expression polymorphisms with expression divergence. In addition, we obtained sequence polymorphism data, based on direct sequencing of upstream fragments from multiple individuals of each population. These data allow us to address general questions on the patterns of gene expression divergence, the correlation with sequence polymorphism and the role of positive selection in causing expression changes.

Materials and methods

Sampling

Individuals of *M. m. domesticus* were caught in Western Germany, individuals of *M. m. musculus* came from the Czech Republic, as described in Ihle et al. (2006) and Voolstra et al. (2007). All animals were caught at localities not closer than 1km from each other to ensure unrelatedness between individual mice. Animals for qRT-PCR were caught in live traps and kept under controlled conditions for 3-5 days in the laboratory. Six males of each subspecies were chosen for gene expression analysis. These males were of similar age according to size and bodyweight. Additional animals from the same populations were used for analyses of sequence polymorphisms.

DNA and RNA extraction

DNA was extracted from ethanol-stored tissue using 7 ml HOM-Buffer (80 mM EDTA, 100mM Tris and 1% SDS) and 40µl Proteinase K (0.2 mg/ml). After incubation overnight 1g of NaCl was added and the tubes were incubated on ice for 10 min. A wash step with 5 ml chloroform was followed by centrifugation for 1 h at 4000 rcf. DNA was precipitated from the upper phase with 2 volumes ethanol. The pellet was dissolved in TE-Buffer (10 mM Tris/1 mM EDTA). For RNA extraction, tissue samples from brain, testis, and liver/kidney were mechanically homogenized in TRIzol (Invitrogen, Carlsbad, CA). RNA was extracted according to the manufacturers' protocol and either further processed for cDNA synthesis or, after resuspension in DEPC-tretaed H_2O, precipitated in 4 M LiCl for storage at -80°C.

cDNA synthesis

cDNA synthesis was done using ThermoScript RT (Invitrogen) with random hexamers (Fermentas K1612) as primers (1-5µg RNA, 200ng random hexamer primer, 10 mM dNTP mix, 5x synthesis buffer, 0.1M DTT, RNaseOUT (40units/µl), ThermoScript (15 U/µl), DEPC-treated H_2O). Before adding enzyme, DTT, primers, and buffer were incubated with RNA and dNTPs at 65ºC for 5 minutes. After 10 minutes at 25ºC the reaction was carried out at 50ºC for 50 minutes. The reaction was terminated by heating to 85ºC for 5 minutes.

Quantitative real-time PCR (qRT-PCR)

An initial total of 39 candidate genes for expression change in at least one tissue between the sub-populations were chosen randomly from a previous microarray data set (Voolstra et al. 2007). Taqman® gene expression assays (ABI) were used for the qRT-PCR analysis on an ABI 7900HT. These assays span always an exon-intron boundary, to ensure that only mRNA is analysed. Probe and primer binding regions of all Taqman® assays used in this study were sequenced at cDNA level and carefully controlled for polymorphism. Assays that were targeted to polymorphic sequences were removed from the analysis (suppl. Fig. 1 and suppl. Table 1), which resulted in 24 genes that were further studied. All further analysis is based on these 24 genes, including the calculation of correction factors (see below) and the correlations in the results part. qRT-PCR was performed according to the manufacturers' instructions. Three technical replicates of each reaction were processed in the same run under the same conditions. *hprt* (hypoxanthine phosphoribosyltransferase) was included as an internal standard on each plate and was used to make plates comparable to each other. However, contrary to the expectations for a house keeping gene, it turned out to have a variable expression level between individuals and it could therefore not be used directly for normalization (suppl. Fig. 2 and suppl. Table 2). Note that this is a general problem in such experiments (Bustin and Nolan 2004; Dheda et al. 2005). We have therefore explored an approach to obtain a correction factor for this problem. For this, we use the expression information that we have for all genes in the tissue of the respective individual. Since these are randomly chosen, they are not expected to be subject to a systematic up- or downshift. Although some of them are expected to show a specific change, this has only a small influence on the median (see suppl. figures 3 and 4 for further justification of this approach).

To obtain the correction factors, gene expression levels were first calculated for each gene in reference to *hprt* with $[G] = 2^{-\Delta Ct}$. To give each gene the same influence on the correction factor, we normalized this absolute measure, by dividing it by the average of all measures of this gene in the same tissue. This yields an expression level relative to a mean of 1. The median of these expression levels was calculated for all genes per mouse per tissue in the study. The variance of this median was small (suppl. Fig. 4). The resulting 36 correction factors (one per individual and tissue) were used to correct all ΔCt measures. All further analyses were performed with the corrected ΔCts.

Comparing expression levels between subspecies

The subspecies means of ΔCts were compared by a Wilcoxon W rank test. We accounted for multiple testing by controlling the FDR, using the QVALUE R library with bootstrapping and robust method options for limited sample size (Storey et al. 2004).

Calculating expression polymorphism and divergence

The standard deviation of ΔCts within a population does not only reflect the biological standard deviation, but is expected to be inflated by the standard error of the measurement. Hence, a set of individuals with a higher uncertainty in measurement will produce a higher within-group variance. To account for variance introduced into the populations by the technical standard error of the measurement, a regression analysis was performed. The standard error of each ΔCt was calculated by Gaussian Error Propagation:

$$SE(\Delta Ct) = \sqrt{SE(Ct(G)) + (SE(Ct(EC)) + (\frac{SE(Cor)}{Cor \cdot \ln(2)})}$$, where SE(Ct(G)) is the standard error of the three technical replicates of the Ct of the target gene and SE(Ct(EC)) of the endogenous control (*hprt*). Cor is the correction factor of *hprt* over all genes and SE(Cor) its 100,000 times bootstrapped standard error. Bootstrapping of SE(Cor) was performed because Cor is a proportion and not normally distributed. The standard deviation of ΔCt (SD(ΔCt)) within the subspecies for each gene and tissue was regressed against the mean standard error of single measurements within the subspecies. The residuals of this regression represent the biological standard deviation of expression levels within a population, as they are purged from technical variance, and are referred to as expression polymorphism. Scaled expression divergence (ED) was calculated by

transforming average subspecies ΔCts to concentrations and dividing the subspecies' absolute difference by the mean of both subspecies. $ED = \frac{|E(musculus) - E(domesticus)|}{\overline{E_{musdom}}}$

PCR, sequencing, and sequence analysis

The Qiagen Multiplex Kit was used according to the manufacturers' protocol to amplify up to 1kb upstream regions of the genes analyzed in this study. One primer of each pair was placed in the 5´UTR of the gene of interest, where possible (see suppl. Table 3 for primer details). Up to 17 animals were included in the analysis (suppl. Table 4), including the ones for which the expression levels were obtained. The resulting PCR products were sequenced on an ABI 3730 DNA-Analyzer (Applied Biosystems) in both directions. ABI Big Dye terminator mix was used according to the manufacturer's protocol. Obtained sequences were analyzed via Codoncodealigner v2.02 (CodonCode Corporation). Watterson´s θ was calculated using DnaSP v4.0 (Rozas et al. 2003).

Linear modeling

Modeling and regression analysis was performed using the R statistics software package V2.6.2. Watterson´s θs (Watterson 1975) equaling zero (three out of 48 cases) were substituted by the smallest θs in the dataset and log transformed prior to modeling. AICc (Akaike's Information Criterion corrected) and BIC (Bayesian Information Criterion) were computed as described in Sugiura (1978) and Schwarz (1978). AICc corrects AIC (Akaike 1974) for small sample sizes compared to the number of parameters and converges to AIC for larger samples.

Results

We studied the correlation between expression divergence and sequence evolution in a set of genes between wildtype populations of *M. m. musculus* and *M. m. domesticus*. A fragment from the immediate upstream region of these genes was sequenced from each population to determine patterns of sequence polymorphism. Quantitative real-time PCR (qRT-PCR) based on Taqman® assays was used to measure expression level polymorphisms in six unrelated males from each population in three tissues (brain, testis and a standardized mixture of liver and kidney). Statistical analysis and linear modeling

was then used to interpret the results. Although this approach seems straight forward, we identified several technical and statistical issues that can significantly affect the conclusions. Because these are of general importance for comparable studies, we treat them in some detail.

Expression measurements

Quantification of expression levels by qRT-PCR may be impaired by two major factors. The first concerns possible polymorphisms in the primer binding sites, the second concerns polymorphic expression of the reference gene used to calibrate the assays. We have encountered both of these problems and found that not addressing them appropriately would significantly change the results.

To assess polymorphisms in the primer binding regions of the Taqman assays, we sequenced the respective regions. We found polymorphisms or splice variants within the binding sites in 15 out of 39 assays tested. We were able to confirm for some of them that expression levels would be incorrectly recorded if these were unrecognized (see suppl. Figure 1). Hence, we restricted our analyses to 24 genes where no such polymorphisms had been found (suppl. Table 1).

We used *hprt* as a reference gene for all qRT-PCR assays. *hprt* could have been considered as a house keeping gene and was therefore expected to be expressed at similar levels across tissues and individuals. However, comparisons with carefully quantified cDNA amounts used in the assays (Libus and Storchova 2006) showed that this assumption can not be upheld. In fact, *hprt* shows major differences between tissues and individuals (see suppl. Fig. 2 and suppl. Table 2). As a consequence, we decided to use a different approach for calibrating the expression measurements against each other and between runs. In this approach, we essentially use the expression of all genes measured for the same RNA sample as reference (see Methods). Evidently, this assumes that the majority of the other genes does not show changes in the same direction as the test gene for this sample, but this seems a realistic assumption for randomly chosen genes. Accordingly, we consider this a general solution for similar studies (see suppl. file Figures 3 and 4 for further justification of this approach). The expression levels obtained in this way are listed in Table 1. Most of the genes analyzed are expressed in all three tissues, albeit mostly at different levels, indicating tissue specific regulation.

Expression variance

Assessment of the expression variance is hampered by the statistical effect of unequal variances (heteroscedasticity) and the need to separate technical variance from biological variance. We used a new approach to effectively address both problems.

Fig. 1A shows that the variance increases when the measured expression levels become lower. This heteroscedasticity effect is expected when the measured values span several orders of magnitude and is also well known from microarray data (Tusher et al. 2001; Manda et al. 2007). To deal with these problems, we used two different variance measures and subtracted these from each other. The *total* variance that includes the technical and the biological variance is reflected in the standard deviations of measurements for each gene within each of the populations (SD_{Pop}). The *technical* variance is reflected in the standard errors of replicated measurements for a single sample. When one regresses these variance measures against each other, one can take the residuals as a measure of the *biological* variance, purged from technically introduced variance. Note that this calculation implies that negative values can be obtained, whereby the smallest values represent the lowest biological variance. Since the heteroscedasticity is expected to be caused by the technical variance, one expects that the remaining biological variance should not correlate with expression level. Figure 1B shows that this is indeed the case for our data, confirming the validity of the approach. Hence, we use these residuals as our measure of biological variance for further analyses.

Significant expression differences

By combining the expression difference and the variance for each gene, it is possible to assess the number of genes that are significantly differently expressed between the subspecies *M. m. domesticus* and *M. m. musculus*. We found that 12 of the genes tested showed a significant difference in at least one of the tissues (Table 1; Figure 2). It should be noted that this result is not based on a simple fold-change measure. The absolute difference can be small and still significant, if the associated variances in each subspecies are small.

Note that these genes were pre-selected from candidate genes that showed significantly different expression levels in microarray experiments (see Methods). The fact that only half of them showed significant differences in the qRT-PCR assays can be ascribed to the general problems with microarray hybridization signals (Pozhitkov et al. 2007) and

the fact that we controlled for nucleotide polymorphisms in the qRT-PCR experiments in a way that is not possible for microarray experiments. It is generally not unusual that only a part of genes identified in microarray experiments to be differentially expressed can be confirmed by other methods.

Although the genes were pre-selected for signs of expression divergence, they represent nonetheless a random selection with respect to the gene functions covered. In this regard, it is interesting to note that we found nine out of these twelve genes to show a significant difference in only one of the three tissues in which they are expressed. Two genes were expressed in only two tissues and showed a shift in the same direction in both of them. One gene was expressed in testis only (Table 1).

Sequence polymorphisms

To assess sequence polymorphisms and possible signs of positive selection for each gene studied, we sequenced up to 1kb from the immediate upstream regions of the 24 genes for up to 17 animals, including the ones for which the expression data were obtained. This upstream region is expected to harbor regulatory elements for the basic transcriptional machinery, but not necessarily the tissue specific enhancer elements. We calculated Watterson´s θ (Watterson 1975) and Tajima´s D (Tajima 1989) from these data (Table 1).

If positive selection (selective sweeps) were involved in shaping the expression differences between the subspecies, one would expect a decrease in genetic variability as measured by Watterson's θ for the respective gene. To examine this question statistically, we used an approach similar to the lnRH statistics (Kauer et al. 2003), by calculating the absolute of the log transformed ratios of Watterson's θ for each gene between the subspecies. We find that these ratios do not differ significantly between the set of genes differently expressed and the genes with the same expression level in both subspecies (p=0.63, Wilcoxon W). Thus, there is no overall evidence for the involvement of selective sweeps, although this does not exclude the action of sweeps for specific cases.

An indicator for a selection effect on a specific gene would be Tajima´s D, but none of the values is significantly negative (Table 1). On the other hand, the power of the test is expected to be small for our dataset, as there are only a small number of segregating sites (Simonsen et al. 1995). But we do not even find spurious traces of a reduction in

Tajima´s *D* for the whole set of differently expressed genes versus non-differently expressed genes (p=0.49 and p=0.74, ANOVA).

Correlation of sequence and expression variance

It was one of our goals to assess to what extent nucleotide diversity correlates with the variance in expression. However, expression levels and their population variances are likely to differ between subspecies, genes and tissues. We therefore included these factors in a multiple linear regression framework to assess the contributions of each factor.

We compared a set of 18 models that included the factors "subspecies", "tissue" and "$\ln\theta$", using all possible combinations of interaction terms (Table 2). Akaike's corrected Information Criterion (AICc; Sugiura 1978) was used to identify the preferred model. We find that a linear dependence of expression polymorphism on $\ln\theta$ as single factor is the preferred model (Table 2), followed by a model including subspecies as factor. In a backward model selection approach we find that "subspecies" drops out early as a non-significant factor (p=0.18, ANOVA comparing an additive model to the model depending on $\ln\theta$ only). This strongly supports the "$\ln\theta$ only" model as the most likely and most parsimonious explanation. The regression analysis between $\ln\theta$ and expression polymorphism (Figure 3) yields a highly significant correlation ($p<0.01$, $r^2=0.073$), suggesting that genes with a higher sequence variance in the immediate upstream region have also a higher biological expression variance on average.

Correlation of expression variance and expression divergence

To assess whether expression polymorphism correlates with higher expression divergence between the subspecies, we calculated the expression divergences as absolute differences of expression levels between populations scaled by their mean (see Methods). We find that for all tissues, expression divergence correlates highly significantly with expression variance ($p<2\times10^{-4}$, $r^2=0.21$, Figure 4A). Tissue (Table 3) ($p>0.75$) drops out as significant factor in determining expression divergence and hence tissues can be considered independent.

