The Use of GC-, Codon-, and Amino Acid-frequencies to Understand the Evolutionary Forces at a Genomic Scale.

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1 Abstract

It is well known that the GC content varies enormously between organisms; this is believed to be caused by a combination of mutational preferences and selective pressure. Within coding regions, the variation of GC is more substantial in position three and smaller in position one and two. Less well known is that this variation also has an enormous impact on the frequency of amino acids as their codons vary in GC content. For instance, the fraction of alanines in different proteomes varies from 1.1% to 16.5%. In general, the frequency of different amino acids correlates strongly with the number of codons, the GC content of these codons and the genomic GC contents. However, there are clear and systematic deviations from the expected frequencies. Some amino acids are more frequent than expected by chance, while others are less frequent. A plausible model to explain this is that there exist two different selective forces acting on the genes; First, there exists a force acting to maintain the overall GC level and secondly there exists a selective force acting on the amino acid level. Here, we use the divergence in amino acid frequency from what is expected by the GC content to analyze the selective pressure acting on codon frequencies in the three kingdoms of life. We find four major selective forces; First, the frequency of serine is lower than expected in all genomes, but most in prokaryotes. Secondly, there exist a selective pressure acting to balance positively and negatively charged amino acids, which results in a reduction of arginine and negatively charged amino acids. This results in a reduction of arginine and all the negatively charged amino acids. Thirdly, the frequency of the hydrophobic residues encoded by a T in the second codon position does not change with GC. Their frequency is lower in eukaryotes than in prokaryotes. Finally, some amino acids with unique properties, such as proline glycine and proline, are limited in their frequency variation.

2 Introduction

The GC-frequency varies significantly between different and within genomes, both in coding and non-coding regions [1]. The reason behind this is not entirely understood. However, it is likely due to a combination of a balance between mutational preferences, selective pressure and evolutionary history [1]. In general mutational preferences decrease GC levels in most organisms [2,3], while GC-biased gene conversion (gBGC) can contribute to higher GC levels [4]. The environment of the organism can also influence the GC level as GC levels are higher in thermophiles [5]. Further, there is a phylogenetic signal so that closely related organisms mostly have similar GC-levels [6]. Finally, differences in DNA polymerase subunit III might correlate with differences in GC [7].

Here, we are not trying to answer the long-disputed origin of the difference in GC content. Instead, we assume that there is some mechanism driving the GC content of a particular organism towards an optimal level. Thereafter, we ask how does this affect the proteomes by examining the frequency of amino acids, nucleotides

1st base	2nd base								3rd base
	T		C		A		G		
T	TTT	Phe (F)	TCT	Ser (S)	TAT	Tyr (Y)	TGT	Cys (C)	T
	TTC		TCC		TAC		TGC		C
	TTA	Leu (L)	TCA		TAA	STOP	TGA	STOP	A
	TTG		TCG		TAG		TGG	Trp (W)	G
C	CTT		CCT	Pro (P)	CAT	His (H)	CGT	Arg (R)	T
	CTC		CCC		CAC		CGC		C
	CTA		CCA		CAA	Gln (Q)	CGA		A
	CTG		CCG		CAG		CGG		G
A	ATT	Ile (I)	ACT	Thr (T)	AAT	Asn (N)	AGT	Ser (S)	T
	ATC		ACC		AAC		AGC		C
	ATA		ACA		AAA	Lys (K)	AGA	Arg (R)	A
	ATG	Met (M)	ACG		AAG		AGG		G
G	GTT	Val (V)	GCT	Ala (A)	GAT	Asp (D)	GGT	Gly (G)	T
	GTC		GCC		GAC		GGC		C
	GTA		GCA		GAA	Glu (E)	GGA		A
	GTG		GCG		GAG		GGG		G

(a) Group

Figure 1. Codon tables with the amino acids encoded according to different properties. (a) The colour is based on the amino acid type (hydrophobic - yellow, Basic - blue, Acidic - red, Polar - green, amphipathic - purple and loop-preferring brown) (b) coloured according to pI-values to be neutral (c) coloured by secondary structure preference and (d) coloured according to disorder preference. The figure is inspired by a figure at Wikipedia at http://www.wikipedia.org/

and codons. A different number of codons encodes the different amino acids. These codons differ in GC content. Therefore, in general, amino acids encoded by more codons are more frequent, and amino acids encoded by GC-rich codons are more frequent in GC-rich genomes [8–10] leading to massive variation in the frequency of amino acids in different organisms [11, 12]. For instance, the positively charged amino acids Arg and Lys vary between 2% and 10% in frequency. Arg is more frequent in GC-rich organism, and Lys is more frequent in GC-poor organisms.

The codon table, see Figure 1, is surprisingly well conserved since early life. The same 61 codons encode the twenty amino acids in most organisms. However, some variations exist. For instance, in eukaryotes, one of the stop codons can encode selenium methionine [13], and other variations exist among Mycoplasma, Spiroplasma, Ureaplasma and Mesoplasma [14]. The redundancy in the codon table means that for many amino acids, the third position does not change the amino acid. Therefore, the overall GC content can change by using different nucleotides in the third position without affecting the proteome. Further, the codons have evolved in such a way that the general properties of the amino acids are determined mainly by the codon in position two [15].

In addition to codon frequency and GC level, there exist many factors that contribute to the frequency of amino acids [16–18]. The cost of amino acid synthesis might affect their frequency [19, 20], and some amino acids, such as serine, can be toxic at high levels [21]. In addition to purifying effects to reduce the frequency of one amino acid, an organism might require a minimum frequency of amino acids with specific properties, while other amino acids, such as alanine, might be allowed to vary more freely [22].

Below, we analyze the frequency of amino acids, codons and nucleotides in different genomes. We show that the codon frequency does not fully explain amino acid frequencies, i.e. other factors also affect the amino acid frequencies. Some amino acids, such as serine, are consistently less frequent than expected, while others, such as glutamate, are more frequent. Further, some amino acids, such as proline, are less dependent on GC than expected, indicating that there are limits to how much they can vary. The picture that emerges is that there on a genomic perspective there exists two selective forces, one that adjusts the GC content to a certain level and one that given a certain GC level adjusts amino acids frequencies. By detailed analysis, we can obtain an understanding of the forces acting on the amino acids.

