Cross-species analysis of genic GC$_3$ content and DNA methylation patterns

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Abstract

Background. The GC-content in the third codon position (GC₃) exhibits a unimodal distribution in many plant and animal genomes. Interestingly, grasses and homeotherm vertebrates exhibit a unique bimodal distribution. High GC₃ was previously found to be associated with variable expression, higher frequency of upstream TATA boxes, and an increase of GC₃ from 5' to 3'. Moreover, GC₃-rich genes are predominant in certain gene classes and are enriched in CpG dinucleotides that are potential targets for methylation. Based on the GC₃ bimodal distribution we hypothesize that GC₃ has a regulatory role involving methylation and gene expression. To test that hypothesis, we selected diverse taxa (rice, thale cress, bee, and human) that varied in the modality of their GC₃ distribution and tested the association between GC₃, DNA methylation and gene expression.

Results. We examine the relationship between cytosine methylation levels and GC₃, gene expression, genome signature, gene length, and other gene compositional features. We find a strong negative correlation (Pearson’s correlation coefficient \( r = -0.67 \), p-value <0.0001) between GC₃ and genic CpG methylation. The comparison between 5'-3' gradients of CG₃-skew and genic methylation for the taxa in the study suggests interplay between gene-body methylation and transcription-coupled cytosine deamination effect.

Conclusions. Compositional features are correlated with methylation levels of genes in rice, thale cress, human, bee and fruit fly (which acts as an unmethylated control). These patterns allow us to generate evolutionary hypotheses about the relationship between GC₃ and methylation and how these affect expression patterns. Specifically, we propose that the opposite effects of methylation and compositional gradients along coding regions of GC₃-poor and GC₃-rich genes are the products of several competing processes.

Keywords
DNA methylation, gene expression, GC₃, grasses, homeotherms, Oryza sativa, Apis mellifera, Homo sapiens, Arabidopsis thaliana
Introduction

The term "epigenetics" was coined in 1957 by Conrad Hal Waddington (Slack 2002). It is defined as the study of changes in gene expression due to mechanisms other than alterations to the DNA sequence; that is, expression modifications are not hard coded into the nucleotide sequence. Consequently, epigenetics explains phenomena, which do not result from standard genetic mutations, like hereditary changes in gene expression under the influence of environmental factors. DNA methylation is one of the most studied epigenetic mechanisms modulating gene expression and has important health implications. For example, the gain or loss of DNA methylation can produce loss of genomic imprinting and results in diseases such as Beckwith-Wiedemann, Prader-Willi and Angelman syndromes (Adams 2008). Changes in the patterns of DNA methylation are also commonly seen in human tumors. Both genome wide hypomethylation (insufficient methylation) and region-specific hypermethylation (excessive methylation) have been thought to play a role in carcinogenesis (Lengauer 2007). DNA hypomethylation contributes to cancer development through an increase in genomic instability, reactivation of transposable elements, and loss of imprinting (Esteller 2002). Hypermethylation-induced silencing of primary transcripts through their CpG island promoters is a common cause of the loss of tumor suppressor miRNAs in cancer (Lengauer 2007; Lopez-Serra & Esteller 2012; Sonkin et al 2013).

Methylation occurs by the addition of a chemical methyl group (-CH3) through a covalent bond to the cytosine bases of the DNA backbone and tends to be more abundant at Cytosine-phosphate-Guanine-(CpG) dinucleotides (Sadikovic 2008). However, methylation can also happen in CHG and CHH contexts (where H indicates any nucleotide other than G). DNA methylation is common in humans and other mammals, where 70 to 80% of CpG dinucleotides are methylated. Interestingly, in some model organisms, such as yeast and fruit fly, there is little or no DNA methylation. Also, DNA methylation in mammals differs from that in plants as it targets CpG sites. In humans and mice, CpG dinucleotides account for roughly three quarters of the total DNA methylation content in their cells (Ziller et al. 2011).

In vertebrates, the methylation process is being catalyzed by members of the enzyme family of DNA methyltransferases (DNMTs), which recognize palindromic sequences with CpG dinucleotides. Thus far, three active DNMTs have been identified in mammals: DNMT1, DNMT3A, and DNMT3B. A fourth similar enzyme (DNMT2 or TRDMT1) is structurally similar to the other DNMTs. However it does not methylate DNA but rather transfers RNA (Goll et al. 2006). DNA methylation of CpG dinucleotides is essential for plant and mammalian development. Methylation mediates the expression of genes and plays a key role in chromosome X inactivation, genomic imprinting, embryonic development, chromosome stability, chromatin structure, and may also be involved in the immobilization of transposons and the control of tissue-specific gene expression (Li et al. 2008).

The relationship between gene expression, nucleotide composition and gene length were the subject of several studies in the past decades. Oliver and Marin (1996) associated the expected length of a reading frame to the CG composition using the property that stop codons (TAG, TAA, and TGA) are biased towards low GC content. They suggested that the longest coding sequences/exons in vertebrates are
GC-rich, while the shortest ones are GC-poor. Subsequently, Xia et al. (2003) described positive correlations between GC content and coding regions (CDS) lengths in 68 genomes. It was later shown that highly expressed rice and human GC-rich genes have significantly more and longer introns than lowly expressed genes, whereas their average exon length per gene is significantly lower. By contrast, GC-poor genes were shown to exhibit similar compactness between highly and lowly expressed genes (Mukhopadhyay and Ghosh 2010).

The relationship between gene-body methylation and gene expression was studied in a number of organisms, and a positive linear correlation was reported (Zemach et al, 2010; Xiang et al 2010). Anastasiadou et al. (2011) reported the relationship between splicing and methylation in the human genome as well as a positive relationship between alternative splicing and methylation. Recently, Flores et al (2012) reported a positive relationship between exon-level DNA methylation and mRNA expression in the honeybee. They also found that methylated genes are enriched for alternative splicing; therefore suggesting that gene-body DNA methylation positively influences exon inclusion during transcription. The authors proposed that DNA methylation and alternative splicing contribute to a longer gene length and a slower rate of gene evolution. However, none of these studies, considered the potential regulatory role of GC$_3$.