In spite of this overall result, there are also a number of genes with high expression divergence and low expression polymorphism (Figure 4A). Such a pattern would be compatible with a recent action of positive selection, having removed the expression

variance. If selective sweeps would have played a general role, one should see a tendency for reduced expression variance in the lineage where expression divergence has occurred. This would increase the absolute difference of expression variances between the two populations for this locus. Figure 4B shows that the absolute difference of expression variances does not correlate with the ratio of expression divergence and expression polymorphism, i.e. there is no indication for the involvement of selective sweeps on average. Although this result does not exclude the possibility of positive selection shaping expression differences in some cases, it implies that positive selection is not the main determinant.

Discussion

Our study was designed to assess whether the patterns of gene expression and correlated sequence polymorphism in natural populations are compatible with a neutral model of change, or whether there is evidence for a major role of positive selection shaping such change. Fay and Wittkopp (2008) have pointed out that the application of selection tests to gene expression data may be confounded by a number of factors, in particular with respect to choosing the appropriate mutation model. However, testing the compatibility with a neutral model should not be much influenced by these problems, as long as polymorphism data within populations are compared with divergence between populations. Khaitovich et al. (2004) were the first to propose that a constant divergence of gene expression over time can be compared with the molecular clock model that posits a constant divergence of protein sequences over time. The divergence rate in the molecular clock model is only determined by the mutation rate and negative selection (Kimura 1983). It excludes positive selection as a major determinant, although it does not exclude that positive selection can happen occasionally.

In a population context, the neutral model of expression change results in two major predictions, namely that signatures of positive selection are rare and that expression variance within populations correlates with divergence between populations. Under the assumption of a predominance of cis-regulation of genes (e.g. Wittkopp et al. 2004, Tirosh et al. 2009) a third prediction would be that sequence variance at a locus correlates with its expression variance. We find our dataset compatible with all three predictions and will discuss them in turn.

(1) Signatures of positive selection should be rare. Using various tests, none of the genes studied showed a signature of selection. However, the power of our test statistics may not be very high, as the numbers of segregating sites are small. To achieve more statistical power, we compared sequences of the whole group of genes showing expression divergence between the populations with the group of genes which did not show divergence. Again no evidence for selection was detected. The comparisons of the ratios of Waterson's θ should be a particularly sensitive indicator for this, but failed to give a significant difference between the two groups of genes.

(2) Expression variance within populations should correlate with divergence between populations. To assess the mutational variance and divergence relationship, we developed an approach that allowed us to subtract technical variance from the expression data and thus to deal specifically with the biological variance. Based on this, we see indeed a strong correlation between expression polymorphism and divergence. This result indicates that random small changes in expression levels can accumulate over time into a larger change. This also implies a reasonable amount of continuity and additivity of alleles affecting gene expression. Rifkin et al. (2005) have studied mutation accumulation lines of *Drosophila* and found a great mutational potential for regulatory changes. Odom et al. (2007) assessed the binding site turnover of four transcription factors on their target genes between mouse and humans and found that 40-90% changed. These studies are in line with continuous small scale changes at the expression level. On the other hand, gene expression divergence is evidently bound by constraints since it can not diverge *ad infinitum* and still keep its function (Rifkin et al. 2005; Whitehead and Crawford 2006a; Bedford and Hartl 2009).

(3) Sequence variance correlates with expression variance. To obtain a reliable measure for sequence polymorphism in the upstream regions of the genes studied, we sequenced an average of 60 chromosomes per locus. However, the qualitative result, namely that there is indeed a significant correlation between sequence polymorphism and expression polymorphism also holds for the subset of animals for which expression information was obtained (see Figure 3 legend). Our sequence data were obtained from putative basal promoter regions, which might directly play a functional role in the expression variance, although tissue specific enhancers further away from this region are also expected to be involved (see below). On the other hand, given an average LD of 20kb in wild populations of mice (Laurie et al. 2007), our sequence data are likely to represent

the sequence polymorphism of the whole gene region on average. In a similar study in *Drosophila simulans* Lawniczak et al. (2008) found also a correlation between sequence polymorphism and expression variance. However, they distinguished between different gene regions in their analysis and found this correlation predominantly in the transcribed regions, but not for the upstream regions as in our study. While our study and their study differs in several technical aspects, it seems possible that different LD patterns between *Drosophila* and mice might cause this difference.

Given that the three major predictions of a neutral model appear to be fulfilled in our data, one can revisit the question how much positive selection could be involved in shaping expression changes. Given that we had 12 genes in our study that showed a significant expression difference between subspecies, but no significant sign of selection, it still seems possible that in the order of 10% of all genes might show such a sign, but would have remained undetected. This estimate is comparable with data from other population surveys (Rifkin et al. 2003; Whitehead and Crawford 2006b) and also in line with indirect evidence for a role of selection in shaping at least some of the expression divergence in *Drosophila* (Nuzhdin et al. 2004; Lemos et al. 2005b; Holloway et al. 2007). In fact, we have previously described a potential case in the mouse, where a different promoter use is correlated with a selective sweep in the region of the gene (Harr et al. 2006), i.e. selection can occasionally play a role in shaping expression divergence.

Blekhman et al. (2008) studied tissue-specific expression differences between three primate species (macaques, chimpanzees and humans) and found a larger number of specific changes in the human lineage compared to the other two lineages. They suggest that this could be due to repeated positive selection on several genes in the human lineage. However, even in a neutral model one would expect fluctuations among lineages and given that both, the number of differences found, as well as the number of lineages evaluated are small, these findings might also be compatible with only a small fraction of genes evolving expression differences under positive selection.

Still, it is of interest to note that frequently repeated rounds of positive selection could also result in patterns comparable to those predicted by the neutral model. Gillespie (2000) has coined the phrase "genetic draft" for this effect. We have addressed the general question of the frequency of positive selection events in population comparisons within each of the subspecies and found that selection cycles occur every 100 generations on average - and this is a conservative estimate (Teschke et al. 2008). Under

these conditions, genetic hitchhiking effects might shape a significant part of the genome, making the distinction between selection and drift more difficult. Bedford and Hartl (2009) have developed a theoretical framework that shows that even very small selection coefficients could potentially shape expression divergence. It will therefore be necessary to focus future studies on expression evolution on comparisons between very closely related populations, to have the chance to capture ongoing events of positive selection, rather than averaging over long time spans.

An independent issue that is evident in our data is that most (9 out of 12) differentially expressed genes show a significant difference in gene expression in only one tissue. This confirms earlier findings that tissue-specific enhancers may evolve independently of each other. Cowles et al. (2002) found tissue specific effects for two out of four genes with *cis*-regulatory expression difference between inbred mouse strains. Blekhman et al. (2008) used statistical approaches on their microarray data set in primates to estimate the fraction of genes with signs of tissue-specific expression changes under directional selection and find that approximately 10% of the genes studied fall into this class. Our data imply that tissue specific changes may be even more prevalent, supporting the notion that the independent regulatory elements driving gene expression in different tissues have the capacity to evolve independently of each other.

There is currently an intensive discussion on the relative role of gene expression changes in the evolution of developmental processes (Hoekstra and Coyne 2007). While there is compelling direct evidence for a number of such cases (reviewed in Carroll 2008), these usually deal with species comparisons that are far beyond an evolutionary distance where the processes that have led to the change can still be inferred. For example, the well studied regulatory element driving differential expression of sexually dimorphic traits in *Drosophila* has a deep evolutionary ancestry, covering a time span of at least 30 million years (Williams et al. 2008). Clearly, any attempt to approach the question of the relative role of gene expression in creating developmental novelties requires the study of much more closely related species or subspecies. A detailed study of an enhancer element affecting pigmentation between closely related *Drosophila* species (Joeng et al. 2008) has not revealed any evidence for positive selection and is fully compatible with a neutral divergence model. On the other hand, it is a general limitation of most studies dealing with the question of the role of expression changes in a microevolutionary perspective that they have so far only looked at changes in

expression level, but not at developmentally relevant changes, i.e. novel expression in different embryonic regions, different tissues or different times in development. There is clearly a need for specific studies addressing this point, before general conclusions on the role of adaptive regulatory changes in driving phenotypic changes can be drawn.

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MGI Gene Symbol			Gene expression						Genetic diversity	
Acc. Num.	Chr	Subsp	Brain		Liver/kidney		Testis		θ	D
			Expr	Res	Expr	Res	Expr	Res		
1110017D15Rik	4	dom	0.02	-0.51	0.002	-0.26	30.319	0.08	0.52	1.48
AK003742.1		mus	0.021	-0.32	0.002	0.31	45.277	-0.27	2.44	0.73
1700125F08Rik	10	dom	n.e.	n.e.	n.e.	n.e.	1.066	0.47	5.37	0.96
AK007277		mus	n.e.	n.e.	n.e.	n.e.	0.883	0.3	1.03	0.25
Cacng2	15	dom	1.448	-1.45	n.e.	n.e.	0.019	-0.55	0.91	1.78
NM_007583		mus	1.448	-0.74	n.e.	n.e.	0.014	-0.38	1.19	1.97
Ccl25	8	dom	0.182	0.69	0.127	0.02	1.513**	-0.49	1.08	-1.45
NM_009138		mus	0.212	0.5	0.109	0.72	0.943**	0.2	1.37	0.71
Cdk5	5	dom	0.390**	0.39	0.448*	-0.5	n.e.	n.e.	0	n.c.
NM_007668		mus	0.286**	-0.38	0.352*	-0.46	n.e.	n.e.	0.27	0.96
Etd	X	dom	0.004	0.88	n.e.	n.e.	0.78	0.53	0.26	-0.76
NM_175147.2		mus	0.002	0.3	n.e.	n.e.	0.603	-0.28	0.99	1.64
Etv2	7	dom	n.e.	n.e.	n.e.	n.e.	0.199*	1.06	1.37	-0.99
NM_007959		mus	n.e.	n.e.	n.e.	n.e.	0.495*	0.13	0.67	-0.77
Flot2	11	dom	0.294	-0.8	0.659*	0.37	2.077	-0.83	0.29	0.57
NM_008028.1		mus	0.321	0.23	0.386*	0.26	1.991	-0.11	1.48	0.63
Gpc6	14	dom	0.173**	-0.1	0.254	-0.59	0.215	0.63	0.45	0.96
NM_011821		mus	0.125**	0	0.266	0.02	0.255	0.69	4.99	-1.05
Hif1a	12	dom	0.602	0.14	2.074	-0.7	2.538	-0.57	2.21	1.35
NM_010431		mus	0.543	0.51	1.741	-0.1	2.205	0.5	1.8	1.25
Kcnd2	6	dom	1.739*	-0.4	0.040*	-0.16	n.e.	n.e.	1.97	0.84
NM_019697		mus	1.488*	-0.17	0.015*	-0.54	n.e.	n.e.	1.12	1.57
Krt2-17	15	dom	0.003	0.63	n.e.	n.e.	n.e.	n.e.	3.29	0.23
NM_010668		mus	0.002	0.11	n.e.	n.e.	n.e.	n.e.	0	n.c.
Mir16	7	dom	0.733	-1	2.869	-1.16	4.329	0.44	0.4	0.57
NM_019580		mus	0.721	-0.33	2.964	-0.15	5.319	-0.29	1.99	-0.87
Nf1	11	dom	0.567	-1.13	0.503	-0.38	3.62	-0.16	0.98	1.25
NM_010897.1		mus	0.54	0.11	0.482	-0.3	3.311	0.65	0	n.c.
PanX1	9	dom	0.072	0.62	0.025	-0.01	0.233	-0.04	3.94	1.58
NM_019482		mus	0.082	0.16	0.038	-0.32	0.221	-0.11	1.32	-0.18
Ppt1	4	dom	1.081	0.22	1.737	-0.07	20.933**	-0.85	1.28	2.43
NM_008917.1		mus	1.208	-0.3	1.795	-0.66	17.291**	-0.97	0.82	-1.5
Rab4b	7	dom	0.188	-1.21	0.203	-1.29	1.211	-1.63	0.75	0.6
NM_029391.1		mus	0.203	-0.48	0.244	-0.29	1.579	0.52	0.75	-0.89
Rarres2	6	dom	0.051**	1.07	3.183	0.97	0.393	0.23	3.04	1.58
NM_027852.1		mus	0.114**	0.6	3.963	1.04	0.423	0.6	2.1	1.91
Rgs16	1	dom	0.143*	0.01	0.768	1.82	0.051	-0.14	1.55	2.38
NM_011267.1		mus	0.194*	0.04	1.807	1.48	0.075	0.2	2.51	-1.31
Scamp5	9	dom	0.55	0.15	0.321	0.24	0.168*	0.04	4.07	-1.73
NM_020270		mus	0.498	0.42	0.28	0.3	0.245*	0.15	0.5	1.61
Sv2c	13	dom	0.974**	0.05	0.002	0.36	0.264	0.06	3	1.77
AK173092.1		mus	0.788**	-0.82	0.003	1.49	0.392	1.62	2.09	0.3

Tcte3	17	dom	0.001	0.05	0.004	0.8	48.593	0.08	1.06	1.51
NM_011560.2		mus	0.001	-0.68	0.007	0.9	48.673	0.45	1.52	-1.21
Tmem24	9	dom	0.42	0.09	0.604	-1.12	0.413	-0.18	0.36	-1.14
NM_027909.1		mus	0.493	0.57	0.707	-0.23	0.305	0.16	0.75	1.4
Tomm40l	1	dom	0.295	0.04	0.344	-0.2	0.498*	0.13	1.37	0.14
AK186544.1		mus	0.281	-1.43	0.293	-0.2	0.340*	0.16	0.82	0.24

Table 1

Summary of expression and sequence polymorphism data for all genes. Expr = linear expression levels based on ΔCt values, Res = residuals of the regression of the variances (see text). θ = Watterson´s θ (Watterson 1975) x 1000, D = Tajima´s D (Tajima 1989). Stars denote value pairs that represent significantly different expression levels between the subspecies, ** = p< 0.01, * = p<0.05

model	k	AICc	ΔAICc	wAICc	BIC
θ	3	233.858	0	0.322	242.119
Subspecies + θ	4	234.154	0.296	0.278	245.099
Subspecies * θ	5	236.254	2.396	0.097	249.847
Tissue * θ	7	237.249	3.391	0.059	256.026
Tissue + Subspecies + θ + Tissue:θ	8	237.663	3.805	0.048	258.973
Tissue + θ	5	237.159	3.301	0.062	250.752
Tissue + Subspecies + θ	6	237.507	3.649	0.052	253.711
Tissue + Subspecies + θ + Subspecies:θ + Tissue:θ	9	239.834	5.976	0.016	263.638
Tissue + Subspecies + θ + Subspecies:θ	7	239.669	5.811	0.018	258.446
Tissue + Subspecies + θ + Tissue:Subspecies + Tissue:θ	10	240.786	6.928	0.01	267.042
Tissue + Subspecies + θ + Tissue:Subspecies	8	240.448	6.59	0.012	261.758
Tissue + Subspecies + θ + Subspecies:θ + Tissue:θ + Tissue:Subspecies	11	242.984	9.126	0.003	271.650
Subspecies	3	241.105	7.248	0.009	249.366
Nullmodel	2	241.13	7.272	0.008	246.672
Tissue * Subspecies * θ	13	246.803	12.945	0	280.157
Tissue + Subspecies	5	244.187	10.329	0.002	257.780
Tissue	4	244.161	10.304	0.002	255.106
Tissue * Subspecies	7	247.061	13.203	0	265.838

Table 2

Linear model comparison of factors determining θ. Models are sorted by goodness of fit with a penalty for number of parameters in the model (AICc)**.** k: Number of parameters in the model, AICc: Akaike's Information Criterion corrected for sample size, wAICc: Akaike weights corrected for sample size (comparable to r^2), BIC: Bayesian Information Criterion or Schwarz Criterion, + indicates additivity, : indicates interaction

model	k	AICc	deltaAICc	wAICc	BIC
Polymorphism	3	-13.339	0	0.866	-7.37141
Polymorphism+Tissue	5	-9.294	4.045	0.115	0.270279
Polymorphism*Tissue	7	-5.483	7.856	0.017	7.333185
Nullmodel	2	-0.748	12.59	0.002	3.302666
Tissue	4	2.791	16.13	0	10.59733

Table 3

Linear model comparison for factors determining expression divergence, abbreviations as in Table 2.

