(MS ID: MBE-12-0129)

Assessing determinants of exonic evolutionary rates in mammals

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Keywords: exon evolution, alternative splicing, structural-functional constraint, gene expression, gene compactness

Running head: Determinants of exonic evolutionary rates

Abstract

From studies investigating the differences in evolutionary rates between genes, gene compactness and gene expression level have been identified as important determinants of gene-level protein evolutionary rate, as represented by nonsynonymous-to-synonymous substitution rate (d_N/d_S) ratio. However, the causes of exon-level variances in d_N/d_S are less understood. Here we use principal component regression to examine to what extent thirteen exon features explain the variance in d_N, d_S, and the d_N/d_S ratio of human-rhesus macaque or human-mouse orthologous exons. The exon features were grouped into six functional categories: expression features, mRNA splicing features, structural-functional features, compactness features, exon duplicability, and other features, including G+C content and exon length. Although expression features are important for determining d_N and d_N/d_S between exons of different genes, structural-functional features and splicing features explained more of the variance for exons of the same genes. Furthermore, we show that compactness features can explain only a relatively small percentage of variance in exon-level d_N or d_N/d_S in either between-gene or within-gene comparison. By contrast, d_S yielded inconsistent results in the human-mouse comparison and the human-rhesus macaque comparison. This inconsistency may suggest rapid evolutionary changes of the mutation landscape in mammals. Our results suggest that between-gene and within-gene variation in d_N/d_S (and d_N) are driven by different evolutionary forces, and that the role of mRNA splicing in causing the variation in evolutionary rates of coding sequences may be underappreciated.

Introduction

The evolutionary rates of different protein-coding genes in a genome can vary by several orders of magnitude (Li 1997). This variation has been extensively studied and is typically explained by differences in mutation rate and selection intensity among genes. In the past few years, data generated by whole-genome sequencing and functional genomic assays have provided biologists an unprecedented opportunity to address this issue systematically. As a result, several biological factors associated with and potentially underlying evolutionary rate variations of protein-coding genes have been identified. These factors include gene essentiality (Hirsh and Fraser 2001; Jordan et al. 2002; Zhang and He 2005; Liao, Scott, and Zhang 2006), gene expression level (Pal, Papp, and Hurst 2001a; Akashi 2003; Subramanian and Kumar 2004; Drummond, Raval, and Wilke 2006), tissue specificity of gene expression (Hastings 1996; Duret and Mouchiroud 2000; Subramanian and Kumar 2004; Winter, Goodstadt, and Ponting 2004; Zhang and Li 2004), presence of a duplicate paralog (Nembaware et al. 2002; Castillo-Davis and Hartl 2003; Yang, Gu, and Li 2003), properties in the protein interaction network (Fraser et al. 2002; Fraser 2005; Hahn and Kern 2005; Kim, Korbel, and Gerstein 2007), tendency to form misinteracting protein complex (Yang et al. 2012), local recombination rate (Pal, Papp, and Hurst 2001b), pleiotropy (He and Zhang 2006), amino acid composition (Xia, Franzosa, and Gerstein 2009), structural features of protein folding (Bloom et al. 2006; Zhou, Drummond, and Wilke 2008; Franzosa and Xia 2009), G+C content (Xia, Franzosa, and Gerstein 2009), gene compactness (Liao, Scott, and Zhang 2006), and subcellular localization (Liao, Weng, and Zhang 2010).

All of the abovementioned studies focused on sequence evolution of protein coding genes as a whole. However, evolutionary rates also differ among regions of the same protein. For example, structurally ordered protein regions evolve more slowly than intrinsically disordered regions (IDRs) (Brown et al. 2002; Brown, Johnson, and Daughdrill 2010; Chen, Pan, and Lin 2011), and protein regions encoded by alternatively spliced exons (ASEs) evolve more rapidly than those encoded by constitutively spliced exons (CSEs) (Chen et al. 2006; Chen et al. 2007; Ramensky et al. 2008; Chen, Pan, and Lin 2011). Thus, exon features have a profound effect on within-gene variation in evolutionary rate. Because exon-intron structure is an important characteristic of multicellular eukaryotic genes that causes complexity (Sorek, Shamir, and Ast 2004; Xing and Lee 2007) and diversity (Xing and Lee 2005; Chen et al. 2006; Chen and Chuang 2007; Keren, Lev-Maor, and Ast 2010; Chen, Pan, and Lin 2011) of proteomes, a systematic analysis to delineate the individual and integrative contributions of exon features to within-gene evolutionary rate variation is necessary to understand the molecular evolution of complex organisms.

To address this issue, we analyzed the effects of exon features (described below) on the variation of exonic evolutionary rates in mammals. We calculated the nonsynonymous substitution rate (d_N), synonymous substitution rate (d_S), and the d_N/d_S ratio for exons in human-mouse and human-rhesus macaque one-to-one orthologous genes. To account for the inter-correlations between evolutionary rate determinants, principal component regression (PCR) was used to analyze the relative contributions of exon features on the variances of d_N, d_S, and d_N/d_S ratio. PCR outperformed multivariate regression and partial correlation in delineating the relationships among multiple inter-correlated factors when the data were noisy (Drummond, Raval, and Wilke 2006). In this study, thirteen exon features that may affect evolutionary rates were analyzed (table 1): weighted exon frequency (WEF, see Materials and Methods), ASE/CSE exon type, exonic expression level, coefficient of variation in exonic expression levels across multiple tissues, exonic expression breadth, percent of IDR, percent of annotated protein domain(s), proportion of amino acids predicted to be solvent-accessible, the lengths of 5' and 3' flanking introns, exon duplicability (Materials and Methods), exon length, and G+C content. We demonstrate that the features related to the splicing and structural-functional constraints of exons are the most important in causing within-protein variation in evolutionary rates in mammals.