Several studies focused on coding regions that are enriched in methylation targets (CpG-rich). For example, Nanty et al. (2011) found an evolutionarily conserved feature in invertebrate genomes separating CpG-poor and CpG-rich genes: CpG-poor genes were associated with basic biological processes, while the latter with more specialized functions. Gavery and Roberts (2010) found that hypo- and hyper-methylated genes differ in both biological function and in the ratio between observed and expected CpG dinucleotides. Coding regions enriched in CpG dinucleotides also exhibit a higher frequency of G or C in the third codon position (GC$_3$). Because mutations in this position lead primarily to synonymous substitutions, the selective pressures affecting its composition are different from those acting on the first two codon positions, making it a valuable tool to study evolution. To name a few, it has been previously shown (Tatarinova et al. 2010; Sablok et al. 2011; Ahmad et al. 2013) that dicot and monocot plant genes with high GC$_3$ have distinctly different properties from genes with low GC$_3$: they contain more targets for methylated GC$_3$-rich genes, and also exhibit more variable expression, possess more upstream TATA boxes, are enriched for certain classes of genes (e.g. stress responsive genes), and have a GC$_3$ content that increases from 5' to 3' (Tatarinova et al. 2010). GC$_3$-rich genes were also shown to be inducible while the GC$_3$-poor are ubiquitously active (Tatarinova et al. 2010). Thus we speculate that GC$_3$ has evolved to be interdependent with gene-body methylation and gene expression so that genes that are GC$_3$-rich or -poor have different expression patterns.

Here, we tested the hypothesis of the regulatory role of GC$_3$ by studying the relationship between GC$_3$, gene-body methylation, and related genomic features in four taxa: rice, arabidopsis, bee, and human. These particular species were chosen because they have well-annotated genomes, rich collections of gene expression measurements, and genome-wide methylation measurements. Comparison with the fruit fly allows us to separate methylation-related effects from other factors. We show that GC$_3$ is
inversely correlated with gene methylation in these four organisms and propose an evolutionary theory to explain these patterns.

**Materials and Methods**

**Gene models** were taken from: MSU (version 6.1) for *Oryza sativa*; TAIR version 7 for *Arabidopsis thaliana*; BeeBase (www.beebase.org) annotation for *Apis mellifera*; NCBI GenBank for *Homo sapiens* (hg18); and dmel_hetr31 from FlyBase (www.flybase.org) as well as Release 5 from Berkeley Drosophila Genome Project (www.fruitfly.org) for *Drosophila melanogaster*.

**Gene expression** data were obtained from the NCBI GEO collection (GSE9415, GSE24177, GSE5624, GSE1647, GSE19700, GSE9646-GPL10978, GSE9646-GP10977, GSE16474, GSE34029, GSE34293, GSM846863, GSE25161, GSE34029, GSE34293, GSE42255, GSE5147, GSE1643, GSE7567, GSE16144, GSE21009-GPL10237).

**Filtering.** We selected gene sets where gene expression, methylation and high-quality annotation data were available: there were 12,577 such genes in *Arabidopsis thaliana*, 14,069 in *Homo sapiens*, 9,607 genes in *Oryza sativa*, and 15,381 genes in *Apis mellifera*. For *Drosophila melanogaster* we used 18,731 coding sequences.

**Methylation** bisulfite sequencing measurements for the four organisms were obtained from previously published studies (Chodavarapu et al. 2010; Foret et al. 2012; Bernal et al. 2012; Chodavarapu et al. 2012). We required a minimum of five reads to call the methylation state of a cytosine. The DNA methylation level was estimated from the fraction of cytosines that failed to undergo bisulfite conversion. Therefore, for each cytosine, the methylation level ranged from 0 to 1. When we computed average gene-body methylation for a given context, we calculated the average methylation for all coding regions, using appropriate gene models for each organism. For *H. sapiens* we used H1 embryonic stem cell line methylation profile. The distributions of gene-body methylation levels are shown in Figure 1B.

\[ GC_3 = \frac{C_3 + G_3}{L/3} \]

For every open reading frame GC3 was computed as \( GC_3 = \frac{C_3 + G_3}{L/3} \), where \( C_3 \) and \( G_3 \) are counts of cytosines and guanines in the third position of the codon and \( L \) is the length of the coding sequence.

**GC3 distributions** are obtained from a histogram of GC3 values, where GC3 values were rounded to hundredths (Figures 2 and 4) or tenth (Figures 5 and 6). We require that all points on the graph were supported by at least 100 observations, criteria which determined the choice of the bin size.

**Standardization of gene expression (Z-statistic).** For a gene \( g \), \( Z_g(\text{Expression}) = \frac{(\bar{E}_g) - e}{\sigma} \), where \( \bar{E}_g \) is the average expression of the gene \( g \) across \( N \) experiments, \( e \) is average expression of all genes, and \( \sigma \) is the standard deviation of gene expression. All expression levels were log-transformed. The genes were
divided into three groups based on their expression level, namely: \( Z_g(\text{Expression}) > 1 \), \(-1 < Z_g(\text{Expression}) \leq 1\), and \( Z_g(\text{Expression}) \leq -1\).

The genome signature (\( \rho_{CG} \)) is defined as the relative abundance of the frequency of di-nucleotides in the genome, so that \( \rho_{CG} = \frac{f_{CG}}{f_X} \) where \( f_X \) is the frequency of a (di) nucleotide. Genomes or genes can thus be compared with respect to their relative abundance of methylation targets and \( \text{GC}_3 \) richness.

**CG\(_3\)-skew.** Following (Tatarinova et al. 2003), \( \text{CG}_3\)-skew was defined as \( \text{CG}_{3}\text{skew} = \frac{C_3 - G_3}{C_3 + G_3} \). We calculated the 5’-3’ \( \text{CG}_3\)-skew gradient patterns in arabidopsis, rice, bee, fruit fly and human by counting the number of Cs and Gs in the third position of codons in the first 200 codons of \( \text{GC}_3\)-rich and \( \text{GC}_3\)-poor genes.

**Expression measures.** We use mean expression value across all collected experiment for every gene, standard deviation of gene expression values across all conditions and coefficient of variation (CV), defined as a ratio or standard deviation and mean gene expression.

**Distinguishing \( \text{GC}_3\)-rich from \( \text{GC}_3\)-poor genes.** Since \( \text{GC}_3 \) varies between organisms, such definitions are organism-specific and depend on the shape of its distribution which can be either unimodal or bimodal (Figure 1A). In the case of unimodal bell-shaped distribution, common to many plant and animal species, the extreme 5% of the genes from the tails of the distributions are denoted as "\( \text{GC}_3\)-rich" and "\( \text{GC}_3\)-poor" genes (Sablok et al. 2011; Ahmad et al., 2013). By contrast, for bimodal distributions that are common to grasses and homeotherm vertebrates (Elhaik et al. 2009; Elhaik & Tatarinova, 2012), the \( \text{GC}_3 \) cutoff is determined based on the position of the "valley" between the two peaks.