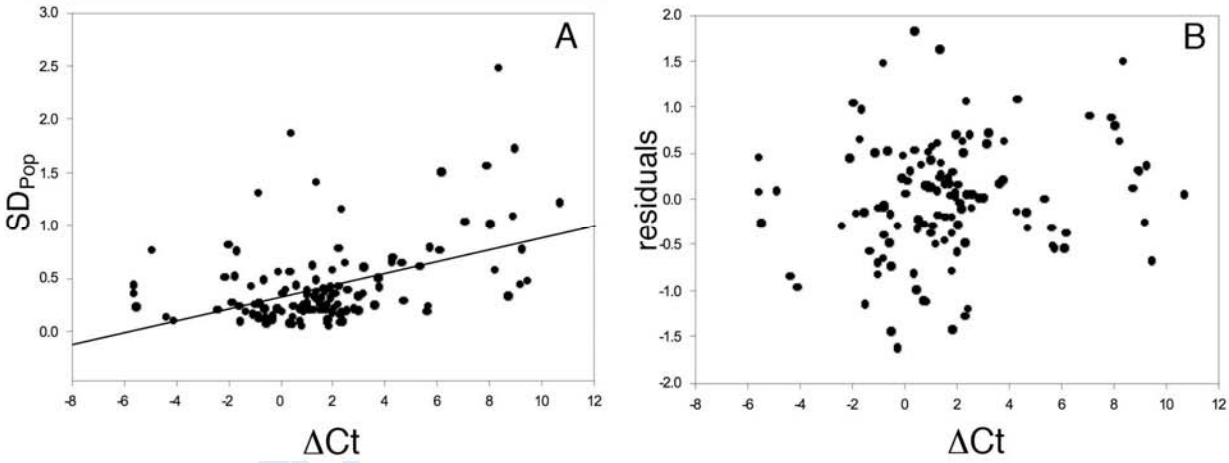

Figure 1

Dependence of measures for expression polymorphism on expression level of a gene in a given tissue (ΔCt) before and after addressing technical variance effects. (A) Heteroscedasticity of ΔCt values. The standard deviation of expression measurements within a population (SD_{Pop}) includes biological and technical effects and depends on expression levels ΔCt ($p<3x10^{-7}$ and $r^2=0.2$, Pearson's correlation). (B) The residuals of the regression of ln(SD) on ln(SE) (referred to in this study as expression polymorphism) are independent of the expression level ($p>0.1$ and $r^2<0.04$, Pearson's correlation).

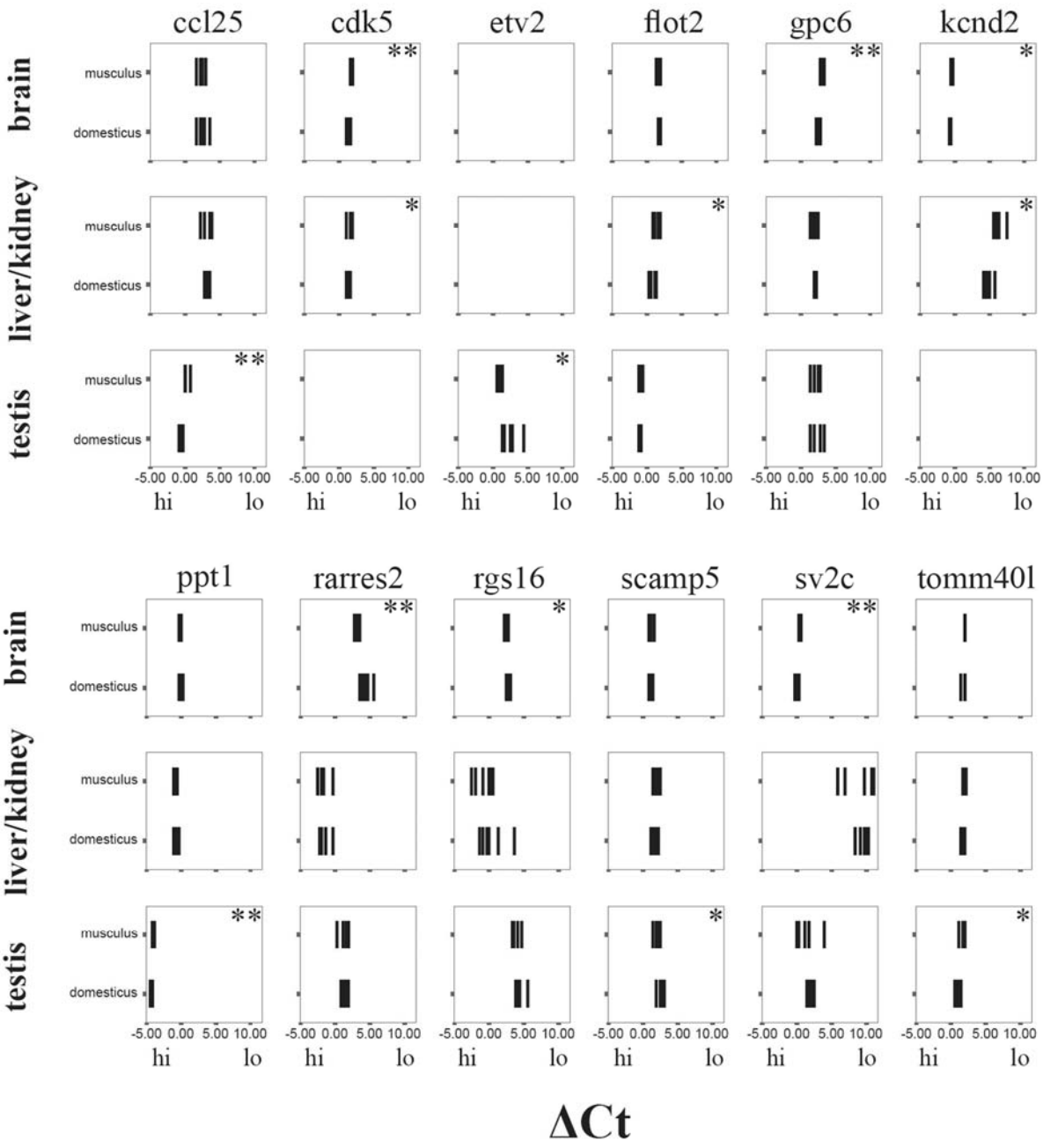

Figure 2

Expression levels of genes differently expressed between house mouse subspecies. Stars denote pairs that represent significantly different expression levels between the subspecies, ** = $p < 0.01$, * = $p < 0.05$. Each vertical bar represents the expression level of an individual mouse represented by the respective ΔCt measurement. Small ΔCts indicate high (hi) expression levels and large ΔCts indicate low (lo) expression levels.

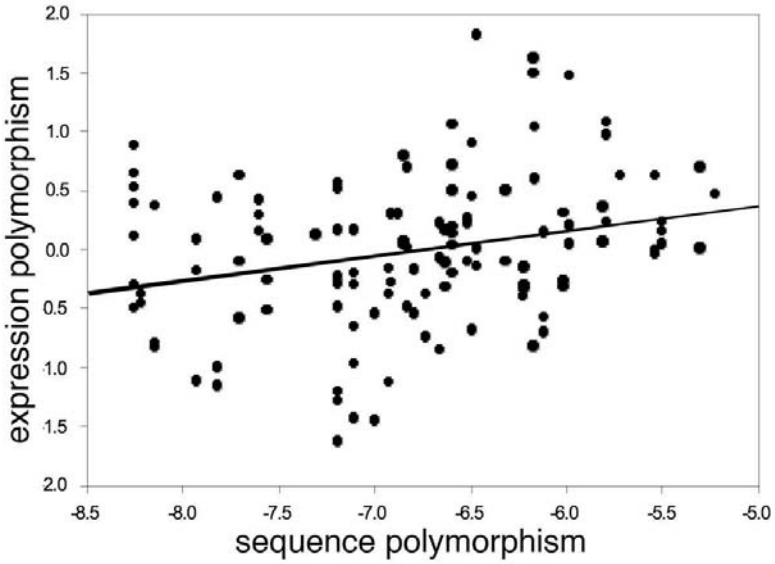

Figure 3

Expression polymorphism (residuals) correlates with sequence polymorphism (ln(θ)). Each dot represents the expression polymorphism in a given tissue in one of the populations (r^2=0.073, $p<0.01$, Pearson's product moment correlation). ln(θ) is based on nucleotide polymorphism in the upstream region of the respective gene in the respective population, based on up to 17 sequenced individuals. The results are similar, if one would use only the sequence information from the six individuals that were used for the expression studies (r^2=0.055, $p<0.01$).

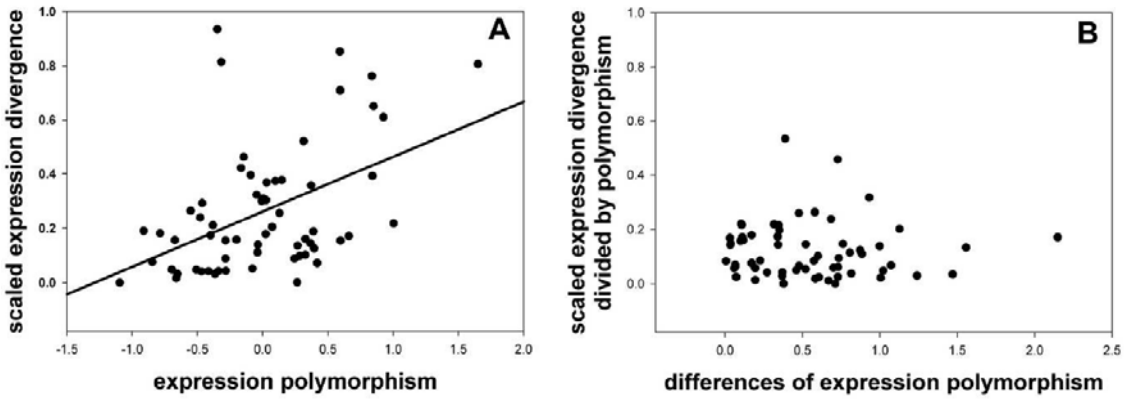

Figure 4

Relationships between expression polymorphism and divergence. (A) Expression divergence between populations (scaled ΔCt values) correlates with expression polymorphism (residuals) within populations. Each dot represents the value for a given gene in a given tissue (r^2=0.21, $p<2x10^{-4}$, Pearson's product moment correlation). (B) The ratio of scaled expression divergence and polymorphism between populations does not correlate with differences of expression polymorphism (absolute difference of residuals) between populations (r^2=-0.029, p=0.89, Pearson's product moment correlation). Lowest polymorphism (residual) was set to equal 1 by adding a constant to all polymorphism measurements in advance.

A test of the neutral model of expression change in natural populations of house mouse subspecies

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key words: *Mus musculus musculus*, *M. m. domesticus*, evolution of gene expression, quantitative real time PCR

Summary

Changes in expression of genes are thought to contribute significantly to evolutionary divergence. To study the relative role of selection and neutrality in shaping expression changes, we analysed 24 genes in three different tissues of the house mouse (*Mus musculus*). Samples from two natural populations of the subspecies *M. m. domesticus* and *M. m. musculus* were investigated using quantitative PCR assays and sequencing of the upstream region. We have developed an approach to quantify expression polymorphism within such populations and to disentangle technical from biological variation in the data. We found a correlation between expression polymorphism within populations and divergence between populations. Furthermore, we found a correlation between expression polymorphism and sequence polymorphism of the respective genes. These data are most easily interpreted within a framework of a predominantly neutral model of gene expression change, where only a fraction of up to 10% of the changes may have been driven by positive selection. Although most genes investigated were expressed in all three tissues analyzed, significant changes of expression levels occurred predominantly in a single tissue only. This adds to the notion that enhancer-specific effects or transregulatory effects can modulate the evolution of gene expression in a tissue-specific way.

Introduction

The relative role of expression changes versus protein coding changes in shaping evolutionary divergence is still a matter of vivid debate (Tautz 2000; Lemos et al. 2005a; Hoekstra and Coyne 2007; Wray 2007; Carroll 2008; Fay and Wittkopp 2008). Although our knowledge on intra- and interspecific variation and change in gene regulation is growing fast, it is still not clear which general role regulatory variation plays in adaptations. Existing estimates of the proportion of genes under directional or stabilizing selection show wide ranges (Rifkin et al. 2003; Lemos et al. 2005a; Whitehead and Crawford 2006a). Khaitovich et al. (2004, 2005) proposed a predominantly neutral model of transcriptome evolution based on microarray data in primates. They argue that an approximately linear accumulation of expression differences over time, as well as a correlation between expression variance within a

species and expression difference between species are signs for evolutionary divergence processes that are best explained in the context of predominantly neutral changes. However, their conclusions have been critizised on the grounds that their calibration was based on the assumption of neutrality of the pseudogene expression, which may not hold (Svensson et al. 2006). On the other hand, this problem would only affect the question of how much purifying selection needs to be taken into account, but does not change the conclusions with respect to the role of positive selection.

Blekhman et al. (2008) have taken a comparative approach along the same lines and report an excess of regulatory changes in the human lineage compared to chimpanzee and rhesus macaques. This would suggest that there is, in addition to neutral and purifying selection patterns, evidence for positive selection in the human lineage. In a different type of study on expression differences within and between *Drosophila* species, Wittkopp et al. (2008) found a predominance of *cis*-regulatory changes in comparisons between species, which would also suggest an involvement of positive selection. However, both of these studies do not directly provide data on the frequency of positive selection versus neutral change.