3 Material and Methods

3.1 Datasets

The dataset used in this study originates from the complete bacterial, archaeal and eukaryotic proteomes in UniProt [23] as of December 2017. All genomes from Mycoplasma, Spiroplasma, Ureaplasma, and Mesoplasma were ignored as they have another codon usage - which influences the expected amino acid frequencies. The final dataset contains 36,098,162 protein sequences from 8,546 genomes, divided into 7,197 bacterial, 351 archaeal, and 998 eukaryotic species. For each genome, the GC content of the genome and the length was obtained from NCBI. Further, the DNA and amino acid sequences of each gene were downloaded. The processed datasets, as well as all scripts, are available from this repository [24].

3.2 Statistics

For each protein, we calculated amino acid-, GC-, codon- and nucleotide-frequencies. Average, maximum and minimum frequencies for each genome in the dataset are presented in Table S1.

ANOVA type 2 F-tests [25] were used to identify the contribution differences between the kingdoms, compensating for differences in GC content, see Table **??**. Using each codon/amino acid/nucleotide as the dependent variable and the GC content is used as the independent variable, the difference between kingdoms was tested. Here, it should be mentioned that even tiny differences are statistically significant, as the dataset is large. Further, differences between eukaryotes and bacteria dominate the ANOVA test as these are the most prominent groups.

3.3 Expected frequencies

It is necessary to define the expected frequency of amino acid (AA^i) to identify any selective pressure. Therefore, we define models to estimate the expected amino acid frequencies (AA^i_X) assuming different scenarios. The simplest model, the *codon* model, assumes that the frequency of an amino acid is solely determined by the number of codons encoding that amino acid:

$$AA^i_{codon} = \frac{Codons^i}{61} \tag{1}$$

where $Codons^i$ is the number of codons for amino acid i and 61 is the number of codons excluding stop codons.

Alternatively, the amino acid frequencies may be dependent on GC (i.e. there exist some other mechanism that determines the GC content of a genome) leading to the expected amino acid frequency AA^i_{GC} at a certain GC level to be:

$$AA^i_{GC}(GC) = (\sum_{Codons^i} \sum_{x=1}^{3} \delta(N(x) \in (A,T)) * (0.5 - \frac{GC}{2})/(1 - AA^{STOP}_{GC}(GC)) \tag{2}$$

where $Codons^i$ represents the codons for amino acid i and x the three nucleotides in that codon, GC is the fraction GC in the genome and $\delta(N(x) \in (A,T))$ is a delta function that is one if the nucleotide $N(x)$ is A or T, and zero if not. Further, $AA_{GC}^{STOP}(GC)$ is the expected frequency of stop-codons given GC as defined here:

$$AA_{GC}^{STOP}(GC) = \sum_{stopcodons} \sum_{x=1}^{3} \delta(N(x) \in (A,T)) * (0.5 - \frac{GC}{2}) \tag{3}$$

where the $\sum_{stopcodons}$ sums over the three stop codons.

However, as we show below there are other parameters that also affect the amino acid frequencies. To take several scenarios into account, we use the following formulae to estimate the amino acid frequency (AA^i) for the amino acid i at a given GC level:

$$AA_{twopar}^i(GC) = W^i * (AA_{GC}^i(GC) - AA_{GC}^i(50\%)) + AA_{GC}^i(50\%) + K^i \tag{4}$$

Here, $AA_{GC}^i(GC)$ is the expected frequency of amino acid i at the GC as defined in equation 2. $AA_{GC}^i(50\%)$ is the expected frequency at GC=50%, and W^i, and K^i are two parameters that are optimized for each amino acid. The reason to use this function, and not simply $AA^i = w^i * GC + k^i$ is to have a consistent definition of the parameters W^i, and K^i. In particular, the parameter K^i is useful to estimate over-, and under-representation of an amino acid.

Using equation 4, we can model different scenarios. If $W^i = 0$ and $K^i = 0$, then equation 4 describes the expected frequency from the number of codons as in equation 1 (the *codon* model). If $W^i = 1$ and $K^i = 0$ the equation describes the expected amino acid frequency at a certain GC level as in equation 2. If $W^i = 0$ while K^i is optimized, this describes the average amino acid frequency in all genomes and then K^i represents a shift from the expected frequency. Finally, we can optimize both W^i and K^i and obtain the amino acid levels using two parameters (the *twopar* model). Here, to be more realistic, we limit W^i to be between 0 and 1. Also here K^i represents the shift from the expected frequency.

To compare the different models to estimate the amino acids, we use the Pearson correlation coefficient [26] and the average error between the estimated and observed frequencies of all twenty amino acids, see Figure S1.

3.4 Linear regressions

To estimate the GC frequency from amino acid frequency, we used sklearn [27]. Given the amino acid frequency of one or more amino acids in a protein or a proteome, the model was trained to predict the GC level of the coding region of a proteome. In addition we trained the same model to predict the GC level from a single protein. Here 25,000 randomly selected proteins were used.

4 Results and Discussion

The GC frequency can vary tremendously between organisms, see Figure 2. In our set of proteomes, the beta proteobacteria *Candidatus Zinderia insecticola* has the lowest GC content with 13.5% and *Geodermatophilus nigrescens* has the highest (75.9%), see Table S1. The mechanism causing these differences is not entirely known, but factors such as mutation rate, crossover rate, thermodynamical stability and phylogenetic memory contribute [4]. Anyhow, in this study, we will not focus on the GC difference. Instead, we will analyze how the difference in GC levels affect the proteomes and use divergence from expected frequencies to analyze the selective pressures at the proteome level.