**Gene Ontology (GO) annotation.** GO annotations were obtained from www.geneontology.org, TAIR (www.arabidopsis.org), and Michigan State University (ftp.plantbiology.msu.edu). Upon division of genes into \( \text{GC}_3\)-rich and –poor classes, we computed \( \chi^2 = \frac{(O-E)^2}{E} \) statistic for each GO category (Tables S4, S5).

**Results**

**\( \text{GC}_3 \), body methylation and gene expression**

Of the guanine and cytosine (GC) content at each codon position (\( \text{GC}_1, \text{GC}_2, \text{GC}_3 \)), the last measure represents the fraction of GC content in the codon’s wobble position that has the most freedom to change without altering amino acid sequence of the gene. \( \text{GC}_3 \) exhibits the strongest Pearson’s correlation with gene-body methylation (\( r_{\text{GC}1} = -0.47, r_{\text{GC}2} = -0.35, r_{\text{GC}3} = -0.67 \)) and variability of gene expression (\( r_{\text{GC}1} = 0.1, r_{\text{GC}2} = 0.14, r_{\text{GC}3} = 0.21 \)) and is correlated with the gene’s GC content (e.g. in rice correlation between genic GC and \( \text{GC}_3 \) is 0.94).
Due to the different shapes of the GC₃ distributions in the studied taxa (Figure 1A) we hypothesized that the GC₃ content has a regulatory role and should be correlated with both CpG methylation and gene expression which, in turn, should also be correlated with one another. To test our hypothesis, we carried detailed analyses of the relationship between GC₃ composition, gene body methylation, and gene expression in rice, arabidopsis, honey bee, and human. As expected, in all four species, GC₃ and genic CpG methylation were negatively correlated and CpG methylation had a consistently negative effect on the variability of gene expression (Table 1). The relationship between GC₃ and average gene expression is nonlinear and saddle-like for all four organisms (Figure 2), but the strength of the dependencies varies from organism to organism.

We compared full and partial correlation coefficients, calculated as in (Kim and Yi, 2007), between GC₃, gene expression variability, and gene body methylation (Table 1). We found that the relationship between gene-body methylation and GC₃ is approximately the same, when controlling for variability of gene expression as compared to the full correlation coefficient. Partial correlations between gene expression variability and methylation and between GC₃ and gene expression variability are much smaller than the full correlation coefficients. These results suggest that the relationship between GC₃ and gene body methylation is the driving force and confounds the two other correlations.

In the following sections, we describe the relationship between GC₃, gene-body methylation and gene expression for each of the four organisms we investigated.

**Oryza sativa**

In rice, distributions of GC₃ and gene-body methylation are both clearly bimodal (Figure 1). Genes can be divided into GC₃-rich and -poor classes using the position of the valley between the two peaks (at GC₃≈0.8) and, similarly, into highly methylated and lowly methylated classes (gene-body methylation ≈0.0178). We have previously shown (Tatarinova et al. 2010) that GC₃-rich genes in rice have more methylation targets ($\rho_{CG}$) that can be used to modulate tissue-specific expression: $\rho_{CG}$(poor) = 0.55 and $\rho_{CG}$(rich) = 1.15.

To estimate the regulatory effects of GC₃ we first calculated its correlation with different genic measures including intron density, the number of introns per 1000 bases and intron fraction, defined as the ratio of intron length to gene length, for GC₃-poor and -rich genes that are highly and lowly expressed (Table 2). Compared to lowly expressed genes, highly expressed genes have an intron density approximately twice as high; with both the average number of exons and average intron length being 1.5 times higher. Remarkably, genic measures for highly and lowly expressed genes varied markedly when compared between GC₃-poor and -rich genes (Table 2). For instance, GC₃-poor genes with high (E>1) and low (E<1) expression values differ in their intron density (6.296 and 3.090, respectively) and number of exons (9.60 and 5.41, respectively). We also found that GC₃ is negatively associated with intron density ($r$=−0.36 p-value <0.0001) and with intron fraction ($r$=−0.40, p-value <0.0001).
We found a significant association between methylation and GC$_3$-richness (Table 3) in agreement with previous studies that described a positive correlation between GC$_3$ content and the variability of gene expression in grasses (Tatarinova et al. 2010). Studying the triangular relationship between methylation, gene expression, and GC$_3$ (Figures 2 and 3) we observe that GC$_3$-rich genes tend to have more variable gene expression and lesser gene-body methylation levels than the GC$_3$-poor genes. Moreover, methylation of CpG in coding regions has a nonlinear relationship with gene expression. Both the most lowly and highly expressed genes have low levels of methylation while medium-expressed genes are more methylated, in agreement with the trends reported by Jjingo et al. (2012). These observations suggest the interplay of two or more forces that affect gene expression. GC$_3$ exhibits a trend from high GC$_3$ and low methylation to low GC$_3$ and high methylation. Highly methylated genes, associated with development, genomic imprinting, or silencing of trans-genes, exhibit low expression levels. These results are consistent with the notion that methylated genes can undergo 5-methylcytosine deamination where mC$\rightarrow$T. In such cases, the third position can often undergo cytosine deamination reducing GC$_3$ without affecting the protein sequence, whereas the first two nucleotides in the codon are less likely to mutate due to selective pressures to conserve amino acid sequences. Hence, methylated genes are expected to be GC$_3$-poor. Consequently, low-methylated genes have high GC$_3$ values and low average expression (Figure 3), where an increase of CpG methylation and high deamination rate lead to a drop in GC$_3$ values; at the same time the average expression reaches the maximum for the broadly expressed genes. A further increase in methylation does not affect GC$_3$, but rather reduces gene expression, leading to a repression of the gene (see Supplementary Materials and Figure S5 for further details).