To infer microevolutionary mechanisms underlying expression evolution, gene expression patterns should be analysed together with sequence polymorphisms and changes. A first study integrating such data was conducted by Brown and Feder (2005). They used microarray data to choose gene sets with gene expression differences between *Drosophila* strains and sequenced the upstream regions of such genes. They did not find a correlation between sequence change and expression change, which would argue for a decoupling of these parameters. However, their data basis was small and Holloway et al. (2007) and Lawniczak et al. (2008) found in more extensive data sets from *Drosophila simulans* strains that sequence polymorphism in *cis*-acting elements may indeed be an important determinant of expression variation and could be subject to adaptive change.

In our study we aimed to test the relative role of positive selection versus a neutral model (including purifying selection) for expression changes at a microevolutionary scale. This includes analysis of gene expression polymorphism and divergence between populations of two subspecies, combined with an analysis of sequence polymorphisms for the same genes and populations. Our study system are house mouse populations that were sampled in the wild. One represents the western subspecies *M. m. domesticus* and the other represents the eastern subspecies *M. m. musculus*. We used quantitative PCR

analysis on a set of genes previously identified in microarray experiments as being differentially expressed between subspecies (Voolstra et al. 2007). We included three different tissues (brain, testis, and liver/kidney) in the study and compared expression polymorphisms with expression divergence. In addition, we obtained sequence polymorphism data, based on direct sequencing of upstream fragments from multiple individuals of each population. These data allow us to address general questions on the patterns of gene expression divergence, the correlation with sequence polymorphism and the role of positive selection in causing expression changes.

Materials and methods

Sampling

Individuals of *M. m. domesticus* were caught in Western Germany, individuals of *M. m. musculus* came from the Czech Republic, as described in Ihle et al. (2006) and Voolstra et al. (2007). All animals were caught at localities not closer than 1km from each other to ensure unrelatedness between individual mice. Animals for qRT-PCR were caught in live traps and kept under controlled conditions for 3-5 days in the laboratory. Six males of each subspecies were chosen for gene expression analysis. These males were of similar age according to size and bodyweight. Additional animals from the same populations were used for analyses of sequence polymorphisms.

DNA and RNA extraction

DNA was extracted from ethanol-stored tissue using 7 ml HOM-Buffer (80 mM EDTA, 100mM Tris and 1% SDS) and 40µl Proteinase K (0.2 mg/ml). After incubation overnight 1g of NaCl was added and the tubes were incubated on ice for 10 min. A wash step with 5 ml chloroform was followed by centrifugation for 1 h at 4000 rcf. DNA was precipitated from the upper phase with 2 volumes ethanol. The pellet was dissolved in TE-Buffer (10 mM Tris/1 mM EDTA). For RNA extraction, tissue samples from brain, testis, and liver/kidney were mechanically homogenized in TRIzol (Invitrogen, Carlsbad, CA). RNA was extracted according to the manufacturers' protocol and either further processed for cDNA synthesis or, after resuspension in DEPC-tretaed H_2O, precipitated in 4 M LiCl for storage at -80°C.

cDNA synthesis

cDNA synthesis was done using ThermoScript RT (Invitrogen) with random hexamers (Fermentas K1612) as primers (1-5µg RNA, 200ng random hexamer primer, 10 mM dNTP mix, 5x synthesis buffer, 0.1M DTT, RNaseOUT (40units/µl), ThermoScript (15 U/µl), DEPC-treated H_2O). Before adding enzyme, DTT, primers, and buffer were incubated with RNA and dNTPs at 65ºC for 5 minutes. After 10 minutes at 25ºC the reaction was carried out at 50ºC for 50 minutes. The reaction was terminated by heating to 85ºC for 5 minutes.

Quantitative real-time PCR (qRT-PCR)

An initial total of 39 candidate genes for expression change in at least one tissue between the sub-populations were chosen randomly from a previous microarray data set (Voolstra et al. 2007). Taqman® gene expression assays (ABI) were used for the qRT-PCR analysis on an ABI 7900HT. These assays span always an exon-intron boundary, to ensure that only mRNA is analysed. Probe and primer binding regions of all Taqman® assays used in this study were sequenced at cDNA level and carefully controlled for polymorphism. Assays that were targeted to polymorphic sequences were removed from the analysis (suppl. Fig. 1 and suppl. Table 1), which resulted in 24 genes that were further studied. All further analysis is based on these 24 genes, including the calculation of correction factors (see below) and the correlations in the results part. qRT-PCR was performed according to the manufacturers' instructions. Three technical replicates of each reaction were processed in the same run under the same conditions. *hprt* (hypoxanthine phosphoribosyltransferase) was included as an internal standard on each plate and was used to make plates comparable to each other. However, contrary to the expectations for a house keeping gene, it turned out to have a variable expression level between individuals and it could therefore not be used directly for normalization (suppl. Fig. 2 and suppl. Table 2). Note that this is a general problem in such experiments (Bustin and Nolan 2004; Dheda et al. 2005). We have therefore explored an approach to obtain a correction factor for this problem. For this, we use the expression information that we have for all genes in the tissue of the respective individual. Since these are randomly chosen, they are not expected to be subject to a systematic up- or downshift. Although some of them are expected to show a specific change, this has only a small influence on the median (see suppl. figures 3 and 4 for further justification of this approach).

To obtain the correction factors, gene expression levels were first calculated for each gene in reference to *hprt* with $[G] = 2^{-\Delta Ct}$. To give each gene the same influence on the correction factor, we normalized this absolute measure, by dividing it by the average of all measures of this gene in the same tissue. This yields an expression level relative to a mean of 1. The median of these expression levels was calculated for all genes per mouse per tissue in the study. The variance of this median was small (suppl. Fig. 4). The resulting 36 correction factors (one per individual and tissue) were used to correct all ΔCt measures. All further analyses were performed with the corrected ΔCts.

Comparing expression levels between subspecies

The subspecies means of ΔCts were compared by a Wilcoxon W rank test. We accounted for multiple testing by controlling the FDR, using the QVALUE R library with bootstrapping and robust method options for limited sample size (Storey et al. 2004).

Calculating expression polymorphism and divergence

The standard deviation of ΔCts within a population does not only reflect the biological standard deviation, but is expected to be inflated by the standard error of the measurement. Hence, a set of individuals with a higher uncertainty in measurement will produce a higher within-group variance. To account for variance introduced into the populations by the technical standard error of the measurement, a regression analysis was performed. The standard error of each ΔCt was calculated by Gaussian Error Propagation:

$$SE(\Delta Ct) = \sqrt{SE(Ct(G)) + (SE(Ct(EC)) + (\frac{SE(Cor)}{Cor \cdot \ln(2)})}$$, where SE(Ct(G)) is the standard error of the three technical replicates of the Ct of the target gene and SE(Ct(EC)) of the endogenous control (*hprt*). Cor is the correction factor of *hprt* over all genes and SE(Cor) its 100,000 times bootstrapped standard error. Bootstrapping of SE(Cor) was performed because Cor is a proportion and not normally distributed. The standard deviation of ΔCt (SD(ΔCt)) within the subspecies for each gene and tissue was regressed against the mean standard error of single measurements within the subspecies. The residuals of this regression represent the biological standard deviation of expression levels within a population, as they are purged from technical variance, and are referred to as expression polymorphism. Scaled expression divergence (ED) was calculated by

transforming average subspecies ΔCts to concentrations and dividing the subspecies' absolute difference by the mean of both subspecies. $ED = \frac{|E(musculus) - E(domesticus)|}{\overline{E_{musdom}}}$

PCR, sequencing, and sequence analysis

The Qiagen Multiplex Kit was used according to the manufacturers' protocol to amplify up to 1kb upstream regions of the genes analyzed in this study. One primer of each pair was placed in the 5´UTR of the gene of interest, where possible (see suppl. Table 3 for primer details). Up to 17 animals were included in the analysis (suppl. Table 4), including the ones for which the expression levels were obtained. The resulting PCR products were sequenced on an ABI 3730 DNA-Analyzer (Applied Biosystems) in both directions. ABI Big Dye terminator mix was used according to the manufacturer's protocol. Obtained sequences were analyzed via Codoncodealigner v2.02 (CodonCode Corporation). Watterson´s θ was calculated using DnaSP v4.0 (Rozas et al. 2003).

Linear modeling

Modeling and regression analysis was performed using the R statistics software package V2.6.2. Watterson´s θs (Watterson 1975) equaling zero (three out of 48 cases) were substituted by the smallest θs in the dataset and log transformed prior to modeling. AICc (Akaike's Information Criterion corrected) and BIC (Bayesian Information Criterion) were computed as described in Sugiura (1978) and Schwarz (1978). AICc corrects AIC (Akaike 1974) for small sample sizes compared to the number of parameters and converges to AIC for larger samples.

Results

We studied the correlation between expression divergence and sequence evolution in a set of genes between wildtype populations of *M. m. musculus* and *M. m. domesticus*. A fragment from the immediate upstream region of these genes was sequenced from each population to determine patterns of sequence polymorphism. Quantitative real-time PCR (qRT-PCR) based on Taqman® assays was used to measure expression level polymorphisms in six unrelated males from each population in three tissues (brain, testis and a standardized mixture of liver and kidney). Statistical analysis and linear modeling

was then used to interpret the results. Although this approach seems straight forward, we identified several technical and statistical issues that can significantly affect the conclusions. Because these are of general importance for comparable studies, we treat them in some detail.

Expression measurements

Quantification of expression levels by qRT-PCR may be impaired by two major factors. The first concerns possible polymorphisms in the primer binding sites, the second concerns polymorphic expression of the reference gene used to calibrate the assays. We have encountered both of these problems and found that not addressing them appropriately would significantly change the results.

To assess polymorphisms in the primer binding regions of the Taqman assays, we sequenced the respective regions. We found polymorphisms or splice variants within the binding sites in 15 out of 39 assays tested. We were able to confirm for some of them that expression levels would be incorrectly recorded if these were unrecognized (see suppl. Figure 1). Hence, we restricted our analyses to 24 genes where no such polymorphisms had been found (suppl. Table 1).

We used *hprt* as a reference gene for all qRT-PCR assays. *hprt* could have been considered as a house keeping gene and was therefore expected to be expressed at similar levels across tissues and individuals. However, comparisons with carefully quantified cDNA amounts used in the assays (Libus and Storchova 2006) showed that this assumption can not be upheld. In fact, *hprt* shows major differences between tissues and individuals (see suppl. Fig. 2 and suppl. Table 2). As a consequence, we decided to use a different approach for calibrating the expression measurements against each other and between runs. In this approach, we essentially use the expression of all genes measured for the same RNA sample as reference (see Methods). Evidently, this assumes that the majority of the other genes does not show changes in the same direction as the test gene for this sample, but this seems a realistic assumption for randomly chosen genes. Accordingly, we consider this a general solution for similar studies (see suppl. file Figures 3 and 4 for further justification of this approach). The expression levels obtained in this way are listed in Table 1. Most of the genes analyzed are expressed in all three tissues, albeit mostly at different levels, indicating tissue specific regulation.

Expression variance

Assessment of the expression variance is hampered by the statistical effect of unequal variances (heteroscedasticity) and the need to separate technical variance from biological variance. We used a new approach to effectively address both problems.

Fig. 1A shows that the variance increases when the measured expression levels become lower. This heteroscedasticity effect is expected when the measured values span several orders of magnitude and is also well known from microarray data (Tusher et al. 2001; Manda et al. 2007). To deal with these problems, we used two different variance measures and subtracted these from each other. The *total* variance that includes the technical and the biological variance is reflected in the standard deviations of measurements for each gene within each of the populations (SD_{Pop}). The *technical* variance is reflected in the standard errors of replicated measurements for a single sample. When one regresses these variance measures against each other, one can take the residuals as a measure of the *biological* variance, purged from technically introduced variance. Note that this calculation implies that negative values can be obtained, whereby the smallest values represent the lowest biological variance. Since the heteroscedasticity is expected to be caused by the technical variance, one expects that the remaining biological variance should not correlate with expression level. Figure 1B shows that this is indeed the case for our data, confirming the validity of the approach. Hence, we use these residuals as our measure of biological variance for further analyses.

Significant expression differences

By combining the expression difference and the variance for each gene, it is possible to assess the number of genes that are significantly differently expressed between the subspecies *M. m. domesticus* and *M. m. musculus*. We found that 12 of the genes tested showed a significant difference in at least one of the tissues (Table 1; Figure 2). It should be noted that this result is not based on a simple fold-change measure. The absolute difference can be small and still significant, if the associated variances in each subspecies are small.

Note that these genes were pre-selected from candidate genes that showed significantly different expression levels in microarray experiments (see Methods). The fact that only half of them showed significant differences in the qRT-PCR assays can be ascribed to the general problems with microarray hybridization signals (Pozhitkov et al. 2007) and

the fact that we controlled for nucleotide polymorphisms in the qRT-PCR experiments in a way that is not possible for microarray experiments. It is generally not unusual that only a part of genes identified in microarray experiments to be differentially expressed can be confirmed by other methods.

Although the genes were pre-selected for signs of expression divergence, they represent nonetheless a random selection with respect to the gene functions covered. In this regard, it is interesting to note that we found nine out of these twelve genes to show a significant difference in only one of the three tissues in which they are expressed. Two genes were expressed in only two tissues and showed a shift in the same direction in both of them. One gene was expressed in testis only (Table 1).

Sequence polymorphisms

To assess sequence polymorphisms and possible signs of positive selection for each gene studied, we sequenced up to 1kb from the immediate upstream regions of the 24 genes for up to 17 animals, including the ones for which the expression data were obtained. This upstream region is expected to harbor regulatory elements for the basic transcriptional machinery, but not necessarily the tissue specific enhancer elements. We calculated Watterson´s θ (Watterson 1975) and Tajima´s D (Tajima 1989) from these data (Table 1).

If positive selection (selective sweeps) were involved in shaping the expression differences between the subspecies, one would expect a decrease in genetic variability as measured by Watterson's θ for the respective gene. To examine this question statistically, we used an approach similar to the lnRH statistics (Kauer et al. 2003), by calculating the absolute of the log transformed ratios of Watterson's θ for each gene between the subspecies. We find that these ratios do not differ significantly between the set of genes differently expressed and the genes with the same expression level in both subspecies (p=0.63, Wilcoxon W). Thus, there is no overall evidence for the involvement of selective sweeps, although this does not exclude the action of sweeps for specific cases.

An indicator for a selection effect on a specific gene would be Tajima´s D, but none of the values is significantly negative (Table 1). On the other hand, the power of the test is expected to be small for our dataset, as there are only a small number of segregating sites (Simonsen et al. 1995). But we do not even find spurious traces of a reduction in

Tajima´s *D* for the whole set of differently expressed genes versus non-differently expressed genes (p=0.49 and p=0.74, ANOVA).

Correlation of sequence and expression variance

It was one of our goals to assess to what extent nucleotide diversity correlates with the variance in expression. However, expression levels and their population variances are likely to differ between subspecies, genes and tissues. We therefore included these factors in a multiple linear regression framework to assess the contributions of each factor.