Materials and Methods

Source data and calculation of evolutionary rates

The human-mouse and human-rhesus macaque one-to-one orthologous genes and the corresponding transcript and peptide sequences were retrieved from Ensembl v59 through the BioMart interface (http://www.biomart.org) (Guberman et al. 2011). To ensure data quality, we retained only full-length transcripts (with start and stop codons) that have known protein products. To avoid unequal weighting between genes, we selected the longest transcript from each human gene as the representative. We then aligned the human peptide sequence against the orthologous mouse/macaque peptide sequences (i.e. peptides derived from one-to-one orthologous gene pairs) using MUSCLE (Edgar 2004). The longest alignable mouse/macaque peptide orthologue was retained. The peptide sequence alignments were then back-translated to nucleotide sequences. The boundaries of "orthologous exons" were defined according to Ensembl human exon annotations. All gaps in the transcript alignments were discarded, so our approach did not consider lineage-specific gains/losses of exons. We calculated the d_N, d_S, and d_N/d_S of each pair of orthologous exons using the *codeml* module of PAML 4 (Yang 2007). To ensure accurate estimates of evolutionary rates, only exons longer than 81 bp were included (Nekrutenko, Makova, and Li 2002; Chen, Pan, and Lin 2011). For the human-mouse comparison, our final dataset included 5,416 human-mouse orthologous gene pairs, comprised of 21,706 orthologous exon pairs (table 2). For the human-rhesus macaque comparison, our final dataset included 4,609 orthologous genes, which were comprised of 14,434 orthologous exon pairs (table 2). Compared to the number of human-mouse orthologous genes, there were fewer human-rhesus macaque orthologous genes in our analysis because the macaque draft genome had not been completely sequenced.

To control for differences in exon features of different genes, we calculated the differences in evolutionary rates (d_N, d_S, and d_N/d_S) and the differences in exon features for all possible two-exon combinations of the same transcript. Using PCR, we examined how much of the variance in exon-level d_N, d_S, or d_N/d_S was explained by exon features. Our dataset for this within-gene analysis included 81,260 human-mouse exon pairs (combinations) and 37,508 human-rhesus macaque exon pairs (table 2).

Measuring exon features

ASE and CSE classification (Shabalina et al. 2010) and WEF calculation were done using in-house PERL scripts. WEF is defined as the length-weighted average of the frequency of an exon (supplementary fig. S1). Here, the frequency of an exon measures its relative importance and is calculated as the percent of transcript isoforms of a gene that include this specific exon. For example, CSEs have an exon frequency of 100% because they always occur in different isoforms. CSEs are considered to be indispensable for the biological functions of their transcript/protein. We assume that an exon’s importance is reflected in how frequently it appears in different transcript isoforms. In the case of partially overlapping exons (supplementary fig. S1), the exon boundaries may be ambiguous, and the frequencies of these exons are hard to define. For such cases, WEF gives a reasonable quantitative measure of the frequency an exon is used in splicing events.

Intrinsically disordered regions were predicted by using Disopred (Ward et al. 2004). Pfam protein domain information was retrieved from the Ensembl Database (http://www.ensembl.org) and the percent of annotated protein domain(s) of each exon was calculated. Solvent-accessible amino acid residues were predicted by using the ACCpro module of the SCRATCH package (Cheng et al. 2005) with a 30% exposure threshold.

5' and 3' intron length, exon length, and G+C content of the exons were calculated using in-house PERL scripts on the genomic sequences downloaded from BioMart. The first and last coding exons of each transcript were excluded because they contain only 3' intron and 5' intron, respectively. Exon duplicability was evaluated by BLAST-aligning each exon to the entire transcriptome. A potential exon duplicate was defined as a BLAST hit that is ≥90% alignable and ≥90% identical to the query exon. The total number of BLAST hits matching these criteria was defined as the duplicability of an exon.

The expression features of the exons were derived from two published human RNA-seq datasets (GSE12946 and GSE13652) (Pan et al. 2008; Wang et al. 2008) archived in Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/). These datasets cover eleven human tissues (adipose, brain, breast, cerebral cortex, colon, heart, liver, lung, lymph node, skeletal muscle, and testes). The 32-mer RNA-seq sequences were mapped to the human genome (hg 19) using SeqMap (Jiang and Wong 2008). Similar to a previously described approach (Sultan et al. 2008; Qian et al.