4.1 GC distributions

First, some notes about the overall GC content. Both prokaryotic kingdoms have a bimodal GC distribution with one peak around 40% and the second at 70% [28], see Figure 2a. In contrast, Eukaryotes have a single,

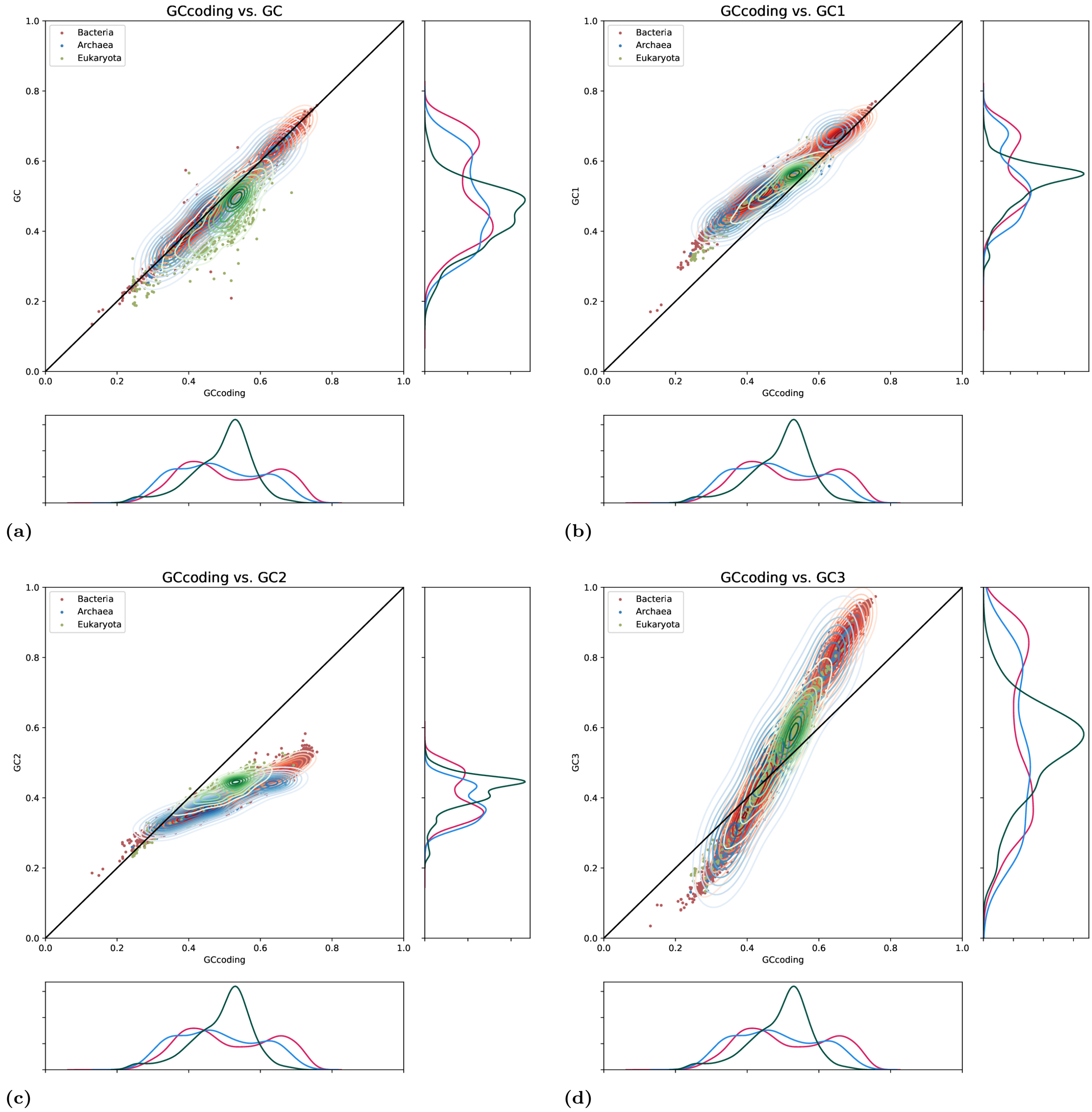


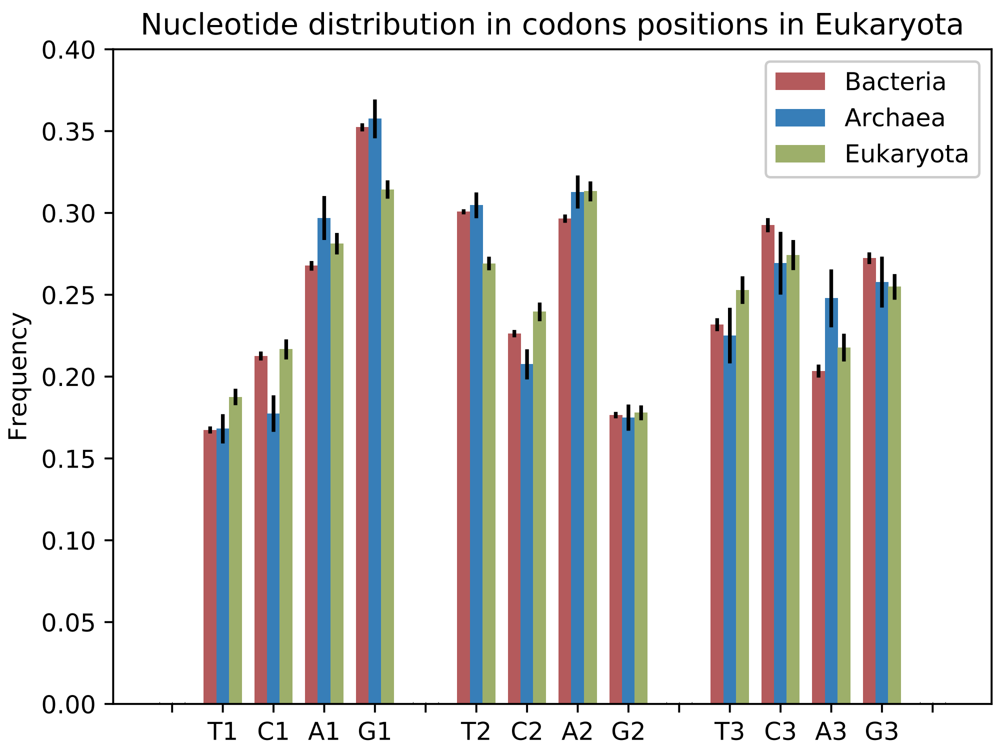
Figure 2. Distribution of GC contents in the three different kingdoms. In (a) the GC content in the whole genome is plotted against the GC content in the coding regions. In (b-d). the GC content in the three codon positions are plotted against the GC in the coding regions.

less wide, peak of GC content. Therefore, the standard deviation in the prokaryotes is larger (12% vs 8%), while the average GC levels are similar (48%-51% for the coding regions), see Table S1.