To examine the effect of alternative splicing on the correlation between methylation and GC$_3$, we next considered the relationship between GC$_3$ and gene-body methylation for intron-containing and intron-less genes. Lyko et al. (2010) discovered that clusters of methylated cytosines are associated with alternatively-spliced exons and that intron containing genes are more methylated than intron-less genes. Intron-less genes are, obviously, not subject to alternative splicing while genes with introns may be alternatively spliced. There are 2,648 intron-less genes in the dataset; for these the average values of GC$_3$=0.63, GC$_2$=0.51, and GC$_1$=0.77, compared to 6,959 intron-containing rice genes with average GC$_3$=0.58, GC$_2$=0.47, and GC$_1$=0.61. Indeed, intron-containing genes are twice more methylated than intron-less genes (0.18 vs. 0.09). As expected, intronContaining genes also exhibit a stronger positive relationship between the average methylation and expression, between the CV of gene expression and GC$_3$, and stronger negative correlation between the CV of gene expression and methylation (Table 4). Interestingly, we observed only a small difference in the correlations between the average methylation and GC$_3$ between intron-less ($r$=−0.6) and intron-containing ($r$=−0.67) genes. Therefore, splicing influences the relationship between methylation, expression and nucleotide composition.

Traditional microarray measurements, which ignore alternative splicing, are not able to fully measure variability of gene expression. This may partially explain why when comparing intron-containing with intron-less genes, the first have higher average expression (1.41 vs. 1.13, respectively) and lower CV of gene expression (0.92 vs. 1.28, respectively). We hypothesize that apparent constitutive expression of hyper-methylated, intron-containing genes can be a complex phenomenon, with different splicing forms
expressed at different developmental stages, tissue types and external conditions. We hypothesize that gene expression variability of hypo-methylated, intron-less genes is achieved by transcriptional regulation. Overall, alternative splicing events may explain the differences in methylation and expression levels between intron-less and intron-containing genes, but not the differences between GC\textsubscript{3} and gene-body methylation.

A more general explanation of the relationship between gene expression and methylation involving the nucleosome was recently proposed by (Jjingo et al. 2012). The authors pointed out that CpG sites occur frequently across gene bodies and that in genes with low levels of expression, methylation is prevented by dense nucleosome packing. By contrast, in genes with average levels of expression these sites are accessible to DNMTs and hence are more likely to be methylated. When expression is high, polymerases and DNMTs compete for the access to the same sites and hence methylation is suppressed again.

*Arabidopsis thaliana*

Arabidopsis has a narrow and unimodal distribution of GC\textsubscript{3} and a bimodal distribution of methylation levels (Figure 1). Despite of the apparent unimodality of the GC\textsubscript{3} distribution, Arabidopsis genes with GC\textsubscript{3}>0.5 are significantly less methylated than genes with GC\textsubscript{3}≤0.5: P(methylation<0.016|GC\textsubscript{3}>0.5)=0.72 and P(methylation<0.016|GC\textsubscript{3}≤0.5)=0.33, suggesting a relationship between GC\textsubscript{3} and methylation. More specifically, the increase of GC\textsubscript{3} composition is negatively correlated with gene body methylation levels (Figure 4A). Of the three methylation contexts, the most pronounced effect is observed for CpG methylation (r=-0.27, p-value <0.0001) (Figure 4), while CHG and CHH methylation levels appear to be less affected by GC\textsubscript{3} composition.

In thale cress, the average relative abundance of the frequency of CG di-nucleotide (genome signature, \(\rho_{CG}\)) for all genes is 0.73. The relative abundance of methylation targets depends on GC\textsubscript{3} richness, for genes with GC\textsubscript{3}>0.5 (mean \(\rho_{CG}=0.91\)) and the remaining genes (average \(\rho_{CG} =0.71\)). There is also a relationship between methylation levels and \(\rho_{CG}\): \(\rho_{CG}(\text{methylation}<0.016)=0.84\) while \(\rho_{CG}(\text{methylation}>0.016)=0.67\). Hence, GC\textsubscript{3}-rich genes have more methylation targets but are less methylated. Therefore, in spite of the unimodality of the GC\textsubscript{3} distribution in *A. thaliana*, the relationship between methylation and GC\textsubscript{3} is similar to the pattern observed for rice. That is, like the other taxa, arabidopsis exhibits a nonlinear, saddle-like dependence, between the strength of gene expression and GC\textsubscript{3} (Figure 2A), but its gene expression variability grows almost linearly with GC\textsubscript{3} (Figure 2B).

To further study the relationship between tissue-specific gene body methylation and tissue-specific expression, we next examined tissue specific patterns across shoots and roots as these exhibit differences in morphology, gene expression activity and function. We investigated 1000 genes from the two tails of the log(shoots/roots) expression distribution (see Materials and Methods) and compared the differences between shoot and root body methylation levels for the two gene groups. We found that the average genic methylation was similar for shoots and roots (0.063 in shoots vs. 0.057 in roots). However, for genes over-expressed in shoots, there was a negligible difference between shoot and root
methylation, whereas for genes over-expressed in roots, on average, the “shoot” genes were 21% more methylated than the “root” genes (p-value =0.003). These results are again in agreement with Jjingo et al. (2012) and highlight the role of methylation in contributing to tissue-specific expression. Interestingly, differences between methylation levels in shoots and roots increase with GC$^3$ for all methylation types (Figure 4B).

In summary, GC$^3$ is positively correlated with both expression variability and variation in genic methylation. There is also an inverse relationship between gene-body tissue-specific methylation and tissue specific gene expression.

**Apis mellifera**

The GC$^3$ distribution of the European honey bee, *Apis mellifera*, is a unimodal right skewed distribution with a long tail of high GC$^3$ values (Figure 1). The honey bee is a GC$^3$–poor organism, but it has a surprising medium and high GC$^3$ tail, containing approximately 25% of its genes with GC$^3$$>$0.5. Based on the current annotation, 2.2% of all *Apis mellifera* genes encode receptors (such as Metabotropic glutamate receptor, Toll-like receptor, Dopamine receptor type D2, D2-like dopamine receptor, Ephrin receptor, SIFamide receptor, Ecdysteroid receptor A isoform, Antennapedia protein, Nicotinic acetylcholine receptor alpha1 subunit, Alpha-glycosidase G-protein coupled receptor, and others). Genes with GC$^3$$>$0.505 are significantly enriched in receptor encoding genes, which account for 5.6% of these compared to 1.3% in genes with GC$^3$$<$0.12 (p-value=2.6E-7). The frequency of CG dinucleotides differ between GC$^3$-rich genes (average $\rho_{CG} = 1.18$) and GC$^3$-poor genes (average $\rho_{CG} = 0.41$) To further study the relationships between GC$^3$-richness, receptor genes and methylation, we compared data from Queen and Worker bees.