2010), the exon-specific transcriptional abundance was defined as the total number of RNA-seq reads uniquely mapped onto the exon divided by the number of unique *n*-mers per exon, where *n*=32. The exon-specific transcriptional abundances were averaged over all of the analyzed tissues to represent the expression level of an exon. To measure the expression breadths of exons, the exon-specific transcriptional abundances were sorted for each tissue, and the top 50% of the exons were defined as expressed in a certain tissue. The expression breadth of an exon was then calculated as the proportion of tissues in which this exon was expressed (transcriptional abundance >0). The coefficient of variation was calculated by dividing the standard deviation of exon-specific transcriptional abundances by the mean of exon-specific transcriptional abundances across the eleven tissues for each exon. The PCR analyses were conducted in R (http://www.r-project.org/) using modified scripts from (Drummond, Raval, and Wilke 2006).

Results

Charicteristics of exons

To evaluate the determinants of exon-level evolutionary rates, we had to control for gene-level differences. For example, expression level may differ by a much larger extent between genes than between exons of the same genes. Therefore, the results from PCR analyses based on pooled exon comparisons may to some extent reflect the gene-level differences. To address this issue, we calculated the between-exon differences in human-mouse evolutionary rates (d_N/d_S, d_N, and d_S) and the thirteen exon features for exons of the same transcript. We performed PCR analyses separately for d_N/d_S, d_N, and d_S against the exon features.

The composition of principal components classified the thirteen exon features into six biologically meaningful categories (table 1): (1) mRNA splicing features: WEF, and ASE/CSE exon type (ASE=0; CSE=1); (2) exon-level RNA expression features: expression level, coefficient of variation in expression level, and expression breadth; (3) structural-functional features: percent of IDR, percent of Pfam protein domain(s), and proportion of amino acid residues predicted to be solvent-accessible; (4) gene compactness features: the lengths of 5’ and 3’ flanking introns; (5) exon duplicability; and (6) other features: exon length and G+C content (Supplementary Table S1).

Structural-functional features and splicing features are the two most important determinants of within-gene d_N/d_S variations

As shown in fig. 1, the primary principal component for exonic d_N/d_S is composed mainly of variation in structural-functional features (component 3 in the left panel in fig. 1A), and the secondary component (component 2) consists mainly of variation in splicing features. Although variation in expression features (component 1) is a well-known determinant of d_N/d_S at the protein-level (Pal, Papp, and Lercher 2006), it ranked sixth in explaining exon-level d_N/d_S variation. A similar trend was observed for d_N (fig. 1B; left panel). Meanwhile, other features dominated the third and the fourth most important components for exonic d_N/d_S. For d_S, the two most important components were composed mainly of splicing features and structural-functional features (fig. 1C; left panel).

We then calculated the total contribution of each feature category to the variance in d_N/d_S. Some feature categories dominated multiple components, so we summed the percentages of variance explained by components that were dominated by the same feature category. A component was dominated by a feature category if the feature category accounted for more than 50% of this component. If none of the feature categories exceeded the 50% threshold, the component was designated as a "mixed" component. For d_N/d_S, structural-functional features, splicing features, and expression features, accounted for approximately 3.4%, 2.8%, and 0.5% of the variance explained, respectively (fig. 1A; right panel). For d_N, the three features accounted for 3.5%, 2.6%, and 0.5% of the variance explained, respectively (fig. 1B; right panel). For d_S, the three features accounted for 1.7%, 1.5%, and 0.2% of the variance explained, respectively. Unexpectedly, other features accounted for a considerable percent of variance explained for d_N/d_S (~2.2%), d_N (~2.3%), and d_S (0.6%) (fig. 1A, 1B, and 1C; right panel). Although compactness features were previously suggested to be a dominant factor affecting gene-level d_N/d_S (Liao, Scott, and Zhang 2006), they accounted for a relatively small percent (<0.1%) of exonic d_N/d_S, d_N, and d_S. Similarly, although gene duplication has a strong effect on evolutionary rates, exon duplicability explained <0.1% of the variance of exon-level evolutionary rates (fig. 1, right panel). (For detailed human-mouse PCR results broken down by each exon feature see supplementary tables S2-S4.)

To evaluate these results across smaller genetic distances, we repeated the analyses for the human-rhesus macaque comparison. We obtained similar results for d_N/d_S and d_N, with splicing features, structural-functional features, and expression features account for ~1.5%, 0.7%, and 0.3% of d_N / d_S variance, respectively (fig. 2A). For d_N, these features accounted for 1.4%, 0.7%, and 0.2%, respectively (fig. 2B). Notably, the percent of variance in evolutionary rates explained by the exon features were generally larger in the human-mouse comparison than in the human-rhesus macaque comparison, possibility due to the relatively low sequence quality of the rhesus macaque genome and the small genetic distance between human and rhesus macaque. The principal components for variation in d_S were different between the two datasets. In the human-rhesus macaque comparison, the importance of splicing and structural-functional features was significantly reduced, whereas the importance of other features increased (fig. 2C). Therefore, the primary determinant of exon-level d_S in mammals remains inconclusive. The detailed human-rhesus macaque PCR results (broken down to thirteen exon features) are given in supplementary tables S5~S7.