We compared a set of 18 models that included the factors "subspecies", "tissue" and "$\ln\theta$", using all possible combinations of interaction terms (Table 2). Akaike's corrected Information Criterion (AICc; Sugiura 1978) was used to identify the preferred model. We find that a linear dependence of expression polymorphism on $\ln\theta$ as single factor is the preferred model (Table 2), followed by a model including subspecies as factor. In a backward model selection approach we find that "subspecies" drops out early as a non-significant factor (p=0.18, ANOVA comparing an additive model to the model depending on $\ln\theta$ only). This strongly supports the "$\ln\theta$ only" model as the most likely and most parsimonious explanation. The regression analysis between $\ln\theta$ and expression polymorphism (Figure 3) yields a highly significant correlation ($p<0.01$, $r^2=0.073$), suggesting that genes with a higher sequence variance in the immediate upstream region have also a higher biological expression variance on average.

Correlation of expression variance and expression divergence

To assess whether expression polymorphism correlates with higher expression divergence between the subspecies, we calculated the expression divergences as absolute differences of expression levels between populations scaled by their mean (see Methods). We find that for all tissues, expression divergence correlates highly significantly with expression variance ($p<2\times10^{-4}$, $r^2=0.21$, Figure 4A). Tissue (Table 3) ($p>0.75$) drops out as significant factor in determining expression divergence and hence tissues can be considered independent.

In spite of this overall result, there are also a number of genes with high expression divergence and low expression polymorphism (Figure 4A). Such a pattern would be compatible with a recent action of positive selection, having removed the expression

variance. If selective sweeps would have played a general role, one should see a tendency for reduced expression variance in the lineage where expression divergence has occurred. This would increase the absolute difference of expression variances between the two populations for this locus. Figure 4B shows that the absolute difference of expression variances does not correlate with the ratio of expression divergence and expression polymorphism, i.e. there is no indication for the involvement of selective sweeps on average. Although this result does not exclude the possibility of positive selection shaping expression differences in some cases, it implies that positive selection is not the main determinant.

Discussion

Our study was designed to assess whether the patterns of gene expression and correlated sequence polymorphism in natural populations are compatible with a neutral model of change, or whether there is evidence for a major role of positive selection shaping such change. Fay and Wittkopp (2008) have pointed out that the application of selection tests to gene expression data may be confounded by a number of factors, in particular with respect to choosing the appropriate mutation model. However, testing the compatibility with a neutral model should not be much influenced by these problems, as long as polymorphism data within populations are compared with divergence between populations. Khaitovich et al. (2004) were the first to propose that a constant divergence of gene expression over time can be compared with the molecular clock model that posits a constant divergence of protein sequences over time. The divergence rate in the molecular clock model is only determined by the mutation rate and negative selection (Kimura 1983). It excludes positive selection as a major determinant, although it does not exclude that positive selection can happen occasionally.

In a population context, the neutral model of expression change results in two major predictions, namely that signatures of positive selection are rare and that expression variance within populations correlates with divergence between populations. Under the assumption of a predominance of cis-regulation of genes (e.g. Wittkopp et al. 2004, Tirosh et al. 2009) a third prediction would be that sequence variance at a locus correlates with its expression variance. We find our dataset compatible with all three predictions and will discuss them in turn.

(1) Signatures of positive selection should be rare. Using various tests, none of the genes studied showed a signature of selection. However, the power of our test statistics may not be very high, as the numbers of segregating sites are small. To achieve more statistical power, we compared sequences of the whole group of genes showing expression divergence between the populations with the group of genes which did not show divergence. Again no evidence for selection was detected. The comparisons of the ratios of Waterson's θ should be a particularly sensitive indicator for this, but failed to give a significant difference between the two groups of genes.

(2) Expression variance within populations should correlate with divergence between populations. To assess the mutational variance and divergence relationship, we developed an approach that allowed us to subtract technical variance from the expression data and thus to deal specifically with the biological variance. Based on this, we see indeed a strong correlation between expression polymorphism and divergence. This result indicates that random small changes in expression levels can accumulate over time into a larger change. This also implies a reasonable amount of continuity and additivity of alleles affecting gene expression. Rifkin et al. (2005) have studied mutation accumulation lines of *Drosophila* and found a great mutational potential for regulatory changes. Odom et al. (2007) assessed the binding site turnover of four transcription factors on their target genes between mouse and humans and found that 40-90% changed. These studies are in line with continuous small scale changes at the expression level. On the other hand, gene expression divergence is evidently bound by constraints since it can not diverge *ad infinitum* and still keep its function (Rifkin et al. 2005; Whitehead and Crawford 2006a; Bedford and Hartl 2009).

(3) Sequence variance correlates with expression variance. To obtain a reliable measure for sequence polymorphism in the upstream regions of the genes studied, we sequenced an average of 60 chromosomes per locus. However, the qualitative result, namely that there is indeed a significant correlation between sequence polymorphism and expression polymorphism also holds for the subset of animals for which expression information was obtained (see Figure 3 legend). Our sequence data were obtained from putative basal promoter regions, which might directly play a functional role in the expression variance, although tissue specific enhancers further away from this region are also expected to be involved (see below). On the other hand, given an average LD of 20kb in wild populations of mice (Laurie et al. 2007), our sequence data are likely to represent

the sequence polymorphism of the whole gene region on average. In a similar study in *Drosophila simulans* Lawniczak et al. (2008) found also a correlation between sequence polymorphism and expression variance. However, they distinguished between different gene regions in their analysis and found this correlation predominantly in the transcribed regions, but not for the upstream regions as in our study. While our study and their study differs in several technical aspects, it seems possible that different LD patterns between *Drosophila* and mice might cause this difference.

Given that the three major predictions of a neutral model appear to be fulfilled in our data, one can revisit the question how much positive selection could be involved in shaping expression changes. Given that we had 12 genes in our study that showed a significant expression difference between subspecies, but no significant sign of selection, it still seems possible that in the order of 10% of all genes might show such a sign, but would have remained undetected. This estimate is comparable with data from other population surveys (Rifkin et al. 2003; Whitehead and Crawford 2006b) and also in line with indirect evidence for a role of selection in shaping at least some of the expression divergence in *Drosophila* (Nuzhdin et al. 2004; Lemos et al. 2005b; Holloway et al. 2007). In fact, we have previously described a potential case in the mouse, where a different promoter use is correlated with a selective sweep in the region of the gene (Harr et al. 2006), i.e. selection can occasionally play a role in shaping expression divergence.

Blekhman et al. (2008) studied tissue-specific expression differences between three primate species (macaques, chimpanzees and humans) and found a larger number of specific changes in the human lineage compared to the other two lineages. They suggest that this could be due to repeated positive selection on several genes in the human lineage. However, even in a neutral model one would expect fluctuations among lineages and given that both, the number of differences found, as well as the number of lineages evaluated are small, these findings might also be compatible with only a small fraction of genes evolving expression differences under positive selection.

Still, it is of interest to note that frequently repeated rounds of positive selection could also result in patterns comparable to those predicted by the neutral model. Gillespie (2000) has coined the phrase "genetic draft" for this effect. We have addressed the general question of the frequency of positive selection events in population comparisons within each of the subspecies and found that selection cycles occur every 100 generations on average - and this is a conservative estimate (Teschke et al. 2008). Under

these conditions, genetic hitchhiking effects might shape a significant part of the genome, making the distinction between selection and drift more difficult. Bedford and Hartl (2009) have developed a theoretical framework that shows that even very small selection coefficients could potentially shape expression divergence. It will therefore be necessary to focus future studies on expression evolution on comparisons between very closely related populations, to have the chance to capture ongoing events of positive selection, rather than averaging over long time spans.

An independent issue that is evident in our data is that most (9 out of 12) differentially expressed genes show a significant difference in gene expression in only one tissue. This confirms earlier findings that tissue-specific enhancers may evolve independently of each other. Cowles et al. (2002) found tissue specific effects for two out of four genes with *cis*-regulatory expression difference between inbred mouse strains. Blekhman et al. (2008) used statistical approaches on their microarray data set in primates to estimate the fraction of genes with signs of tissue-specific expression changes under directional selection and find that approximately 10% of the genes studied fall into this class. Our data imply that tissue specific changes may be even more prevalent, supporting the notion that the independent regulatory elements driving gene expression in different tissues have the capacity to evolve independently of each other.

There is currently an intensive discussion on the relative role of gene expression changes in the evolution of developmental processes (Hoekstra and Coyne 2007). While there is compelling direct evidence for a number of such cases (reviewed in Carroll 2008), these usually deal with species comparisons that are far beyond an evolutionary distance where the processes that have led to the change can still be inferred. For example, the well studied regulatory element driving differential expression of sexually dimorphic traits in *Drosophila* has a deep evolutionary ancestry, covering a time span of at least 30 million years (Williams et al. 2008). Clearly, any attempt to approach the question of the relative role of gene expression in creating developmental novelties requires the study of much more closely related species or subspecies. A detailed study of an enhancer element affecting pigmentation between closely related *Drosophila* species (Joeng et al. 2008) has not revealed any evidence for positive selection and is fully compatible with a neutral divergence model. On the other hand, it is a general limitation of most studies dealing with the question of the role of expression changes in a microevolutionary perspective that they have so far only looked at changes in

expression level, but not at developmentally relevant changes, i.e. novel expression in different embryonic regions, different tissues or different times in development. There is clearly a need for specific studies addressing this point, before general conclusions on the role of adaptive regulatory changes in driving phenotypic changes can be drawn.

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MGI Gene Symbol			Gene expression						Genetic diversity	
Acc. Num.	Chr	Subsp	Brain		Liver/kidney		Testis		θ	D
			Expr	Res	Expr	Res	Expr	Res		
1110017D15Rik	4	dom	0.02	-0.51	0.002	-0.26	30.319	0.08	0.52	1.48
AK003742.1		mus	0.021	-0.32	0.002	0.31	45.277	-0.27	2.44	0.73
1700125F08Rik	10	dom	n.e.	n.e.	n.e.	n.e.	1.066	0.47	5.37	0.96
AK007277		mus	n.e.	n.e.	n.e.	n.e.	0.883	0.3	1.03	0.25
Cacng2	15	dom	1.448	-1.45	n.e.	n.e.	0.019	-0.55	0.91	1.78
NM_007583		mus	1.448	-0.74	n.e.	n.e.	0.014	-0.38	1.19	1.97
Ccl25	8	dom	0.182	0.69	0.127	0.02	1.513**	-0.49	1.08	-1.45
NM_009138		mus	0.212	0.5	0.109	0.72	0.943**	0.2	1.37	0.71
Cdk5	5	dom	0.390**	0.39	0.448*	-0.5	n.e.	n.e.	0	n.c.
NM_007668		mus	0.286**	-0.38	0.352*	-0.46	n.e.	n.e.	0.27	0.96
Etd	X	dom	0.004	0.88	n.e.	n.e.	0.78	0.53	0.26	-0.76
NM_175147.2		mus	0.002	0.3	n.e.	n.e.	0.603	-0.28	0.99	1.64
Etv2	7	dom	n.e.	n.e.	n.e.	n.e.	0.199*	1.06	1.37	-0.99
NM_007959		mus	n.e.	n.e.	n.e.	n.e.	0.495*	0.13	0.67	-0.77
Flot2	11	dom	0.294	-0.8	0.659*	0.37	2.077	-0.83	0.29	0.57
NM_008028.1		mus	0.321	0.23	0.386*	0.26	1.991	-0.11	1.48	0.63
Gpc6	14	dom	0.173**	-0.1	0.254	-0.59	0.215	0.63	0.45	0.96
NM_011821		mus	0.125**	0	0.266	0.02	0.255	0.69	4.99	-1.05
Hif1a	12	dom	0.602	0.14	2.074	-0.7	2.538	-0.57	2.21	1.35
NM_010431		mus	0.543	0.51	1.741	-0.1	2.205	0.5	1.8	1.25
Kcnd2	6	dom	1.739*	-0.4	0.040*	-0.16	n.e.	n.e.	1.97	0.84
NM_019697		mus	1.488*	-0.17	0.015*	-0.54	n.e.	n.e.	1.12	1.57
Krt2-17	15	dom	0.003	0.63	n.e.	n.e.	n.e.	n.e.	3.29	0.23
NM_010668		mus	0.002	0.11	n.e.	n.e.	n.e.	n.e.	0	n.c.
Mir16	7	dom	0.733	-1	2.869	-1.16	4.329	0.44	0.4	0.57
NM_019580		mus	0.721	-0.33	2.964	-0.15	5.319	-0.29	1.99	-0.87
Nf1	11	dom	0.567	-1.13	0.503	-0.38	3.62	-0.16	0.98	1.25
NM_010897.1		mus	0.54	0.11	0.482	-0.3	3.311	0.65	0	n.c.
PanX1	9	dom	0.072	0.62	0.025	-0.01	0.233	-0.04	3.94	1.58
NM_019482		mus	0.082	0.16	0.038	-0.32	0.221	-0.11	1.32	-0.18
Ppt1	4	dom	1.081	0.22	1.737	-0.07	20.933**	-0.85	1.28	2.43
NM_008917.1		mus	1.208	-0.3	1.795	-0.66	17.291**	-0.97	0.82	-1.5
Rab4b	7	dom	0.188	-1.21	0.203	-1.29	1.211	-1.63	0.75	0.6
NM_029391.1		mus	0.203	-0.48	0.244	-0.29	1.579	0.52	0.75	-0.89
Rarres2	6	dom	0.051**	1.07	3.183	0.97	0.393	0.23	3.04	1.58
NM_027852.1		mus	0.114**	0.6	3.963	1.04	0.423	0.6	2.1	1.91
Rgs16	1	dom	0.143*	0.01	0.768	1.82	0.051	-0.14	1.55	2.38
NM_011267.1		mus	0.194*	0.04	1.807	1.48	0.075	0.2	2.51	-1.31
Scamp5	9	dom	0.55	0.15	0.321	0.24	0.168*	0.04	4.07	-1.73
NM_020270		mus	0.498	0.42	0.28	0.3	0.245*	0.15	0.5	1.61
Sv2c	13	dom	0.974**	0.05	0.002	0.36	0.264	0.06	3	1.77
AK173092.1		mus	0.788**	-0.82	0.003	1.49	0.392	1.62	2.09	0.3

Tcte3	17	dom	0.001	0.05	0.004	0.8	48.593	0.08	1.06	1.51
NM_011560.2		mus	0.001	-0.68	0.007	0.9	48.673	0.45	1.52	-1.21
Tmem24	9	dom	0.42	0.09	0.604	-1.12	0.413	-0.18	0.36	-1.14
NM_027909.1		mus	0.493	0.57	0.707	-0.23	0.305	0.16	0.75	1.4
Tomm40l	1	dom	0.295	0.04	0.344	-0.2	0.498*	0.13	1.37	0.14
AK186544.1		mus	0.281	-1.43	0.293	-0.2	0.340*	0.16	0.82	0.24