4.1.1 GC coding vs non-coding

For both prokaryotes, the genomic GC level (from NCBI) and the GC level of the coding regions (from Uniprot) are almost identical and perfectly correlated (CC=0.998), while for Eukaryotes the levels differ slightly but are still strongly correlated (CC=0.89), see Table S1. Eukaryotes have a higher GC content in coding regions (49% vs 44%), see Figure 2a. The GC level in the coding regions is similar to the average level observed in prokaryotes. Therefore, we believe that comparing features with the GC content of the coding region is more appropriate. Further is also simplifies the analysis of codon and nucleotide frequencies.

4.2 The selective pressure at the GC level.



(a)

Figure 3. Average composition of nucleotides in different codon positions.

In the codon table, seven (Phe, Leu, Val, Pro, Thr, Ala, and Gly) out of the twenty amino acids are determined by position one and two, see Figure 1. Further, the two first bases and a combination of TC or AG in position three determines eight other amino acids (Tyr, His, Gln, Asn, Lys, Asp, Glu, and Cys). Two amino acids (Met and Trp) have only one codon, and Ile uses the three ATX codons not encoding Met. The remaining two amino acids, serine and arginine, are encoded by two groups of codons with different nucleotides in position one and two. Finally, there are three stop codons that all have a T in its first position (T1).

Given the position in the codon table for amino acids with similar properties, it is clear that in particular, position two determines the properties of the amino acid [15]. For instance, all codons with T2 encode hydrophobic amino acids, while both negatively charged amino acids have A2.

4.2.1 GC in different positions.

The GC content differs between the three codon positions, see Figure 2 and Table S1. In all positions, the GC content is strongly correlated with the overall GC content ($Cc > 0.93$). The average GC content is lower in position two than in the other two positions. Further, in position one and two, the variation in GC content is much more restricted than in position three. The highest GC level in position three is 97% and the lowest 3%, compared to 18% and 58% in position two. The difference between the positions means that the GC variation in position three is significantly higher than in the other parts of the genome.

A model to explain the variation of GC in the three positions can be formulated as follows: In an organism, there exists a selective pressure to have a certain optimal GC content (in the coding regions). For some organisms, this optimal level is very high or very low, i.e. extreme. However, the selective pressure acting on amino acids frequencies makes it impossible to have extreme GC levels in position one and two. Therefore, to obtain extreme overall GC levels in these organisms, it is necessary to over-compensate in position three. In theory, if the GC content is limited to 50% in position one and two but varying in position three, this allows the genomic GC to vary between 33 and 67%. However, the GC content in one and two also varies, and amino acid frequencies also change; therefore, the overall GC content can vary between 13% and 76%.

Although the average GC content is similar in all three positions, it is clear that the nucleotide frequencies are not, see Figure 3, 4 and Table S1. The differences are largest for position one and two. In position one, G1 and A1 are more frequent than the other two nucleotides, while in position two A2 and T2 are most frequent. For a more detailed understanding of these differences, we will analyze the frequencies of each nucleotide in each position, starting with position one.

4.2.2 Position 1

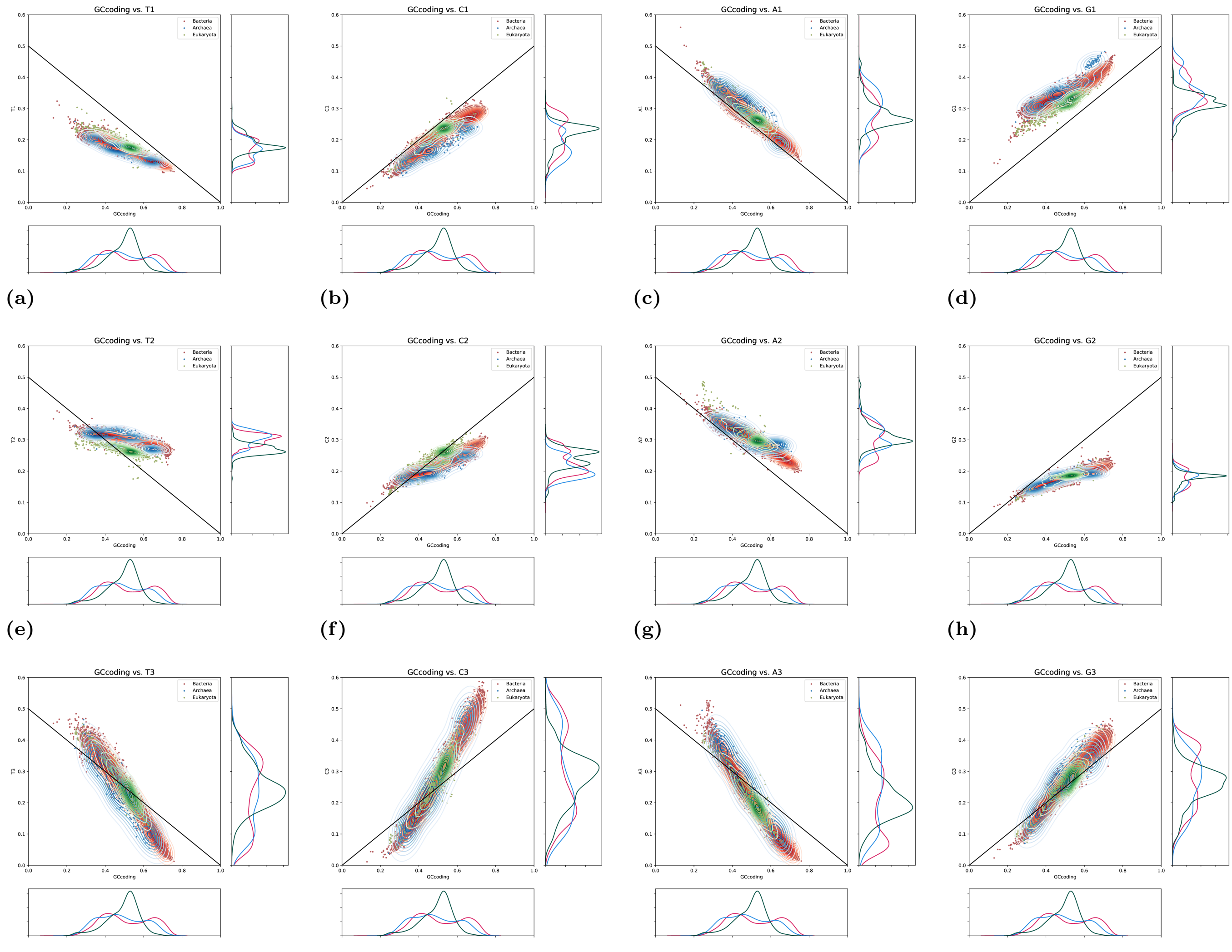
In position one, it can be seen that G1 is most frequent, and T1 is least frequent (average frequency is 16.7%). However, it should be remembered that all three stop codons have a T1, so the expected T1 frequency is not 25% but only 20.3%. In addition, serine, which has four out of six codons with T1, is one of the most underrepresented amino acids, as we have described before [12]. G1 encodes for VADEG, these amino acids are all over-represented compared to random, see Figure 5.