The effects of gene-level characteristics on the evolutionary rates of exons

Unlike previous findings based on analyses of full-length mammalian proteins (Liao, Scott, and Zhang 2006; Drummond and Wilke 2008), expression features and compactness features accounted for only a small percent of variance in exon-level d_N/d_S and d_N. This is possibly due to that these two feature categories, especially expression features, differed to a greater extent between genes than between exons of the same genes. Therefore, the effects of these two features were less significant in the intragenic analyses. To examine this possibility, we randomly selected 81,260 human-mouse and 37,508 human-rhesus macaque exon pairs from different genes without replacement and conducted a between-gene PCR analysis. We then summed the contributions of each of the feature categories as described above. We repeated this analysis on randomly selected exon sets for 500 times and generated boxplots of percent variance explained for each feature category (figs. 3 and 4). In the human-mouse comparison (fig. 3), expression features are more important in affecting the variances in d_N/d_S and d_N than splicing features and structural-functional features (fig. 3A and 3B). For the variance in exon-level d_S, splicing features were the most important, followed by structural-functional features and expression features (fig. 3C).

For the human-rhesus macaque comparison, the results were similar for d_N/d_S

and d_N (fig. 4A and 4B). For d_S, splicing features remained relatively important. However, the contributions of structural-functional features and mixed features varied to a large extent. This is because in many cases, structural-functional features accounted for either slightly less or slightly more than 50% of a component. In the former case, the component was designated as dominated by structural-functional features, whereas in the latter case, it was considered a mixed component. These variations in component designation caused the large variations in fig. 4C.

Discussion

In this study, we analyzed the contributions of thirteen exon features (table 1) to exonic evolutionary rates using both within- and between-gene comparisons. The thirteen features of exons were classified into six major components based on the principal component analysis: splicing features, expression features, structural-functional features, gene compactness features, duplicability, and other features composed of G+C content and exon length. Although other features contributed to d_N, d_S, and d_N / d_S at an appreciable level (figs. 1-4), a biological interpretation of this component is currently lacking and requires further exploration. We cannot exclude the possibility that our datasets contain noises that cannot be easily eliminated using PCR analyses. Alternatively, some important exon features might not have been included, leaving a considerable proportion of variances in evolutionary rates unexplained.

The within-gene analyses (figs. 1 and 2) controlled for between-gene differences in exon features and indicated that structural-functional features and splicing features are the two most important determinants of exon-level d_N/d_S and d_N. By contrast, between-gene analyses (figs. 3 and 4) indicated that expression features have larger effects on exon-level d_N/d_S and d_N variance than structural-functional and splicing features. Taken together, our results suggest that the differences in gene-level biological features (especially expression features) may set the coarse background of protein evolution at the gene level, upon which exon features fine-tune the within-gene variations in protein evolutionary rates. Because between-gene variation in expression features has strong effects on d_N/d_S and d_N, controlling for the expression features revealed the significant effects of exon-level features, such as splicing features and structural-functional features.

Gene compactness was identified as more important than expression level in affecting d_N/d_S at the gene level (Liao, Scott, and Zhang 2006). However, at the exon level, gene compactness only has minor contributions to d_N/d_S (figs.1~4). By contrast, expression features were an important contributor to exonic d_N/d_S variations in the between-gene analysis (figs. 3 and 4). The increased importance of gene expression and decreased importance of gene compactness on exon-level d_N/d_S variance may reflect differences in the source data between the gene-level and exon-level analyses. Notably, the gene-level study incorporated microarray expression data, whereas the present exon-level study incorporated RNA-seq expression data (Pan et al. 2008). For the microarray data, probes were not located within all exons of a gene. As a result, microarrays do not precisely measure mRNA abundance, especially for alternatively spliced genes. Furthermore, the expression signals measured by hybridization methods are affected by probe-target affinity, which can vary for probes within the same transcript (Liao and Zhang 2006). Therefore, sequencing-based methods, such as RNA-seq, may have better accuracy and resolution than array-based methods in measuring exon-level expression properties. Another potential reason for the decreased effect of gene compactness on d_N/d_S might be the result of including splicing features (which have been overlooked in previous studies) in the present analyses.

It is unexpected that splicing features and structural-functional features are more important than expression features and compactness features in affecting within-gene exon-level d_N/d_S differences (figs. 1 and 2). Many unicellular organisms, such as yeast, contain few introns and rarely implement alternative splicing. By contrast, complex multicellular organisms implement alternative splicing as a mechanism for gene regulation. Thus, lineage-specific properties, such as splicing features, can be an important determinant in affecting within-gene variation in protein evolutionary rate.