Queen and Worker bees share the same genome but differ in size, appearance and life span. While there is little difference between the whole-genome methylation level of the Worker and Queen bees (around 1% of cytosines in CpG contexts are methylated in both), their genes differ significantly in methylation levels (Figures 5A-B). This finding is in agreement with a previous report that Worker and Queen bees differ in the methylation of ~550 genes (Lyko et al. 2010). Lyko et al. (2010) also reported that unmethylated genes are enriched in receptors. The methylated genes encode proteins showing a higher degree of conservation than proteins encoded by non-methylated genes (Foret et al 2009). Of the three methylation contexts, we observed that the average fraction of CG methylation per gene was associated with GC$^3$ composition (Figures 5C-D) in support of a putative GC$^3$ regulatory role. In other words, increases in GC$^3$ in bees are associated with a decrease in gene-body methylation levels, which are enriched for receptor encoding genes. Follow up analyses of bee methylation patterns can be found elsewhere (Lyko et al. 2010; Foret et al. 2012).

In addition to these relationships, we also found that differences between methylation levels in Worker and Queen bees depend on the nucleotide composition of coding regions. We analyzed the relationship between gene body-methylation and GC$^3$ for Queen and Worker bees. The relative difference between
gene-body methylation in queen and worker bees, defined as \( \frac{(Q-W)}{Q+W} \), depends on the methylation context (CpG, CHH or CHG) (Figure 5A). The relative difference in CpG and in total methylation is low for the GC\textsubscript{3}-poor genes and increases substantially when GC\textsubscript{3} approaches 0.4 after which methylation stays roughly the same for genes with GC\textsubscript{3}>0.4 (Figure 5A). Relative difference between CHH and CHG methylation decreases with the increase of GC\textsubscript{3}. The transition between the compositional environments may be related to changes in the regulatory role of each region. The difference between CpG methylation levels between Queen and Worker is negative for low GC\textsubscript{3} genes (Queen bee is less methylated) and becomes positive with an increase of GC\textsubscript{3} (Figure 5B). Overall, GC\textsubscript{3} poor genes are more methylated than GC\textsubscript{3} rich genes (Figures 5C and 5D). As compared to the worker bees, queen bees have lower body methylation levels for GC\textsubscript{3}-poor class (enriched for ubiquitously expressed genes) and higher for GC\textsubscript{3}-rich class (enriched for receptor-encoding genes) (Figure 5B). Since queen and worker bees play drastically different roles in the beehive, they activate and rely onto different sets of genes (Aamodt, RM 2009). Higher social role of the queen bee may require more elaborate interaction with environment, which necessitates more regulation of the GC\textsubscript{3}-rich receptor-encoding genes through methylation. Our observations agree with Foret et al (2009) and Elango el al (2009), who pointed out that ubiquitously expressed critical genes are methylated at the germ-line, while cast-specific genes lack methylation. Caste-specific genes remain unmethylated to allow for greater epigenetic flexibility and regulatory control (Elango et al., 2009). Greater degree of flexibility is important for certain classes of genes in other invertebrates: according to Gavery and Roberts (2010) and Roberts and Gavery (2012), the ubiquitously expressed housekeeping genes tend to be hyper-methylated while tissue-specific and inducible genes are hypomethylated.

**Homo sapiens**

Coding regions of *Homo sapiens* have a broad bimodal distribution of GC\textsubscript{3} values (Figure 1A) and a unimodal distribution of genic methylation levels, with a long tail towards low methylation levels (Figure 1B) (Chodavarapu et al. 2010). As in the other three species, the relative abundance of CpG dinucleotides differs for GC\textsubscript{3}-rich and poor genes: \( p_{CG}(rich) = 0.68 \) and \( p_{CG}(poor) = 0.29 \). Overall, the *H. sapiens* genome is more methylated than bee, rice, and arabidopsis (Figure 1B). Although the nonlinear dependence between GC\textsubscript{3} and gene expression is apparent (Table 1, Figure 2A and Figure 2B), its shape differs compared to the other three species we analyzed. In human, CpG methylation is negatively correlated with GC\textsubscript{3} and CHH and has no significant correlation with CHG methylation (Table 1 and
Figure 6). The weak correlation between GC₃ expression and methylation suggests the existence of other evolutionary forces affecting gene expression in the human genome.

The compositional environment and gene-body methylation paradox

A pronounced pattern that emerged from all our analyses is that GC₃-rich genes are, on average, under methylated, despite their enrichment of CpG dinucleotides. To further illustrate this trend, we compared the GC₃ gradient (Figure S1) and the CG₃-skew (Figure S2) across all tested taxa with gradients of methylation levels using the same groups of GC₃-rich and GC₃-poor genes (Figure S3). The positive 5’-3’ gradient of body-methylation, where methylation increases toward the mid-portion of the transcribed part of the gene can be attributed to a gene experiencing "boundary effects" from the attachment of transcriptional and translational machinery. At the 5’-end methylation needs to be low to enable attachment of proteins. Deamination of methylated cytosines in broadly expressed and highly methylated GC₃-poor genes leads to the decrease in C nucleotides and negative CG₃-skew in the middle of the gene (Figure S2). Although GC₃-rich genes are enriched in methylation targets, they are under-methylated compared to GC₃-poor genes. In fact, GC₃-rich genes were so hypomethylated that we had to log-transform the methylation levels to be able to plot the two trends on the same figure. Additional evidence of the different regulatory roles GC₃-poor and GC₃-rich genes assume in methylation can be found by looking at the competing process of cytosine deamination reducing methylation targets.

GC₃-rich and GC₃-poor genes exhibit different body-methylation levels and different gradients of methylation in coding regions (see Supplementary Materials, Figures S1-S3). The variation in compositional gradients may explain the under methylation observed in GC₃-rich genes. Methylation level of GC₃-poor genes experiences steep growth in the first 100 codons (300 nucleotides) and then stays approximately constant (Figure S3). With the exception of H. sapiens H1 cell line, methylation levels of GC₃-rich genes are position-independent. As shown by Tatarinova et al. (2010) and by Sablok et al. (2011), towards the middle of the gene, GC₃-rich genes continuously become more C-rich (positive CG₃-skew), whereas GC₃-poor genes become G-rich (negative CG-skew); GC₃-rich genes become even more GC₃-rich towards the middle of the gene, and GC₃-poor genes become more GC₃-poor. We hypothesize that for the broadly expressed and highly methylated GC₃-poor genes, the decrease in C nucleotides may be due to cytosine deamination (mC→T transitions).