4.2.3 Position 2

Position two is the most conserved position when it comes to GC content. It is also clear that A2 and T2 are more frequent than G2 and C2, on average about 30% vs 20%, see Table S1 and Figure 3. Further, T2 is almost independent of the GC content in all genomes, but consistently lower in eukaryotes than prokaryotes, see Figure 4. The constant level of T2 guarantees a stable amount of the non-polar, and β-sheet forming amino acids (FLIVM). G2 is rare and have a limited range. G2 encodes several amino acids with unique properties, such as glycine (the smallest amino acid) and cysteine (that can form disulphide bonds), but also arginine, tryptophan and one of the stop codons. The rareness can be contributed to the 40% (6 out of 15) of the non-stop G2 codons that encode arginine, and the frequency of arginine is underrepresented, see Figure 5. Finally, A2, that encodes primarily charged and polar amino acids (YHQNEDK), and C2, that encodes APST are allowed to vary more freely than the other two nucleotides in position 2.

4.2.4 Position 3

In general, it is believed that position three in a codon is not under selective pressure as it only rarely affects the amino acid, Figure 1. However, if no selective pressure acted on position three random drift would make all



(a) (b) (c) (d)

(e) (f) (g) (h)

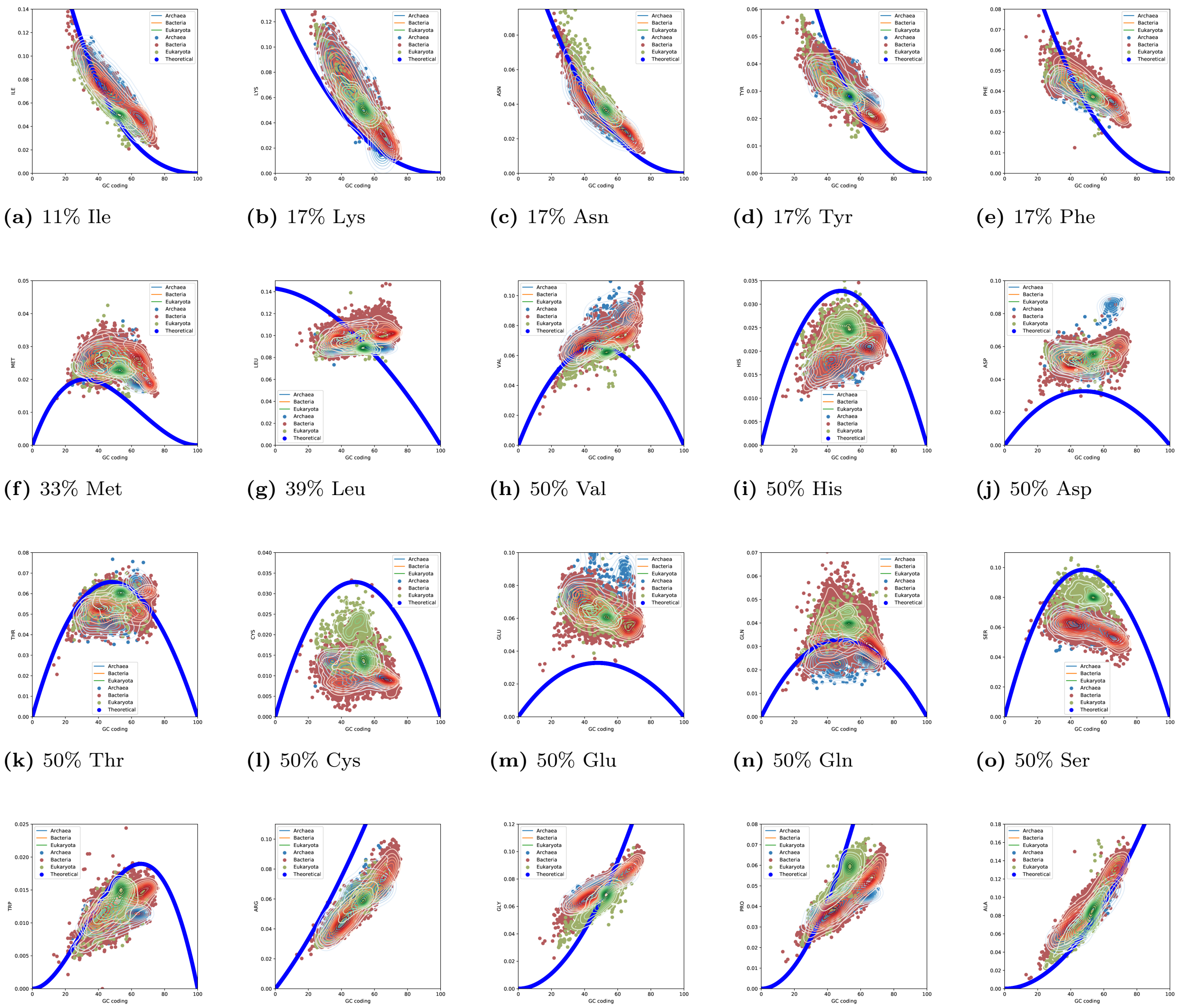
(i) (j) (k) (l)