In a previous PCR study that examined gene-level evolutionary rates in yeast, the percent of d_N/d_S variation accounted for by the most influential factors (expression level, codon bias, and protein abundance) were as large as ~40% (Drummond, Raval, and Wilke 2006). By contrast, the contributions to exon-level evolutionary rate variation of the most influential categories are smaller (~3%, fig. 1). There are several possible reasons for this difference in explainable variance. First, the significantly reduced effective population sizes in multicellular organisms have led to a decrease in the efficiency of selection, thereby weakening the correlation between d_N/d_S and the examined exon features. Second, in multicellular organisms, tissue differentiation results in genes that are expressed in multiple tissues and subject to complex regulation and selection pressures. In mammals, natural selection may have targeted not only individual biological factors, such as exon features, but also factors associated with spatial-temporal interactions (Gu and Su 2007). Thus, the relatively small percent of explainable d_N/d_S variance may reflect our limited knowledge of the targets of selection in complex organisms. Consistent with this notion, previous studies showed that the percent of variance in d_N/d_S explained by a single biological factor is smaller in mammals than in yeast (Liao, Scott, and Zhang 2006; Liao, Weng, and Zhang 2010). Third, although we filtered out short exons (Materials and Methods), the average length of the analyzed exons are shorter than the lengths of genes. Therefore, the estimates of exonic evolutionary rates and other exon features may be less accurate and subject to large variation. In other words, exon-level data may be noisier than gene-level data. In addition, the within-gene differences in biological features and evolutionary rates can be fairly small. The signal-to-noise ratio in the exon-level analysis is thus limited, which may have reduced the explaining power of the exon features. Regardless of the amount of variance explained, the human-mouse and human-rhesus macaque comparisons yielded consistent results for how exon features explain variation in d_N/d_S and d_N.

By analyzing the effects of thirteen exon features on exon-level evolutionary rate, we demonstrate the predominant roles of splicing features and structural-functional features in determining d_N/d_S and d_N of mammalian exons. Our results clearly demonstrate that gene-level and exon-level variations in d_N/d_S and d_N are affected by different biological properties of DNA. Our findings thus may shed new lights on the sources of evolutionary rate variations within mammalian genes.

Acknowledgement

This study was supported by the intramural funding of National Health Research Institutes, Taiwan, to both F.-C.Chen and B.-Y. Liao and research grant (NSC 99-2311-B-400-003-MY2) from the National Science Council, Taiwan, to B.-Y. Liao. We thank Dr. Pei-Shen Lin for statistical advice. We also thank two anonymous reviewers for constructive comments.

Figure legends

Figure 1

The principal components that affect human-mouse within-gene exonic evolutionary rate variations (left panel) and the percent of variance explained by each category of exon features (right panel) for (A) d_N/d_S ratio; (B) d_N; and (C) d_S. Note that only the six most important components are shown.

Figure 2

The principal components that affect human-macaque within-gene exonic evolutionary rate variations (left panel) and the percent of variance explained by each category of exon features (right panel) for (A) d_N/d_S ratio; (B) d_N; and (C) d_S. Note that only the six most important components are shown.

Figure 3.

The distributions of percent variance in human-mouse evolutionary rates explained by different categories of exon features as generated by 500 random sets of exon pairs from different genes: (A) d_N/d_S ratio; (B) d_N; and (C) d_S. Upper quartile, median, and lower quartile values are indicated in each box. Bars outside the box indicate 1.5-fold interquartile range from the upper and lower quartile.

Figure 4.

The distributions of percent variance in human-macaque evolutionary rates explained by different categories of exon features as generated by 500 random sets of exon pairs from different genes: (A) d_N/d_S ratio; (B) d_N; and (C) d_S. Upper quartile, median, and lower quartile values are indicated in each box. Bars outside the box indicate 1.5-fold interquartile range from the upper and lower quartile.

Supplementary figure S1 The calculation of weighted exon frequency (WEF). The WEF of an exon is the length-weighted average of the frequency of occurrence of this exon in all of the alternatively spliced transcripts of the gene of interest.

Table 1. Exon features included in PCR.

Exon features	(1) Splicing features	Weighted exon frequency
		ASE/CSE exon type
	(2) Expression features	Average expression level
		Coefficient of variation of expression level
		Expression breadth
	(3) Structural-functional features	Proportion of intrinsically disordered region
		Proportion of Pfam domain
		Proportion of solvent accessible region
	(4) Compactness features	Length of 5’ flanking intron
		Length of 3’ flanking intron
	(5) Exon duplicability	Exon duplicability
	(6) Other features	Exon length
		G+C content

Table 2. The human-mouse and human-rhesus macaque orthologous genes and exons for within-gene and between-gene analyses.

	Human-Mouse	Human-Rhesus Macaque
Number of genes	5,416	4,609
Number of exons	21,706	14,434
Number of exon pairs[a]	81,260	37,508

[a] The total number of within-gene exon combinations.