To this end, we next looked at genes of Drosophila melanogaster, which belongs to the so-called "Dnmt2 only" organisms that do not contain any of the canonical DNA methyltransferases (Dnmt1 and Dnmt3) (Krauss and Reuter 2011). The levels of DNA methylation in the fruit fly are significantly lower than in other organisms (Lyko et al. 2000). In the fruit fly GC₃ content is positively associated with strength of gene expression (Figure S4D). For the 300 genes with GC₃<0.55, average expression across 71 conditions is 2.12 on the log10 scale, vs. average expression of 3.62 for the 300 genes with GC₃>0.8. Surprisingly, variability of fruit fly gene expression does not seem to be affected by GC₃. In addition, average genome signatures , for both GC₃-rich and -poor fly genes are even ($\rho_{CG} = 0.9$).
We compared the 5' to 3' gradients of CG skew in bee, thale cress, rice and human (where a significant degree of gene body methylation exists) to those in the fruit fly. In the first four taxa, we observed drastically different 5' to 3' gradients of CG skew in both GC$_3$ rich and GC$_3$ poor genes (Figure S2), whereas in the fruit fly these trends are absent (Figure S4C). Decreased methylation of 5' regions of was previously described by Roberts and Gavery (2013). In other words, the unmethylated fly genes exhibit similar GC$_3$ 5'-3' gradients (Figure S4B) to those of the other taxa. However, due to the absence of cytosine deamination there are even levels of Cs and Gs for both fly GC$_3$-rich and -poor genes, whereas in the other taxa cytosine deamination reduces the number of Cs for the highly methylated GC$_3$-poor genes in a position-specific manner (Figure S2).

DISCUSSION

Gene-body methylation and gene expression exhibit complex relationships with one another and with sequence composition. For example, DNA methylation in coding and non-coding regions have opposite effects on gene expression: in promoters, cytosine methylation often makes transcription factor binding sites inaccessible to transcription factors and is responsible for transcriptional repression in A. thaliana (Chan et al. 2005) while gene-body methylation is reported to be positively correlated with gene expression in H. sapiens (Hellman and Chess 2007). Generally, these relationships exhibit similarity across diverse taxa, but may vary for particular genes. For example, Aceituno et al. (2008) noted that in A. thaliana housekeeping genes that have broad and steady expression levels were more body-methylated than expected based on whole genome methylation levels ($p=1.5E-35$). Only 8% of the hyper-variable genes, such as stress response or tissue specific genes with high values of gene expression coefficient of variation) were found to be body-methylated. Aceituno et al. (2008) also reported that gene body-methylation is negatively correlated ($r=-0.89$) with the variability of gene expression on a genome-wide scale, implying that housekeeping genes having low expression variability have higher methylation levels and vice versa. This report follows Bird et al.’s (1995) hypothesis that gene-body methylation could be responsible for the repression of spurious transcription within genes and hence lead to more reliable transcription, which results in a positive correlation between gene expression and gene-body methylation. This relationship was previously described as exhibiting a bell-shaped distribution (Zilberman et al. 2007; Zemach et al. 2010).

To better understand the regulatory role of gene-body methylation and its relationship with sequence composition, we studied the role of GC$_3$ in four taxa: rice, thale cress, bee, and human. We showed that GC$_3$ richness and methylation are negatively correlated, which leads to a seeming paradox: if GC$_3$-rich genes are enriched in methylation targets, why are they under methylated compared to GC$_3$-poor genes? One reason for this negative correlation may be due to the prevalence of ubiquitously expressed genes in the GC$_3$-poor class that use body-methylation as one of the mechanisms to maintain broad expression. Association between alternative splicing, gene expression and methylation allows us to hypothesize that the alternatively spliced intron-containing genes and oppositely, the intron-less achieve gene expression variability via different mechanisms. Hypo-methylation of intron-less, high GC$_3$ genes and abundance of methylation targets allows achieving higher regulatory control. Hyper-
methylated, intron-containing, low GC$_3$ genes can express different splicing forms and be expressed at different developmental stages, tissue types and external conditions. It is thus not surprising that GC$_3$-rich, hypo-methylated genes have higher genetic diversity as compared to the GC$_3$-poor, hyper-methylated genes (Tatarinova et al. 2009; Lyko et al., 2010; Roberts and Gavery, 2012).

We propose that the opposite effects of methylation and compositional gradients along CDS of GC$_3$-poor and GC$_3$-rich genes (Figure S3), are the products of two or more competing processes. The first driver is transcriptional efficiency. There may be a "universal pressure" to increase the fraction of C-ending codons from the 5' to the 3' end of the gene that can be explained by the need to increase the speed of transcription in this direction. This is especially important for stress-specific genes (that are frequently GC$_3$-rich) (Tatarinova et al. 2010), since they are expressed as a response to a certain environmental condition, likely at a high level, for a limited amount of time resulting in a large number of RNA polymerases (RNAPs) that move simultaneously along the same track. Hence, it is necessary to avoid RNAP congestion and increase the speed of transcription. There is no such pressure for ubiquitously expressed genes (frequently GC$_3$-poor), since RNAP congestion effects are not likely to occur.

The competing process may be cytosine deamination, which affects more methylated genes and genes that are expressed at relatively constant levels across tissues. GC$_3$-rich genes are less methylated and are likely to have limited tissue-specific and stress-specific expression patterns that require less time in the transcriptional bubble. Therefore, the effect of cytosine deamination is less pronounced in GC$_3$-rich genes. For GC$_3$-rich genes, transcriptional kinetics is the winning driver.

Takuno and Gaut (2012) hypothesized that "body-methylated genes would be both longer and more functionally important than unmethylated genes." The authors suggested that methylation has a functional role, such as maintaining transcriptional accuracy and splicing efficiency, thus explaining why the GC$_3$-poor housekeeping genes are overall highly methylated. This agrees with our findings (Table 2) that GC$_3$ poor genes are longer (e.g., in rice, GC$_3$-rich genes are on average 1031 nt long and GC$_3$-poor genes are on average 1648 nt long) and have more exons (e.g., in rice, GC$_3$-rich genes have on average 2.38 exons and GC$_3$-poor genes have on average 8.57 exons). Takuno and Gaut (2012) also found that "body-methylated genes evolve more slowly than unmethylated genes, despite the potential for increased mutation rates in methylated CpG dinucleotides." This is also consistent with our observation (Tatarinova et al. 2010) of faster evolution of unmethylated GC$_3$-rich genes as compared to methylated GC$_3$-poor genes. Finally, we have shown that methylated genes have a lower proportion of CpG nucleotides, which supports the deamination hypothesis.