Figure 4. Position specific nucleotide frequencies plotted against the GC frequency.

nucleotides equally frequent in that position, and clearly, they are not, see Figure 4. In contrast, the nucleotides in position three vary much more than in the other positions. The frequency of most nucleotides varies between 1% and 60%, supporting the idea that the genomic GC preference governs nucleotide frequencies. C3 is most frequent in position three but least frequent in the other two positions, see Figure 3.

4.3 Amino acid frequency vs GC

To be able to identify the selective pressures acting on amino acid frequencies, it is necessary to estimate the expected amino acid frequencies without any selective pressure. Therefore, it is necessary to have a model to describe the expected amino acid frequency for a genome. Following the speculations above we do assume that: There exist some evolutionary process that strives the GC content of a genome to be adapted, but that is independent of the selective pressure acting at the amino acid level. It is then possible to model the expected



(a) 11% Ile (b) 17% Lys (c) 17% Asn (d) 17% Tyr (e) 17% Phe

(f) 33% Met (g) 39% Leu (h) 50% Val (i) 50% His (j) 50% Asp

(k) 50% Thr (l) 50% Cys (m) 50% Glu (n) 50% Gln (o) 50% Ser

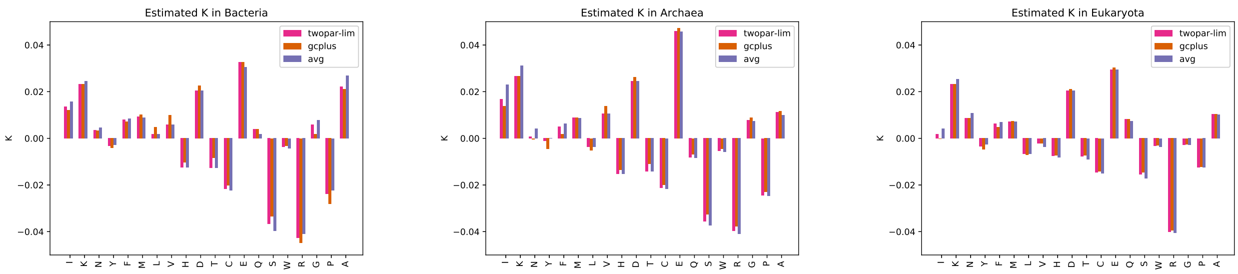
(p) 67% Trp (q) 72% Arg (r) 83% Gly (s) 83% Pro (t) 83% Ala

Figure 5. Frequency of different vs GC of the genomes amino acids are sorted by the GC content of the codons. The amino acids are sorted by their TOP-IDP scores. The number represent the fraction of GC among the codons. The blue line represent the expected fraction according to the codon frequency. The purple lines represent the expected fraction from the codon position GC content.

amino acid frequencies assuming that protein-coding regions would be random in the absence of any selective pressure at the amino acid level.

A simple explanation of the variation of amino acid frequency would be that it is just decided by the number of codons coding for an amino acid as in equation 1. Nevertheless, a better agreement is observed when taking the GC into account and calculate the expected amino acid frequencies, given the GC of the genome, as in equation 2. Below, we use this equation to estimate the expected frequencies of the amino acids.

Figure 5 shows the amino acid frequencies of each amino acid against the GC content of the coding regions



(a) Bacteria **(b)** Archaea **(c)** Eukaryota

Figure 6. Combined frequency plots

with the blue lines representing the expected amino acid frequencies according to equation 2. The sorting of the amino acids is based on the GC content in their codons.

4.3.1 Frequency of low GC amino acids depends strongly on GC

The frequency of all the amino acids with less than one-third of GC in their codons, i.e. Ile, Lys, Asn, Phe and Tyr, show a strong correlation with GC, see the top row in Figure 5. The frequency of these amino acids vary from 1-2% at high GC up to 19% at low GC and the correlation with GC is 0.83 to 0.93, see Table S1. The lowest correlations against GC are for Tyr and Phe, which have a flatter distribution than expected from the GC frequency alone.

4.3.2 The frequencies of amino acids low GC dependency are independent of GC

Next, there are 11 amino acids with a GC content in their codons between one- and two-thirds. None of these shows a strong dependency of GC, but the correlations with GC are rather high for Valine (CC=0.72) and Trp (CC=0.74). More notably, some of these amino acids are more frequent than expected from the codons and some less.