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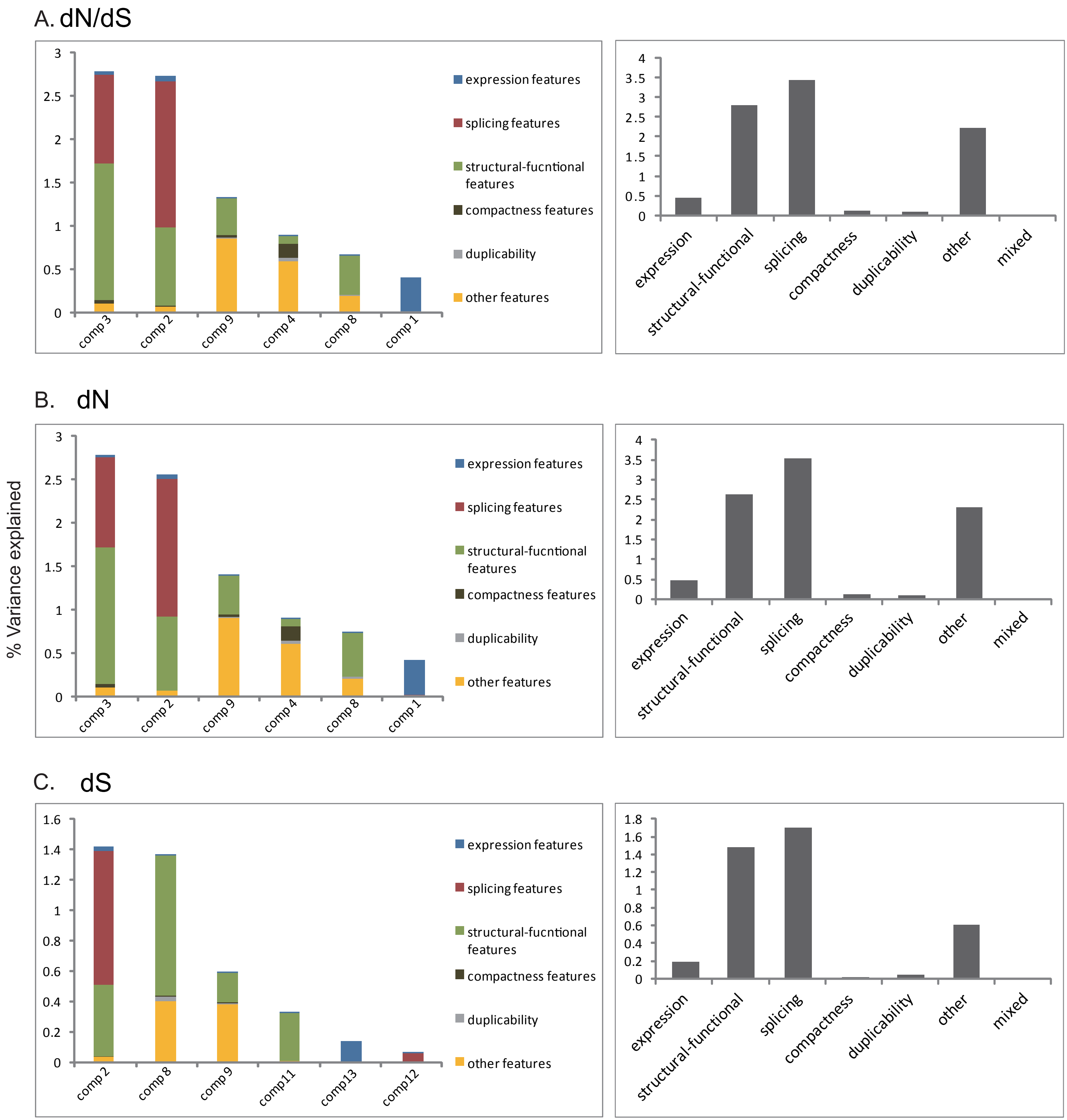
A. dN/dS
B. dN
C. dS
% Variance explained
3
2.5
2
1.5
1
0.5
0
comp 3
comp 2
comp 9
comp 4
comp 8
comp 1
expression features
splicing features
structural-fucntional features
compactness features
duplicability
other features
4
3.5
3
2.5
2
1.5
1
0.5
0
expression
structural-functional
splicing
compactness
duplicability
other
mixed
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
comp 2
comp 8
comp 9
comp11
comp13
comp12
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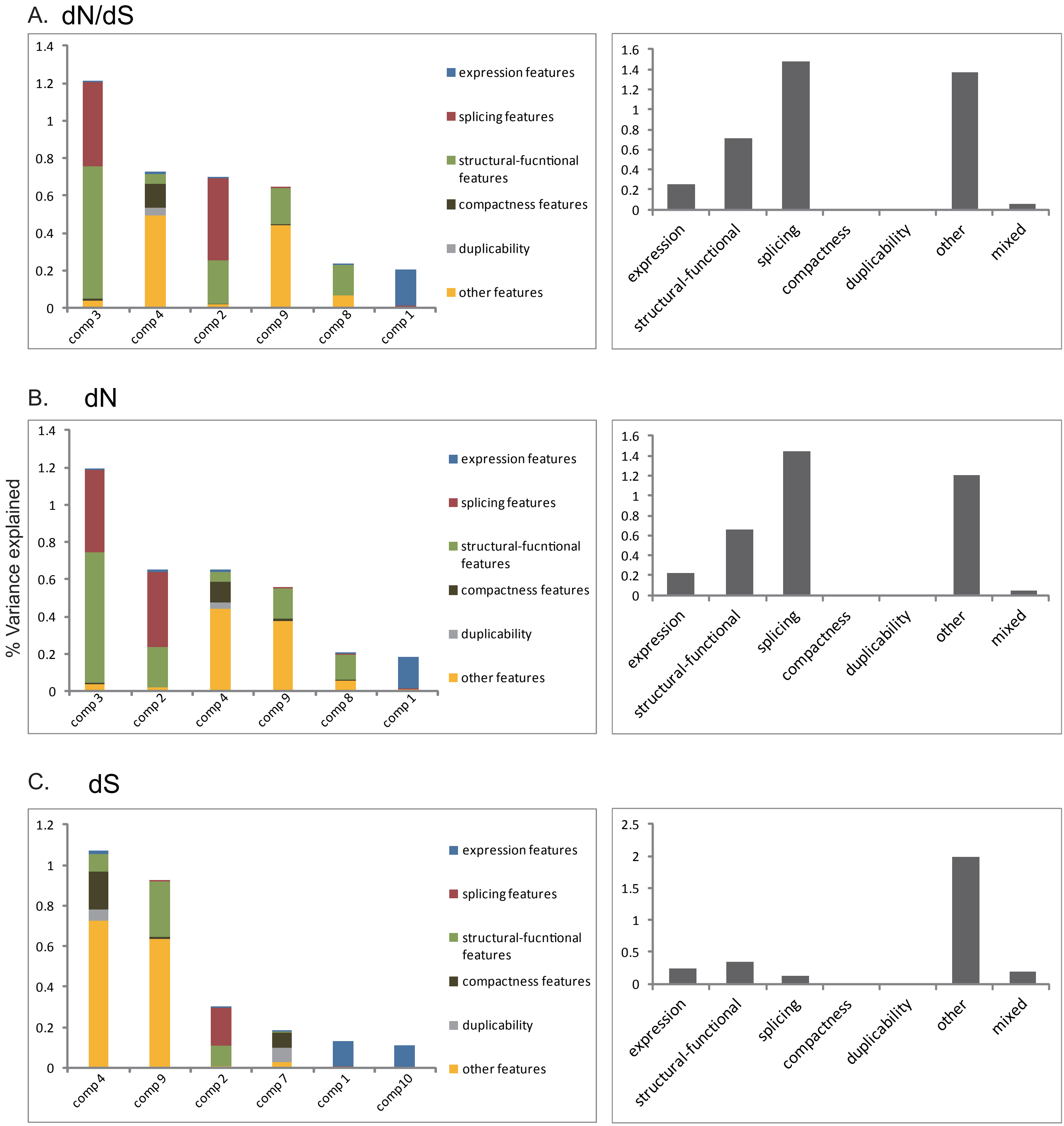
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0