Overall, our work supports and expands recent findings by Takuno and Gaut (2012) and Roberts and Gavery (2012). We propose several possible explanations to the question of why GC$_3$-rich genes are enriched in CpG dinucleotides compared to GC$_3$-poor genes: first, these sites may have played a regulatory role in the past and are maintained in the genome to allow phenotypic plasticity by increasing the number of transcriptional opportunities (Roberts and Gavery, 2012). Second, these sites may have an active regulatory role that has yet to be determined. Third, we suggest considering the problem from
a different angle—that while GC3-poor genes have less CpG sites than GC3-rich genes, they are more body-methylated because as methylation increases in the 5’→3’ direction, there is more chance for mC→T mutation towards the middle of the gene. Most of the GC3-poor genes are ubiquitously expressed; therefore, the sense strand spends more time unprotected during transcription (Tatarinova et al. 2003). The cytosines are therefore lost in the deamination processes and the CG3-skew value is reduced. Since the third position in the codon is not under pressure to conserve the protein sequence, the mC→T mutations are manifested as gene’s GC3-poorness. In support of this view, the 5’ end of genes has a lower level of methylation and positive gradient of CG3-skew for both GC3-rich and GC3-poor genes, which can be explained by transcription/translation initiation requirements.

If methylation is associated with transcription then the ubiquitously active genes should lose GC3 due to deamination while the inducible ones should not. Looking at the gene body methylation and GC3 composition as a function of the normalized average gene expression in rice (Figure S5), methylation and GC3 have opposing trends: where GC3 increases, methylation decreases and vice versa. Normalized gene expression between -1 and +1 contains many of the ubiquitously expressed genes, and in this region a decrease in GC3 is accompanied by an increase in methylation. Methylation and GC3 of inducible genes, having low average expression (below -1 in Figure S5) are not affected by the change in gene expression.

Our observation that the unmethylated fly genes exhibit similar GC3 5’→3’ gradients to those of the other taxa but different patterns of CG3-skew supports the significance of cytosine deamination. In the fruit fly, due to the absence of cytosine deamination, levels of Cs and Gs for both GC3-rich and -poor genes are approximately the same, whereas in the other taxa cytosine deamination reduces the number of Cs for the highly methylated GC3-poor genes.

We note that in addition to the processes described here, there are two major forces affecting GC3. One is GC-biased gene conversion (BGC) (Duret 2008), which is common to all our model species (Duret and Arndt 2008; Duret and Galtier 2009; Katzman et al. 2011; Kent et al. 2012; Günther et al. 2012; Muyle et al. 2011). The other is selection on codon usage, which has been shown to occur in Arabidopsis (Muyle et al. 2011; Günther et al. 2012). It has been suggested that recombination hotspots can create strong substitution hotspots that are correlated with gene density that drive the evolution of GC content (Duret and Arndt 2008; Tatarinova et al., 2010). Affecting both coding and non-coding regions, BGC may lead to enrichment in GC-content in genomic regions of high recombination compared to regions of low recombination and may explain the patterns observed in human. Coding regions may also be susceptible to codon usage bias that directly affects the frequency of GC3. The complex interplay between these forces and their relative effect on methylation and gene expression in different species remains unclear and provides a fertile area for future studies.

**Conclusions**
We report strong negative correlations between CpG methylation and the GC₃ content of genes in rice, bees, Arabidopsis and humans. We propose several explanations for the triangular relationship between GC₃, methylation, and expression patterns. The negative correlation between GC₃ and methylation can be explained by the prevalence of ubiquitously expressed genes in the GC₃-poor class that use body-methylation as one of the mechanisms to maintain broad expression. Positive 5’-3’ gradient of body-methylation, where methylation levels rise toward the mid-portion of the transcribed part of the gene, can be attributed to a gene experiencing "boundary effects" from the attachment of transcriptional and translational machinery. We propose that the opposite effects of methylation and compositional gradients along CDS of GC₃-poor and GC₃-rich genes are the products of two or more competing processes. The first driver is transcriptional efficiency. The competing process may be cytosine deamination, which affects more methylated genes and genes that are expressed at relatively constant levels across tissues. GC₃-rich genes may be enriched in CpG dinucleotides as compared to GC₃-poor genes for a number of reasons: firstly, these sites may have played a regulatory role in the past and are maintained in the genome to allow phenotypic plasticity. Secondly, these sites may have an active regulatory role that has yet to be determined. Thirdly, cytosine deamination may reduce the frequency of CpG dinucleotides in ubiquitously expressed (GC₃-poor) genes.

**List of abbreviations**

GC₃ - the percentage of cytosines and guanines in third codon positions in a gene.
CDS - coding DNA sequence.
CpG, CpHpG, and CpHpH – motifs consisting of Cysteine (C), phosphate (p), Guanine (G), and H any nucleotide except guanine (H).

**Competing interests**

The author(s) declare that they have no competing interests.

**Authors' contributions**

TT and EE designed the study and carried out all analyses. MP conceived of the study, and participated in its implementation. All authors were involved in preparation of the manuscript; they read and approved the final version of it.

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Figures

Figure 1: Distributions of GC₃ content for rice, Arabidopsis, bee and human.
Figure 2: GC$_3$ vs. Expression for 4 organisms: Bee (green), Rice (blue), Arabidopsis (red), and Human (purple). (A) shows relationship between standardized values of GC$_3$ and average expression, (B) shows gene expression variability as a function of GC$_3$. Every point represents a mean across at least 100 genes and the standard error of the mean does not exceed 0.1 (plot A) and 0.06 (plot B).
Figure 3: *Oryza sativa*: Relationship between GC$_3$ (purple triangles), gene expression strength (blue diamonds), expression variability (red squares) and methylation. Standard error of the mean is below 0.03 (GC$_3$), 0.11 (expression), 0.03 (expression variability).