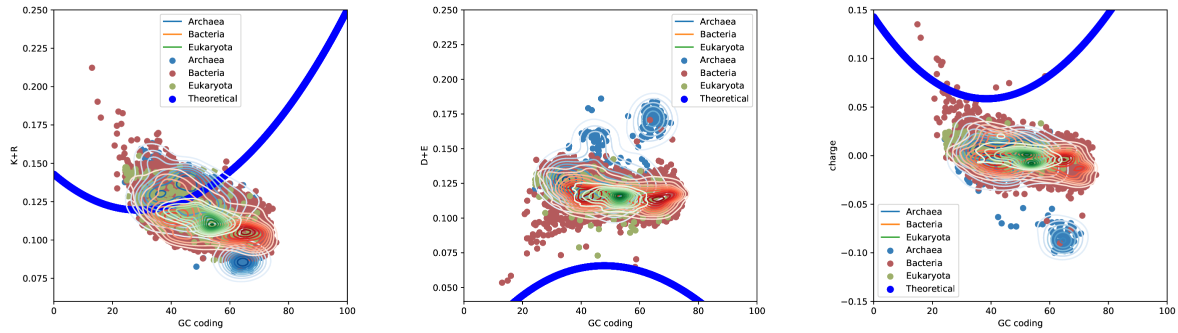
4.3.3 Frequency of all high GC codons strongly depends on GC

Finally, the amino acids with more than two-third of GC in their codons are also strongly dependent on the GC content (CC> 0.85). Shifts can be seen as Arg is less frequent than expected. The frequencies of Gly and Pro also appears to be limited to be within a specific range.

4.4 Systematic shifts

From the studies above, it is clear that there exist systematic divergences of amino acid frequencies for some amino acids. In general, the divergences are (a combination) of two types, shifts and decreased GC dependency. A shift refers to that the amino acid frequency is consistently over- or under-represented (as for serine), while the decreased GC dependency refers to a decreased dependency of GC, i.e. a flatter distribution (as for proline), see Figure 5. It is tempting to speculate that a shift would indicate that there exist a selective pressure for that amino acid to be more or less frequent, while a decreased GC dependency indicates that there exists a selective pressure to keep that amino acid at a constant level.

To identify systematic shifts, we have used equation 4 (with different limitations to the parameters). Here, K describes a shift up or down from what is expected by random and W describes the strength of the



(a) K+R vs GC **(b)** D+E vs GC **(c)** Charge vs GC

Figure 7. Frequencies of groups of amino acids vs GC.

dependency with GC (one is perfectly correlated, and zero indicates no dependency). The parameter W is, therefore, only relevant for the amino acids with GC-rich or GC-poor codons, see Figure S3.

Figure 6 shows that the shifts (K) are consistent independent of what model is used. Arginine, serine, cysteine and proline are under-represented while glutamate, aspartate, lysine and alanine are over-represented, see Figure 6. These shifts are also clearly observable in Figure 5. The variation between the kingdoms is small, but the shifts are in general smaller in Eukaryotes. The average error for the GC model, equation 2, is 1.4% in Eukaryotes vs 1.9% in Bacteria and 2.1% in Archaea.

4.5 The intricate balance of charged residues.

The positively charged amino acids Lys and Arg are like Siamese twins, one has GC-rich codons, and one GC-poor, both are positively charged, and they can often (but not always) perform similar roles in a protein. One notable difference is that six codons encode arginine compared with two for lysine, i.e. arginine should be three times as frequent at 50% GC. However, arginine is consistently less frequent than expected from GC while lysine is more frequent, compensating for the difference in codons, see Figure 5 and 6. The total number of Arg+Lys is rather constant but decreases slightly with GC, see Figure 7.

The negative amino acids (Asp and Glu) are, in contrast, not very GC dependent and are constant in GC, see Figure 5. Notably, as a group, the negatively charged amino acids are much more frequent compared to what is expected by random, see Figure7 and 6. The shifts are therefore most likely a consequence of that there are eight codons for positively charged amino acids compared to only four for the negatively charged amino acids and that the overall charge of the proteome is close to neutral independent on GC content, see Figure 7.

4.6 Limited frequency ranges.

In addition to amino acids consistently over- or under-represented, there exist amino acids that are limited in their variation. In Figure 5 and S3, it can be seen that five amino acids are less dependent on GC than expected. Isoleucine, tyrosine and phenylalanine are all less frequent than expected at low GC and more frequent at high GC. Similarly, Pro and Gly are both more frequent than expected at low GC and less frequent at high GC. Given the unique properties of Tyr/Phe (aromatic) and Gly/Pro (secondary structure breakers), it is not surprising that there exist boundaries to their frequency variations.

Feature	F-test	P-value	Bacteria	Archaea	Eukaryota
SER	7926	0.000E+00	0.059	0.061	0.081
T2	6876	0.000E+00	0.301	0.305	0.269
PRO	3405	0.000E+00	0.043	0.041	0.053
ILE	2652	0.000E+00	0.065	0.072	0.053
CCA (Pro)	2623	0.000E+00	0.007	0.010	0.015
GLY	2595	0.000E+00	0.073	0.073	0.063
CYS	2376	0.000E+00	0.010	0.011	0.018
G1	2360	0.000E+00	0.352	0.357	0.314
TGT (Cys)	1944	0.000E+00	0.004	0.005	0.008
TCT (Ser)	1674	0.000E+00	0.009	0.010	0.015
CCT (Pro)	1670	0.000E+00	0.008	0.009	0.014

Table 1. ANOVA tests for comparison between kingdoms. The most significant features when comparing all three kingdoms are listed here, for all other comparisons, see supplementary Table **??**. The average frequencies feature in the three kingdoms are shown. Note that the average feature reported here does not compensate for differences in GC contents as done in the ANOVA test.

4.7 Differences between kingdoms

Although most amino acids and codon frequencies are similar in the three different kingdoms, there exist some differences to be noted. We have earlier reported that serine and proline are more frequent in eukaryotes, and that isoleucine is less frequent [12]. Here, we confirm that these differences are among the most significant differences between the kingdoms using an ANOVA test, see Table 1. However, other differences can also be detected.

In Table 1, it can be seen that two features dominate the difference between the kingdoms, increased serine frequency in eukaryotes and decreased T2 frequency in eukaryotes. As mentioned above, T2 codes for the hydrophobic amino acids phenylalanine, leucine, isoleucine, methionine and valine.