Figure 1



A. dN/dS

B. dN

C. dS

Figure 2

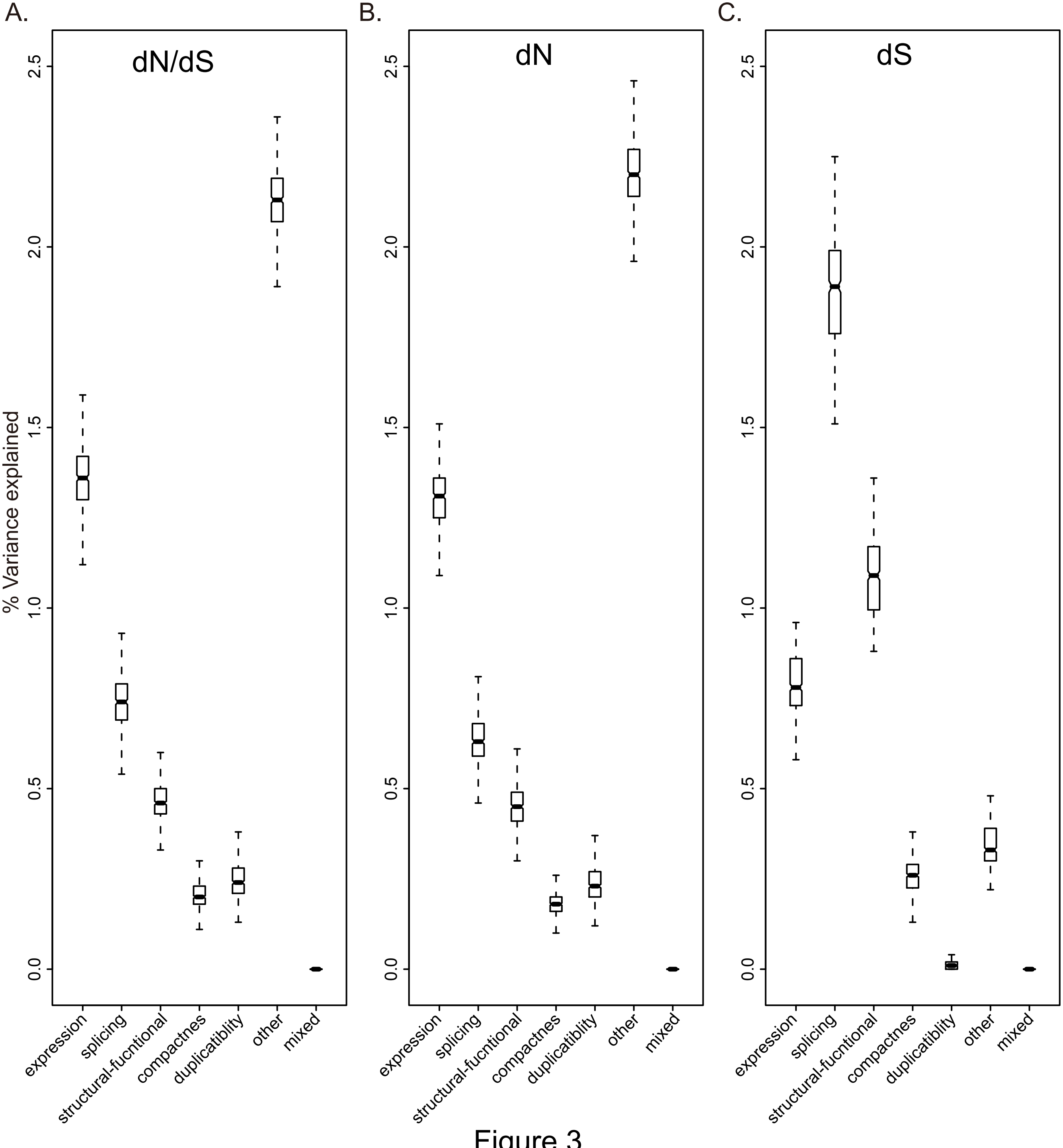

A.
B.
C.
dN/dS
dN
dS
% Variance explained
0.0
0.5
1.0
1.5
2.0
2.5
expression
splicing
structural-fucntional
compactnes
duplicatiblity
other
mixed