![Graph showing relationship between GC$_3$, gene expression strength, expression variability, and methylation.](image-url)
Figure 4: *Arabidopsis thaliana*. Methylation level in Arabidopsis as a function of GC$_3$ (A) and differential methylation between shoots and roots (B). Blue diamond: CG, Red square: C, Green triangle: CHG, violet cross (CHH). Every point represents an average across 100 or more genes. The absolute relative difference is calculated as $\frac{|\text{Meth}_{\text{shoots}} - \text{Meth}_{\text{roots}}|}{\text{Meth}_{\text{shoots}} + \text{Meth}_{\text{roots}}}$. Standard error of the mean does not exceed 0.05 for the mean methylation levels calculation.
Figure 5: *Apis mellifera*: (A) Relative difference in gene body methylation levels $\frac{Q-W}{Q+W}$ as a function of GC$_3$ between worker and queen bee. (B) Difference in gene body methylation between worker and queen bee as a function of GC$_3$. (C) Queen and (D) Worker bee methylation as a function of GC$_3$. Every point represents an average of at least 228 genes. Standard error of the mean for methylation levels was below 0.006.
Figure 6: *Homo sapiens*: Methylation as a function of $\text{GC}_3$. Every point represents a mean across at least 100 genes, with standard error of the mean not exceeding 10% of the mean.
### Tables

**Table 1: Pearson’s correlation coefficients between CpG methylation, GC\textsubscript{3} and gene expression variability for *O. sativa*, *A. thaliana*, *A. mellifera*, and *H. sapiens*.** Top numbers in each cell represent Pearson’s correlation coefficients and bottom numbers represent partial correlation coefficients.

<table>
<thead>
<tr>
<th>Correlation between</th>
<th><em>O. sativa</em></th>
<th><em>A. thaliana</em></th>
<th><em>A. mellifera</em></th>
<th><em>H. sapiens</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>CpG methylation and GC\textsubscript{3}</td>
<td>-0.67</td>
<td>-0.27</td>
<td>-0.65</td>
<td>-0.23</td>
</tr>
<tr>
<td></td>
<td>-0.65</td>
<td>-0.23</td>
<td>-0.62</td>
<td>-0.23</td>
</tr>
<tr>
<td>CpG methylation and gene expression variability (CV)</td>
<td>-0.18</td>
<td>-0.18</td>
<td>-0.24</td>
<td>-0.02</td>
</tr>
<tr>
<td></td>
<td>-0.06</td>
<td>-0.13</td>
<td>-0.04</td>
<td>-0.06</td>
</tr>
<tr>
<td>Gene expression variability (CV) and GC\textsubscript{3}</td>
<td>0.21</td>
<td>0.16</td>
<td>0.34</td>
<td>-0.16</td>
</tr>
<tr>
<td></td>
<td>0.12</td>
<td>0.12</td>
<td>0.22</td>
<td>-0.16</td>
</tr>
</tbody>
</table>

**Table 2: Compactness of rice genes, stratified by expression and GC\textsubscript{3}**

<table>
<thead>
<tr>
<th>GC\textsubscript{3}</th>
<th>EXON LENGTH</th>
<th>EXONS</th>
<th>INTRON DENSITY (per 1000nt)</th>
<th>INTRON LENGTH</th>
<th>INTRON FRACTION (length)</th>
<th>NUMBER OF ORFs</th>
<th>EXPRESSION (standardized)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC\textsubscript{3}&gt;0.800</td>
<td>767</td>
<td>2.47</td>
<td>2.301</td>
<td>1683</td>
<td>62.4%</td>
<td>428</td>
<td>E&gt;1</td>
</tr>
<tr>
<td></td>
<td>1132</td>
<td>2.21</td>
<td>1.132</td>
<td>1085</td>
<td>41.9%</td>
<td>1215</td>
<td>E&lt;-1</td>
</tr>
<tr>
<td>GC\textsubscript{3}&lt;0.491</td>
<td>1503</td>
<td>9.60</td>
<td>6.296</td>
<td>4249</td>
<td>73.3%</td>
<td>924</td>
<td>E&gt;1</td>
</tr>
<tr>
<td></td>
<td>1587</td>
<td>5.41</td>
<td>3.090</td>
<td>3116</td>
<td>60.1%</td>
<td>386</td>
<td>E&lt;-1</td>
</tr>
</tbody>
</table>

**Table 3: Four classes of (n=9,607) rice genes by GC\textsubscript{3} and methylation.** Yates’s χ\textsuperscript{2} = 4267.237

<table>
<thead>
<tr>
<th></th>
<th>GC\textsubscript{3}-rich</th>
<th>GC\textsubscript{3}-poor</th>
</tr>
</thead>
<tbody>
<tr>
<td>High methylation</td>
<td>289</td>
<td>4787</td>
</tr>
<tr>
<td>Low methylation</td>
<td>3161</td>
<td>1370</td>
</tr>
</tbody>
</table>
Table 4: Pearson’s correlation coefficients between GC$_3$, methylation (AVG_MET), average expression (AVG_EXP), standard deviation of gene expression (STD_EXP), coefficient of variation of gene expression (CV_EXP) and gene length (LENGTH) for intron-less genes and genes with introns. Calculated for 2,648 intron-less genes and 6,959 intron-containing genes. 95% CI is shown in square brackets below each correlation value.

<table>
<thead>
<tr>
<th>Type</th>
<th>AVG_MET and GC$_3$</th>
<th>AVG_EXP and GC$_3$</th>
<th>STD_EXP and GC$_3$</th>
<th>CV_EXP and GC$_3$</th>
<th>LENGTH and GC$_3$</th>
<th>AVG_MET and AVG_EXP</th>
<th>AVG_MET and CV_EXP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intron-less genes</td>
<td>-0.602 [-0.626, -0.577]</td>
<td>0.103 [0.065, 0.141]</td>
<td>0.187 [0.149, 0.223]</td>
<td>-0.075 [-0.113, -0.037]</td>
<td>-0.235 [-0.270, -0.198]</td>
<td>0.038 [-0.001, 0.075]</td>
<td>-0.017 [-0.055, 0.021]</td>
</tr>
<tr>
<td>Intron-containing genes</td>
<td>-0.671 [-0.684, -0.657]</td>
<td>-0.230 [-0.252, -0.208]</td>
<td>0.000 [-0.023, 0.024]</td>
<td>0.245 [0.222, 0.267]</td>
<td>-0.307 [-0.328, -0.286]</td>
<td>0.233 [0.211, 0.255]</td>
<td>-0.209 [-0.231, -0.186]</td>
</tr>
</tbody>
</table>