Table 1

Summary of expression and sequence polymorphism data for all genes. Expr = linear expression levels based on ΔCt values, Res = residuals of the regression of the variances (see text). θ = Watterson´s θ (Watterson 1975) x 1000, D = Tajima´s D (Tajima 1989). Stars denote value pairs that represent significantly different expression levels between the subspecies, ** = p< 0.01, * = p<0.05

model	k	AICc	ΔAICc	wAICc	BIC
θ	3	233.858	0	0.322	242.119
Subspecies + θ	4	234.154	0.296	0.278	245.099
Subspecies * θ	5	236.254	2.396	0.097	249.847
Tissue * θ	7	237.249	3.391	0.059	256.026
Tissue + Subspecies + θ + Tissue:θ	8	237.663	3.805	0.048	258.973
Tissue + θ	5	237.159	3.301	0.062	250.752
Tissue + Subspecies + θ	6	237.507	3.649	0.052	253.711
Tissue + Subspecies + θ + Subspecies:θ + Tissue:θ	9	239.834	5.976	0.016	263.638
Tissue + Subspecies + θ + Subspecies:θ	7	239.669	5.811	0.018	258.446
Tissue + Subspecies + θ + Tissue:Subspecies + Tissue:θ	10	240.786	6.928	0.01	267.042
Tissue + Subspecies + θ + Tissue:Subspecies	8	240.448	6.59	0.012	261.758
Tissue + Subspecies + θ + Subspecies:θ + Tissue:θ + Tissue:Subspecies	11	242.984	9.126	0.003	271.650
Subspecies	3	241.105	7.248	0.009	249.366
Nullmodel	2	241.13	7.272	0.008	246.672
Tissue * Subspecies * θ	13	246.803	12.945	0	280.157
Tissue + Subspecies	5	244.187	10.329	0.002	257.780
Tissue	4	244.161	10.304	0.002	255.106
Tissue * Subspecies	7	247.061	13.203	0	265.838

Table 2

Linear model comparison of factors determining θ. Models are sorted by goodness of fit with a penalty for number of parameters in the model (AICc)**.** k: Number of parameters in the model, AICc: Akaike's Information Criterion corrected for sample size, wAICc: Akaike weights corrected for sample size (comparable to r^2), BIC: Bayesian Information Criterion or Schwarz Criterion, + indicates additivity, : indicates interaction

model	k	AICc	deltaAICc	wAICc	BIC
Polymorphism	3	-13.339	0	0.866	-7.37141
Polymorphism+Tissue	5	-9.294	4.045	0.115	0.270279
Polymorphism*Tissue	7	-5.483	7.856	0.017	7.333185
Nullmodel	2	-0.748	12.59	0.002	3.302666
Tissue	4	2.791	16.13	0	10.59733

Table 3

Linear model comparison for factors determining expression divergence, abbreviations as in Table 2.

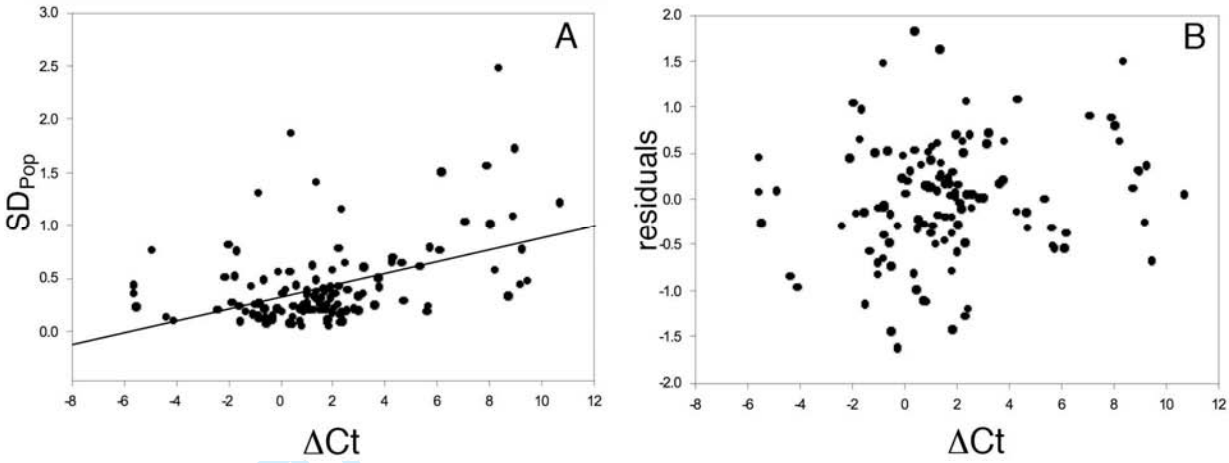


Figure 1

Dependence of measures for expression polymorphism on expression level of a gene in a given tissue (ΔCt) before and after addressing technical variance effects. (A) Heteroscedasticity of ΔCt values. The standard deviation of expression measurements within a population (SD_{Pop}) includes biological and technical effects and depends on expression levels ΔCt ($p<3x10^{-7}$ and $r^2=0.2$, Pearson's correlation). (B) The residuals of the regression of ln(SD) on ln(SE) (referred to in this study as expression polymorphism) are independent of the expression level ($p>0.1$ and $r^2<0.04$, Pearson's correlation).

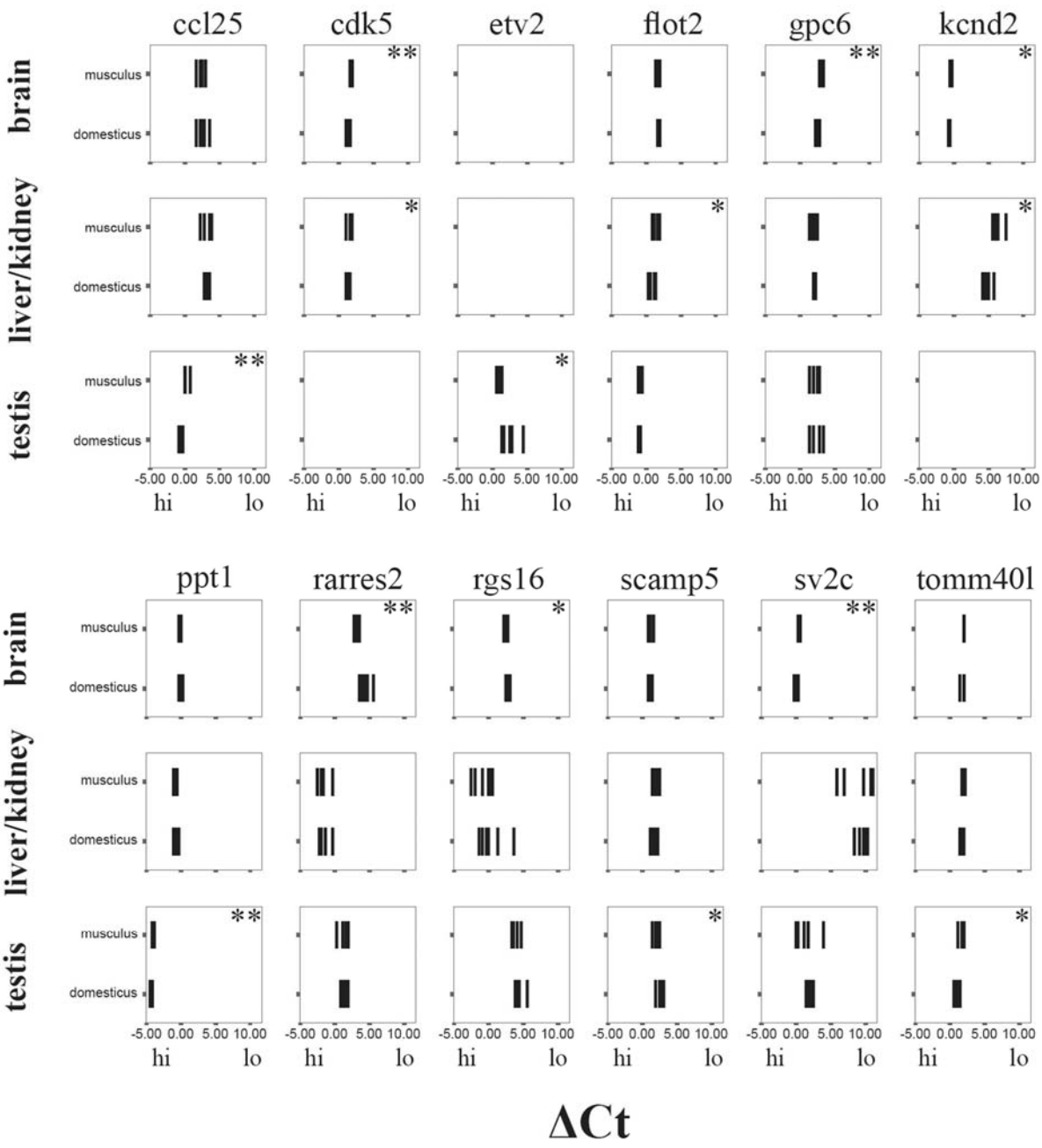


Figure 2

Expression levels of genes differently expressed between house mouse subspecies. Stars denote pairs that represent significantly different expression levels between the subspecies, ** = $p < 0.01$, * = $p < 0.05$. Each vertical bar represents the expression level of an individual mouse represented by the respective ΔCt measurement. Small ΔCts indicate high (hi) expression levels and large ΔCts indicate low (lo) expression levels.

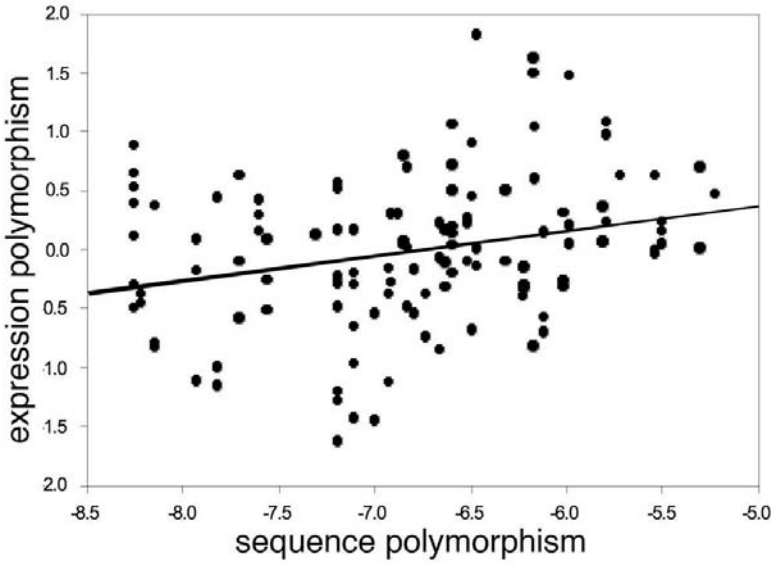


Figure 3

Expression polymorphism (residuals) correlates with sequence polymorphism (ln(θ)). Each dot represents the expression polymorphism in a given tissue in one of the populations (r^2=0.073, $p<0.01$, Pearson's product moment correlation). ln(θ) is based on nucleotide polymorphism in the upstream region of the respective gene in the respective population, based on up to 17 sequenced individuals. The results are similar, if one would use only the sequence information from the six individuals that were used for the expression studies (r^2=0.055, $p<0.01$).

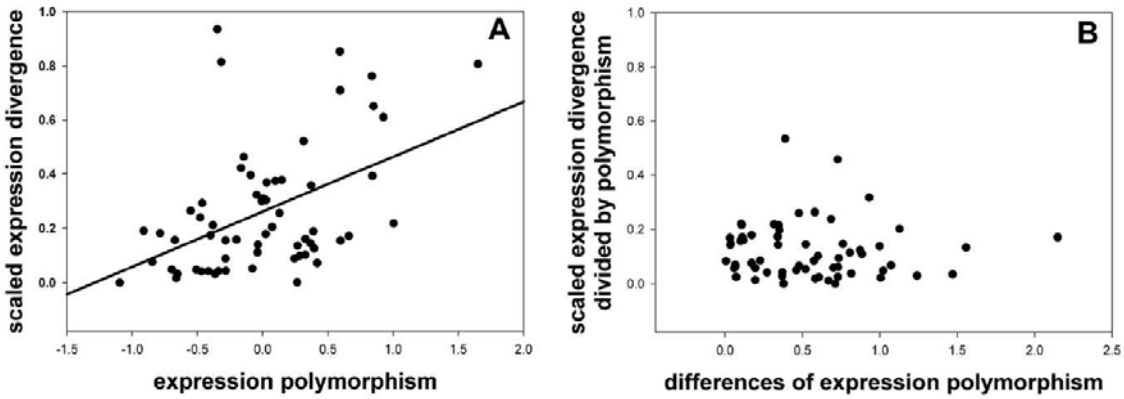


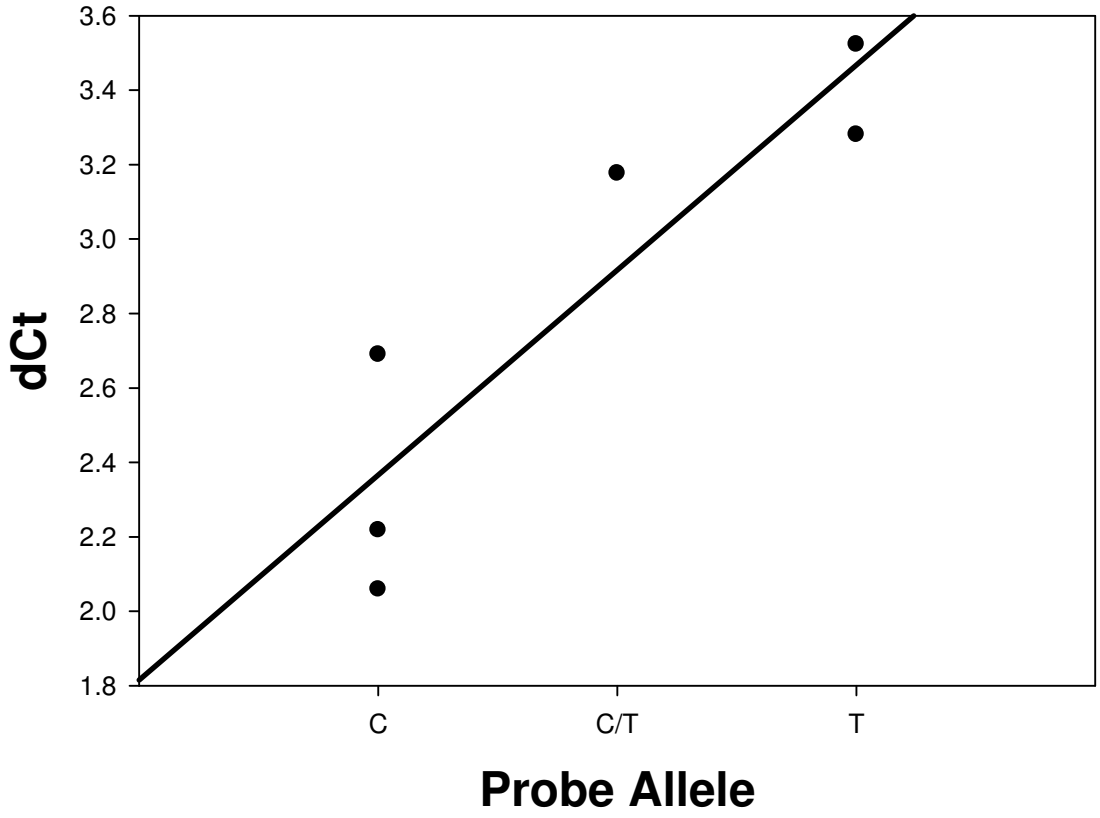
Figure 4

Relationships between expression polymorphism and divergence. (A) Expression divergence between populations (scaled ΔCt values) correlates with expression polymorphism (residuals) within populations. Each dot represents the value for a given gene in a given tissue (r^2=0.21, $p<2x10^{-4}$, Pearson's product moment correlation). (B) The ratio of scaled expression divergence and polymorphism between populations does not correlate with differences of expression polymorphism (absolute difference of residuals) between populations (r^2=-0.029, p=0.89, Pearson's product moment correlation). Lowest polymorphism (residual) was set to equal 1 by adding a constant to all polymorphism measurements in advance.