Figure 3

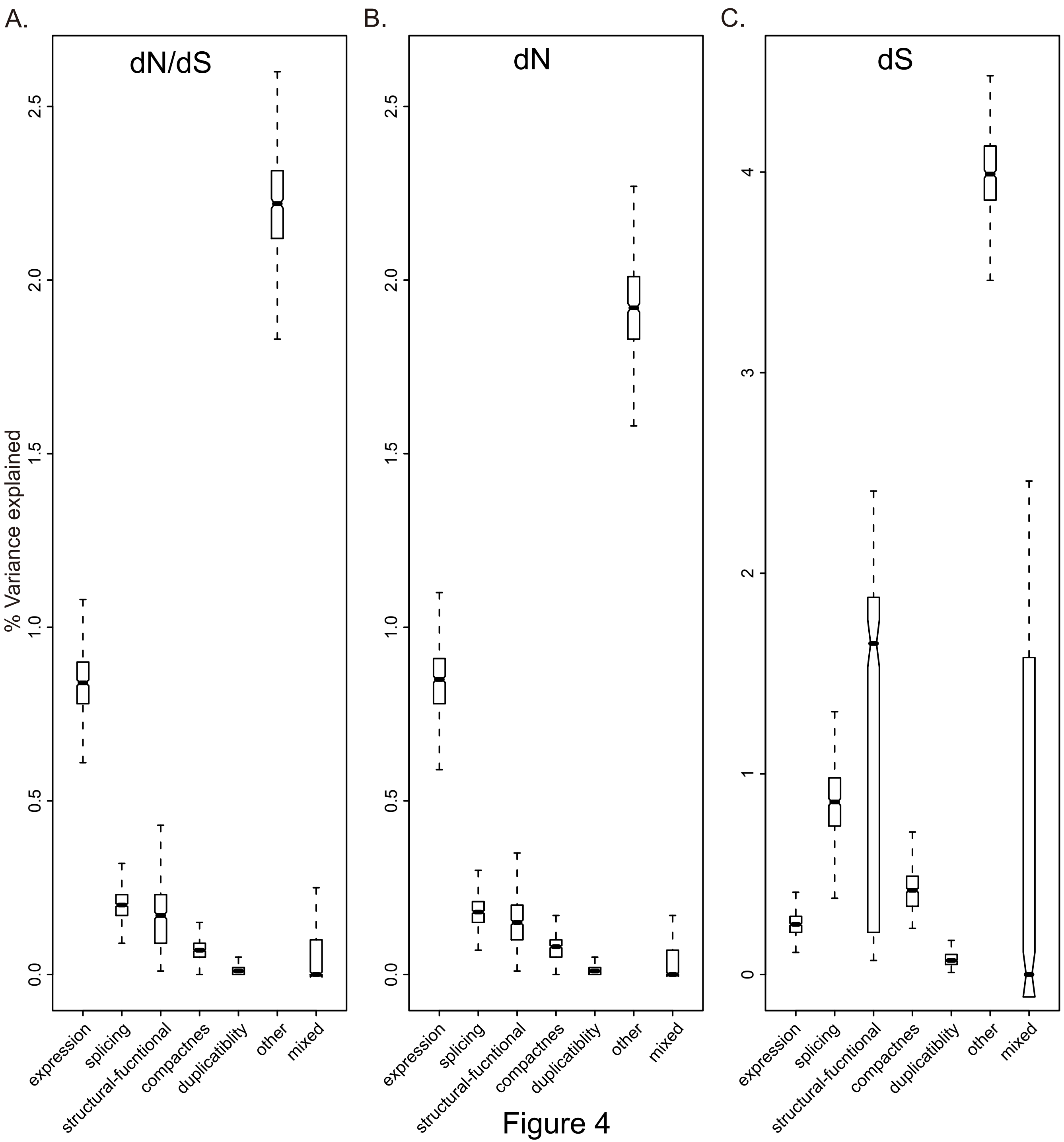

A.
dN/dS
% Variance explained
0.0
0.5
1.0
1.5
2.0
2.5
B.
dN
0.0
0.5
1.0
1.5
2.0
2.5
C.
dS
0
1
2
3
4
expression
splicing
structural-fucntional
compactnes
duplicatiblity
other
mixed

Figure 4