Analysis of gene expression differences between natural populations of house mouse subspecies supports a predominantly neutral model of evolutionary change

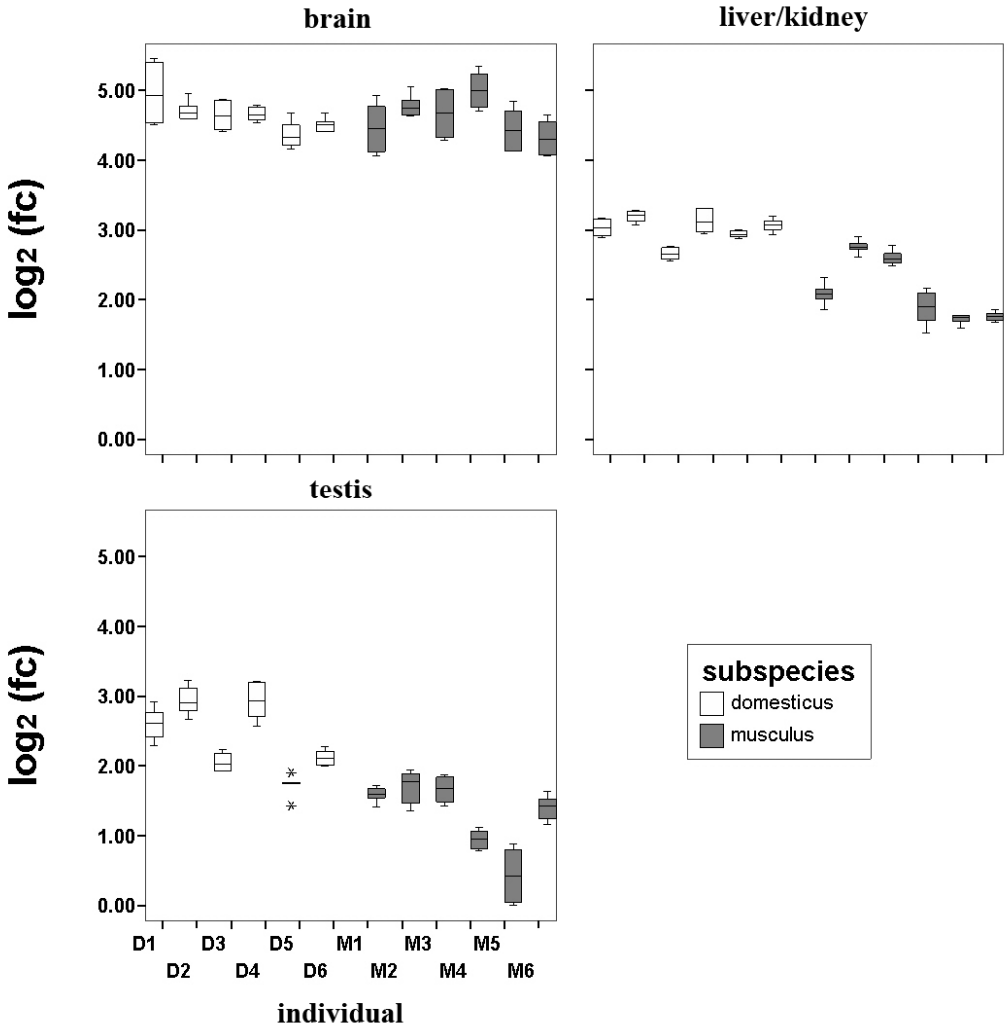
Fabian Staubach, Meike Teschke, Christian R. Voolstra, Jochen B. W. Wolf and Diethard Tautz

Supplementary data



Supplementary Figure 1

Example of probe polymorphism effect on expression measurement. The probe of assay Mm00661819_m1 binds to a region which contains a SNP in *M. m. domesticus*. Measured Expression levels for the six individuals strongly correlate with the allelic state of the SNP ($r^2>0.9$, $p<0.05$, Pearson's correlation).



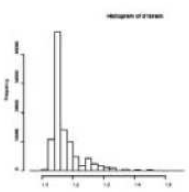
Supplementary Figure 2

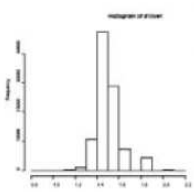
hprt expression differences between individuals and tissues, based on absolute quantification of RNA.

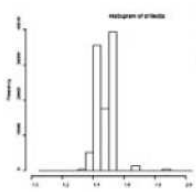
Log_2 fold-changes of *hprt* expression relative to the smallest value are plotted on the y-axis. Each box represents *hprt* expression in an individual mouse and tissue. Error bars represent the 95% confidence interval based on six technical replicates. Apart of considerable differences between individuals there are systematic differences between subspecies in expression level as well as variance (see Suppl. Table 2 below). RNA concentrations were measured with an Agilent Bioanalyzer and adjusted to the same level prior to reverse transcription. cDNA concentrations were adjusted to the same level using RiboGreen fluorescent dye on a Nanodrop 3300 Fluorometer according to Libus and Storchova (2006) prior to quantitative PCR.

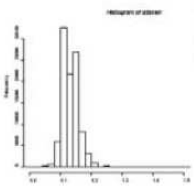
Libus, J. and H. Storchova (2006). Quantification of cDNA generated by reverse transcription of total RNA provides a simple alternative tool for quantitative RT-PCR normalization. *Biotechniques* **41**: 156-164

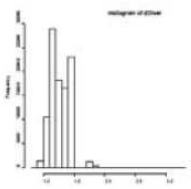
domesticus

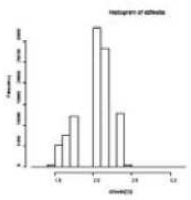

d1 brain
median:1.15
sd:0.045

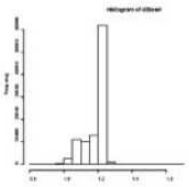

d1 liver/kidney
median:1.49
sd:0.111

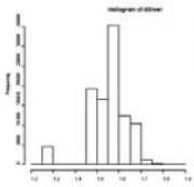

d1 testis
median:1.46
sd:0.063

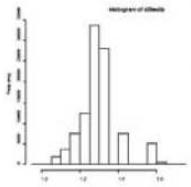

d2 brain
median:1.13
sd:0.034

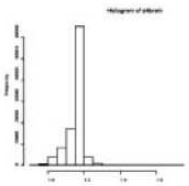

d2 liver/kidney
median:1.24
sd:0.132

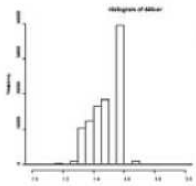

d2 testis
median:2.05
sd:0.220

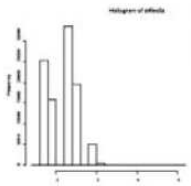

d3 brain
median:1.21
sd:0.055

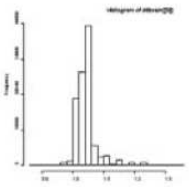

d3 liver/kidney
median:1.57
sd:0.086

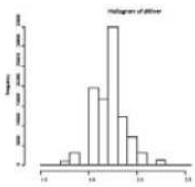

d3 testis
median:1.30
sd:0.095

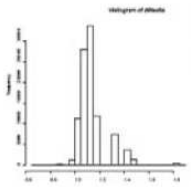

d4 brain
median:1.18
sd:0.045

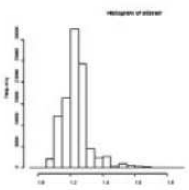

d4 liver/kidney
median:1.49
sd:0.122

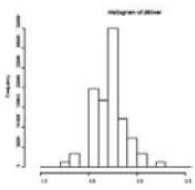

d4 testis
median:2.24
sd:0.345

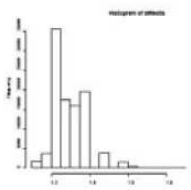

d5 brain
median:1.23
sd:0.090

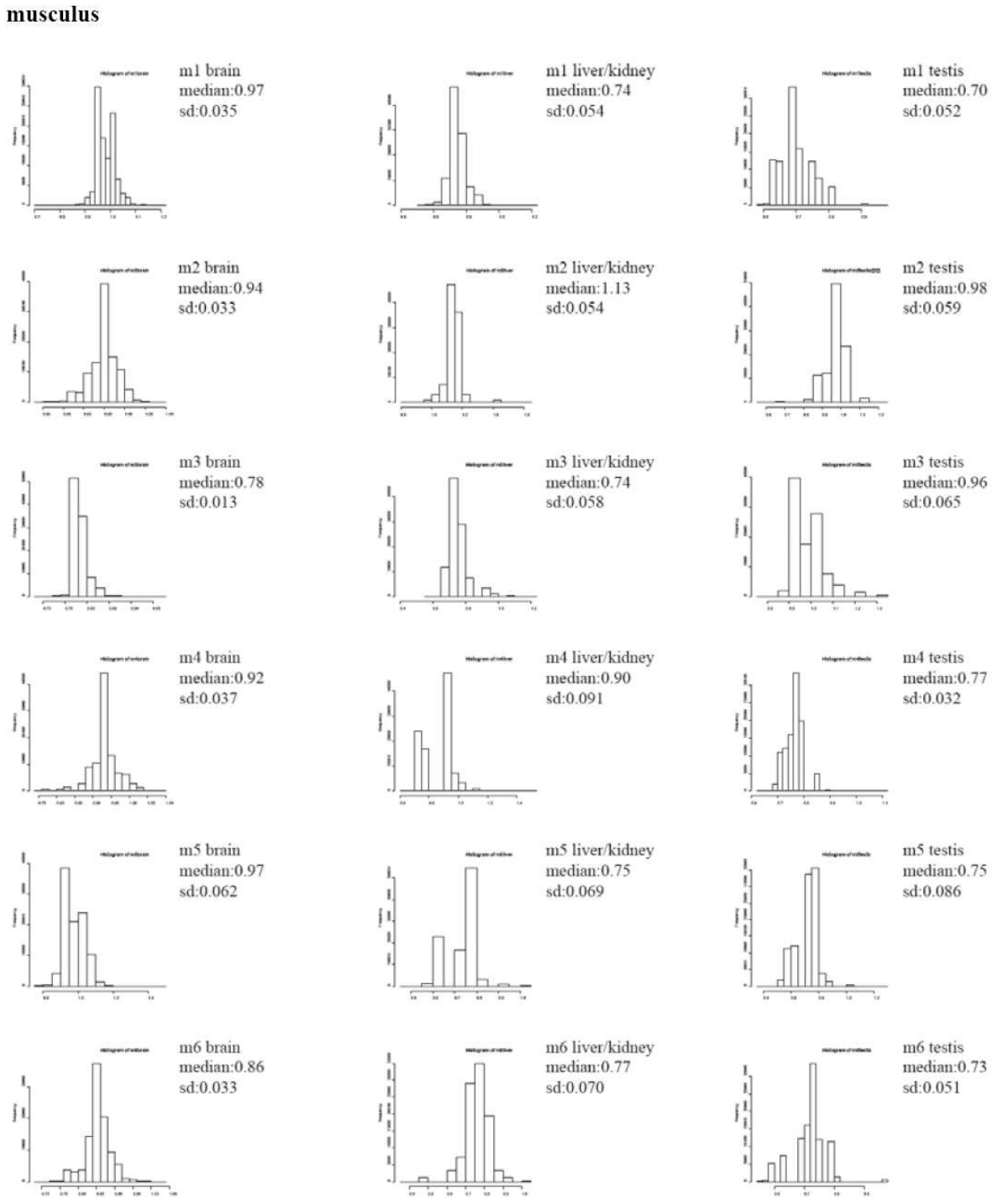

d5 liver/kidney
median:1.60
sd:0.134


d5 testis
median:1.12
sd:0.119


d6brain
median:1.04
sd:0.032


d6 liver/kidney
median:1.76
sd:0.164

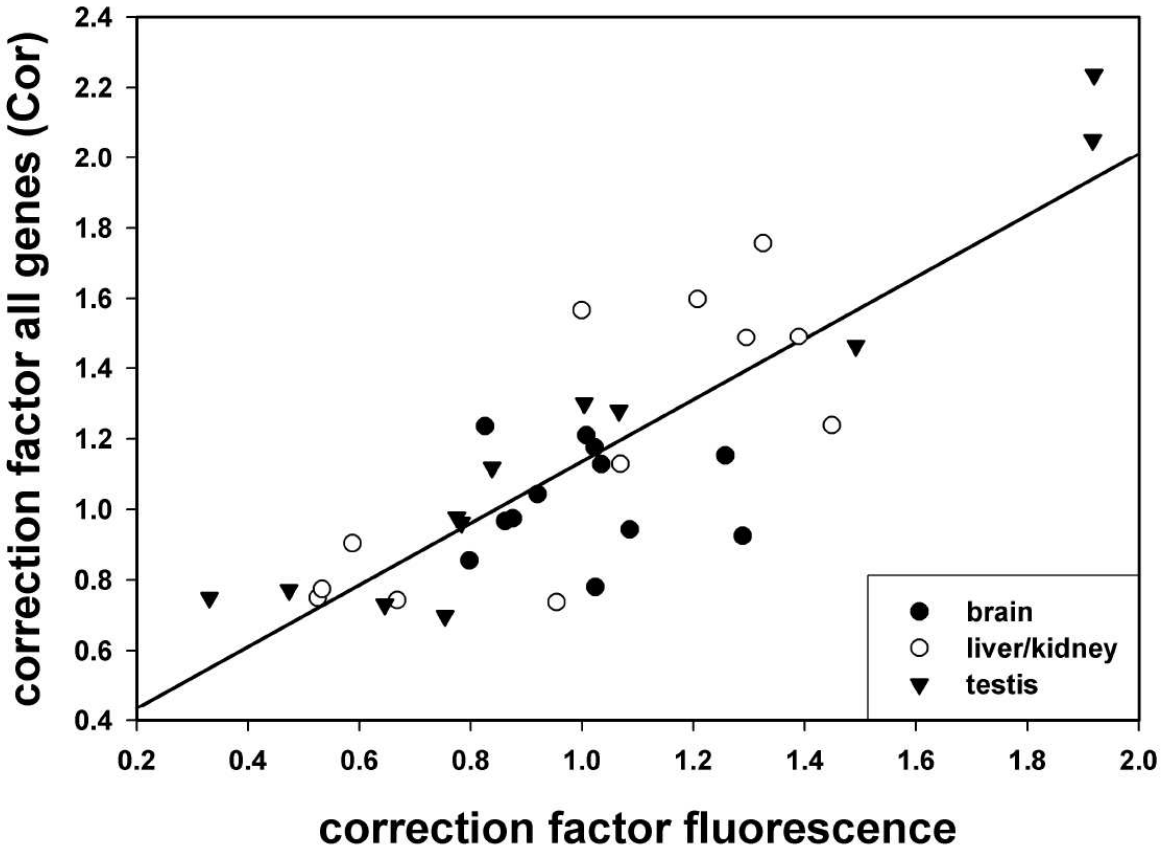

d6 testis
median:1.28
sd:0.083



Supplementary Figure 3

Assessment of the suitability of using the whole gene set tested for each mouse and tissue in calculating the correction factors for qPCR normalization. Histograms of 100,000 times bootstrapped correction factors over all genes for each mouse and tissue (Cor). The stadarddeviations (sd) are small (mean sd = 0.082). Each histogram represents the bootstrapped distribution of correction factors for an individual mouse (domesticus = d1-d6; musculus = m1-m6) in a given tissue across all genes analyzed in that respective tissue. This shows that the

median of the correction factors is stable across all genes within a tissue and an individual indicating that the median does not depend much on which of the 24 genes are included.



Supplementary Figure 4

Assessment of using the correction factors obtained across all genes per mouse per tissue with those obtained by direct measurements of cDNA amounts used in the qPCR reaction (see legend suppl. Fig. 2 for details). The 36 correction factors calculated over all genes for each mouse and tissue (Cor) correlate strongly with the factors calculated using the cDNA concentration measures ($r^2 = 0{,}71$, $p = 1{,}53 \times 10^{-10}$, Pearson's Correlation).

Taqman assays	assay ID	MGI gene symbol	status
Included	Mm01217369_m1	1110017D15Rik	no polymorphism detected
	Mm01172741_g1	1700125F08Rik	no polymorphism detected
	Mm00432248_m1	Cacng2	no polymorphism detected
	Mm00436443_m1	Ccl25	no polymorphism detected
	Mm00432437_m1	Cdk5	no polymorphism detected
	Mm00558327_s1	Etd	no polymorphism detected
	Mm00468389_m1	Etv2	no polymorphism detected

	Mm00514956_m1	Flot2	no polymorphism detected
	Mm00516235_m1	Gpc6	no polymorphism detected
	Mm00468869_m1	Hif1a	no polymorphism detected
	Mm00498065_m1	Kcnd2	no polymorphism detected
	Mm01300291_m1	Krt2-17	no polymorphism detected
	Mm00450997_m1	Mir16	no polymorphism detected
	Mm01298523_m1	Nf1	no polymorphism detected
	Mm01290707_g1	Tomm40l	no polymorphism detected
	Mm00450900_m1	PanX1	no polymorphism detected
	Mm01192227_m1	Ppt1	no polymorphism detected
	Mm00453021_m1	Rab4b	no polymorphism detected
	Mm00503581_gH	Rarres2	no polymorphism detected
	Mm00803317_m1	Rgs16	no polymorphism detected
	Mm00491014_m1	Scamp5	no polymorphism detected
	Mm01282622_m1	Sv2c	no polymorphism detected
	Mm00843984_s1	Tcte3	no polymorphism detected
	Mm01168596_m1	Tmem24	no polymorphism detected
Excluded	Mm01217598_g1	4833411C07Rik	fixed difference probe
	Mm00661819_m1	AI604832	polymorphic probe domesticus
	Mm00731639_m1	Crisp1	shared polymorphism F primer
	Mm01174266_m1	Dscaml1	polymorphic F primer musculus
	Mm00834825_g1	Edf1	fixed difference R primer
	Mm00784689_s1	MGC118210;Xmr;Xmr	multicopy gene
	Mm00435145_m1	Nkx2-9	polymorphic probe domesticus
	Mm00439358_m1	Nr4a1	fixed difference probe
	Mm00510343_m1	Ppil3	fixed difference F primer
	Mm00499682_m1	Ppp1r11	duplicated F primer binding region domesticus
	Mm00839568_m1	Spt1	polymorphic probe domesticus
	Mm01352176_m1	Tmem16k	polymorphic R primer musculus
	Mm02017439_g1	Tmsb10	polymorphic probe domesticus
	Mm00840578_g1	Tnfrsf13c	different splice variants
	Mm00441325_m1	Sema3f	shared polymorphism R primer

Supplementary Table 1

Taqman assays assessed for this work. Assays, which bind to polymorphic regions, were excluded from further analysis. The status column provides a short note on the reason of exclusion

Tissue	Subspecies	Mean ΔCt	Std. Deviation	Wilcoxon W Test p-value	Levene's Test p-value
Brain	domesticus	27.12	0.20	0.94	0.34
	musculus	27.16	0.26		
Liver/kidney	domesticus	28.75	0.19	0.004	0.04
	musculus	29.62	0.44		
Testis	domesticus	29.36	0.51	0.002	0.77
	musculus	30.48	0.50		

Supplementary Table 2

Expression differences of *hprt* between subspecies.

MGI Gene Symbol /Acc. Num.	PCR primers		sequencing primers	
direction	*F*	*R*	*F*	*R*
1110017D15Rik	CATTCTGCAGGGTCTCTTCCCC	CCTCAGCAGCTCCCTGTTCTCC		
AK003742.1	GTGCTGAAATTTGAAGCCAGGC	CACACTCGTGCACGCGTGCACG		
1700125F08Rik	GCCACAAACCTACGTGTGGTGG	CTGTACACGCAGGCTTGGCAGC	TTCCACCATTAGGGTTCCACTG	GTCGTGACTGCTTGGCAAACCT
AK007277				
Cacng2	CTGGAATTTACACCCAAGGAAC	AAACCTCTCTACTATGAGCA	ATCGCCAGCTACGCCTTCCTCCCA	GTGTTTCTTCCAGTTCAGCCT
NM_007583	TCAATCTCATTTATGAATGAC	CCCACCTACTGCGTCGCCGTGGT		
Ccl25	GGCCAGGACAGAGCAAGAGAGC	ATGCCTTTCTGGTCCTGAGAGC		
NM_009138	ACTGCAGGGTGGGGCTCTGACT	CATCTACCTCCAGTAGTACCAG		
Cdk5	TGAAAGACCCTTGCGTGTCATC	AGCGATCAACTCCAGGGACTCG	CGCTGGAGTCCGGTGGGTTTCG	GACCTCACAGGGACCAGCTGAC
NM_007668				
Etd	GAGGGAAATCCCAGGAAGGATG	CTCTGCAGCATTTCTCCACAAC		
NM_175147.2	GAAGCAGGTATTTCTATTCTCA	TGCATCAGCTGAAAGACTTCCT		
Etv2	TGTCACGACTTCTCGGACCCAG	TCCCAAGCCACAGCAGCTTACC	GCAACTTGACCCAGGCTGCGAC	AGTTCGTGGCTCACCTCTGGCA
NM_007959	AAGCCAAGGTTCGACAAGACTT	CAGGAAGAGGGATTTCCGGCCA		
	GATGTTATTTTGATTATGGTTG	AGCCAGGGGGGTTTCAGCCCCAT		
Flot2	GCGCCCACTGGCTTGGCTGGTG	TTACAGTGTCTCTTAGGAAGTA		
NM_008028.1	ACCTCGGAACCTCTTGTCATGT	AGCCCCGCCCGCCTGCGCCCCTCT		
Gpc6	TCCCTGGCTTTGTGTTAGGTAC	AGCTCGCCTATCCAGTGTGCG		
NM_011821	CTCCAACGATTTTCTACCGGAG	CAGCCCGGATCCAAGAAGGCAT		
Hif1a	ACTGGAAACTCGGGCGGGATGG	GAGGGAAAAGCCGAGGGGTGCG		
NM_010431	CCAGCATAGCCGGTGAGCAGTC	GCCAACTTTGCGTTCTGGCAGC		
Kcnd2	CTGGGATCTGGCTGCTCGGGAG	GCAACAGGCATCCACCCAATGG	AGTACAGGCGGCCAGCGGACTC	AGGGAAAGTCAACCAGTCAGTG
NM_019697				
Krt2-17	ACCGGAACCTTGACCTGGACAG	TCCACATCCAGGGACAGCTTGG	GAAGCTGTGTCTCCAACCACTG	GAAGCTGTGTCTCCAACCACTG
NM_010668	GAGCTTCACTAACGGGGTCACA	ACCTGCTTCTTCACATGTGATA		
Mir16	TCTGGAAGAGCAGCCAGTACTC	GCAAGGGCTGTTTCCACCAGGA		
NM_019580	AATGCAGACATGTGTCCTGGTG	CAAGTCCGTTTTCAGGGTTCTG		
Nf1	GAAGCCCCATCGACTGCGT	AGCGAGTCCTCCTGGAGGTGAC		
NM_010897.1	GCAGCAGGCCCCTTCCCTCTCG	CGGCGAGCCGAGCGGCTGAGGA		
PanX1	ACTACTGCGAGACCCAACCGAG	CATTTACGGCAAACGGCCTTGG	CAGCACTCCATAGCCATCTGGA	CTGCACAGCCAGCAACCAGCAC
NM_019482	TGCCTTTCGAGGAACAGACAGG	TGCCCAGCCCTTTGCCCTTTCC		
	GAACAGAACCCGGATTGCACCC	GCTCTTCGCTACAGCTGCCCGC		
Ppt1	TTCATATGTCGCTCTCTACAAG	GGACGACGCCATCTTAGCAATC	CCTTCCCCAGTCCCGACACTGA	GTGAGAAATTAGGTAAGGTCCT
NM_008917.1				
Rab4b	ATACCCGGGATACAACAGTGAAC	CTGTACGTTCTATGCGCTTCTC	GTTGTTGGTATGTACCCAAGCT	TTAGCTTCCAGTACTGCACCCT
NM_029391.1	TGTAACGTCAGTGGCTACTAGG	AGCTCCTTTCCACTTCTGGACC		
	AGCCCTTTGCTCCAAGGTAAGG	GCCACTTCCGCCTTCACCGCCC		
Rgs16	CCTTACTGCATAGAATTCCATA	ATGAGAGACCTTAGAGACTCCA		
NM_011267.1	TGCTCCCTGGGGAAGTCCAG	GGTCAGAACAGGTACCATCCCA		
Scamp5	AACTGGCTTTGAACTTGCACTG	GTAGTGCCAGCAACTAGTGCCT		
NM_020270	TTCCCGGTACATCCCACAGGTC	GCCCTGCCTGCTGCAACCACTC		
Sv2c	CAGAGCTGGCCCATATGGCTCA	CAAACTGGACTCAGAGCAGGTG		
AK173092.1	TCACAATCATAATCACTCCT	CAGCAGCTCGGCTGCAACAGCA		
	GGTGAGTTGGATGAGGCTAAGG	ATAATGTACACTGTGCACCAGG		
Tcte3	TGAATATTTCTCCTCTGATTTA	TGGAACTGCGGTATAATAAAGG		

NM_011560.2	AATCAGAGATACTTTACAGGGA	GCCTACGCCGCGTGCAGCCGCG		
Tmem24	AAAAGGGTACATCCAGAGGTTG	TTACTCCTTGACTGAAGCTAGCT		
NM_027909.1	TTCTCGCCGCCGTTGACATTAG	CGGCACCGGCTCCGGGAGGGCT		
Tomm40l	GTCGTTGAGACAGGGTTTCTCT	CTTCCATATGGTTCAATGCTAG	ATAGGCAAAATGCTCCCTCTAG	GAGTTTGTGGGTGGCCCTTTAG
AK186544.1	TGAGCCACTATGTGATTGCTGG	GCAGGCCCCAGGGCTTCAGTCC		

Supplementary Table 3

List of PCR and sequencing primers used to assess polymorphisms in the upstream regions of the genes analysed. The PCR primers column provides primers used for PCR and sequencing. Rows indicate the forward primer (F) and reverse primer (R) pairs used. Additional sequencing primers were used in several cases in addition to the PCR primers on the same PCR product. These are listed in the sequencing primers column.

MGI Gene Symbol	Subspecies	bp	#Chromosomes
1110017D15Rik	domesticus	968	30
AK003742.1	musculus	968	26
1700125F08Rik	domesticus	752	30
AK007277	musculus	752	28
Cacng2	domesticus	846	28
NM_007583	musculus	846	30
Ccl25	domesticus	920	32
NM_009138	musculus	920	14
Cdk5	domesticus	894	28
NM_007668	musculus	894	34
Etd	domesticus	984	30
NM_175147.2	musculus	984	34
Etv2	domesticus	753	30
NM_007959	musculus	753	30
Flot2	domesticus	841	34
NM_008028.1	musculus	841	32
Gpc6	domesticus	539	34
NM_011821	musculus	539	34
Hif1a	domesticus	594	26
NM_010431	musculus	594	24
Kcnd2	domesticus	870	34
NM_019697	musculus	870	34
Krt2-17	domesticus	999	30
NM_010668	musculus	999	34
Mir16	domesticus	614	34
NM_019580	musculus	614	34
Nf1	domesticus	498	34
NM_010897.1	musculus	499	34
PanX1	domesticus	869	34
NM_019482	musculus	830	22
Ppt1	domesticus	593	30
NM_008917.1	musculus	593	34
Rab4b	domesticus	716	24
NM_029391.1	musculus	716	24
Rarres2	domesticus	997	30
NM_027852.1	musculus	997	26

Rgs16	domesticus	487	30
NM_011267.1	musculus	487	34
Scamp5	domesticus	488	32
NM_020270	musculus	488	34
Sv2c	domesticus	770	28
AK173092.1	musculus	770	24
Tcte3	domesticus	483	28
NM_011560.2	musculus	483	34
Tmem24	domesticus	689	32
NM_027909.1	musculus	686	28
Tomm40l	domesticus	618	20
AK186544.1	musculus	618	30

Supplementary Table 4

Sequence information obtained from the different genes. The sequence length that was surveyed for each gene is given in bp - the value corresponds to the number of basepairs that could be unequivocally determined. #Chromosomes stands for the number of animals for which sequence information was obtained, with 2 chromosomes per animal. Note that heterozygous positions had always to be clearly identified on both strands sequenced to be included.