If we ignore differences in codons, following next in importance is the increase in eukaryotic proline frequency and decrease in isoleucine frequency [12]. These are then followed a decrease in glycine and an increase in cysteine in the eukaryotes. Finally, G1 is less frequent in eukaryotes than in prokaryotes. G1 encodes valine, alanine, aspartate, glutamate and glycine, and all these are slightly less frequent in eukaryotes than in the prokaryotes.

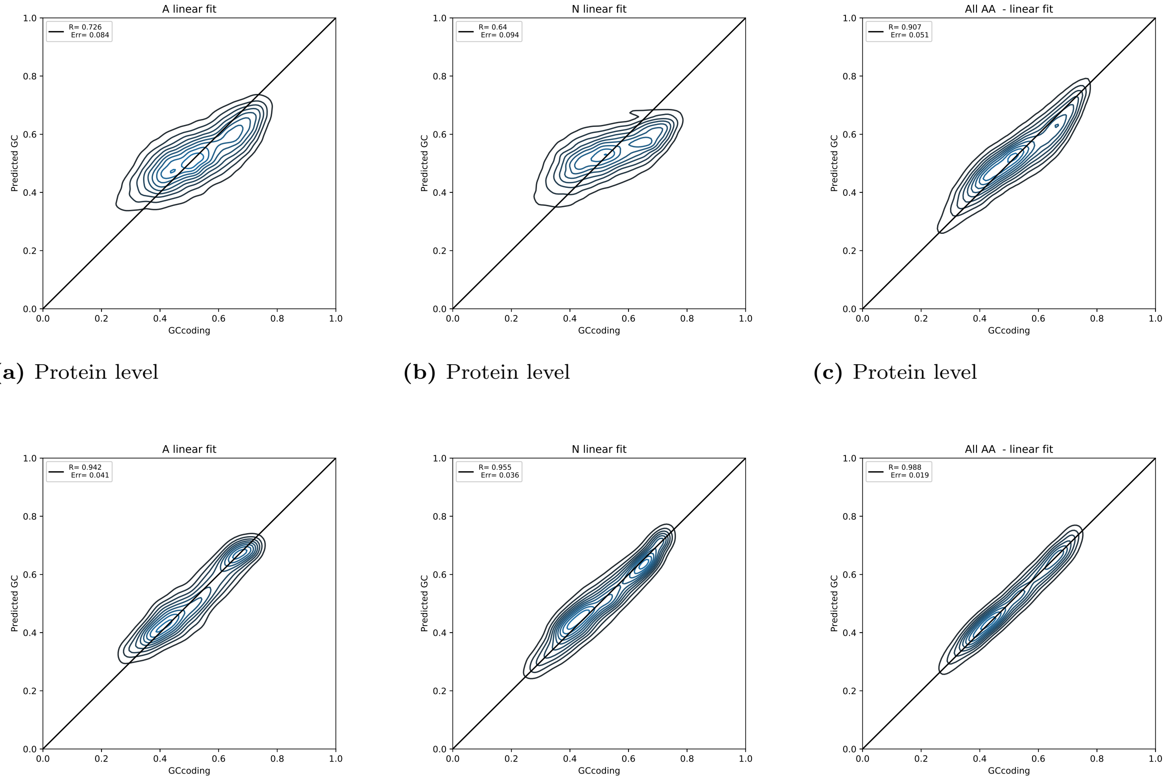
All the codons that are highest ranked in the ANOVA test are coding for one of the amino acids discussed above. It is interesting to note that CCA codon explains the most of the proline increase.

4.7.1 Archaea

For many features, such as glutamate and aspartate frequencies, it can be seen that the archaea kingdom is divided into two groups. Brief analysis indicates that this roughly correlates with the phylum Euryarchaeota and other archaea. Euroarchaeota have more proteins (2170 vs 1620), higher GC (50% vs 45%) and more Asp (6.3% vs 4.9%) and Glu (8.2% vs 7.2%) but less Lys (7.3% vs 5.8%). Although interesting, a detailed analysis of these differences is beyond the goals of this study.

4.8 Predicting GC from amino acid frequencies

Is it possible to predict the GC frequency from amino acid frequencies? We show that even the frequency of one amino acids, such as asparagine or alanine, in the proteome, can predict the GC level with an error of less than 5% and a correlation coefficient of 0.95, see Figure 8. If the frequency of all twenty amino acids is included, the error drops below 2%, and the correlation coefficient is 0.99.



(a) Protein level **(b)** Protein level **(c)** Protein level

(d) Proteome level **(e)** Proteome level **(f)** Proteome level

Figure 8. Predicting GC content from amino avid frequencies.

Even the frequency of amino acids for a single protein is informative of the GC level of the entire proteome. The sequence of a single protein can predict the GC level with an average error of 5% and a correlation coefficient above 0.90. This can, for instance, be used to detect laterally transferred genes directly from amino acid sequences if the genomic sequence was not available.

5 Conclusions

Here, we study the relationship between GC content of organisms and frequencies in their coding regions. We highlight that amino acid frequencies differ significantly in high and low-GC genomes and that their frequencies are primarily dependent on the number of codons and the GC content of their codons. But there are also significant differences.

To explain this, we propose that there exist an (unknown) mechanism acting to maintain the GC level in an organism. This can be seen by the fact that the third position varies much more than the others and by the differences in nucleotide frequencies in the different codon positions. Next, we propose that there is also a selective pressure changing amino acid frequencies from what is expected by chance. This mechanism decreases the frequencies of arginine and serine in all organisms, while lysine, aspartate and glutamate are more frequent than expected by chance. Further, this mechanism limits the influence of GC on the frequency of tyrosine, phenylalanine, glycine, proline and isoleucine.

We also note that the selective pressure acts to; (i) Keep a balance of negatively and positively charges amino acids in all genomes (except some Euroarchaeota). This is maintained by an intriguing by the underrepresentation of arginine and overrepresentation of negatively charged amino acids. (ii) Maintaining the hydrophobic residues at a constant level by keeping a constant fraction of Thymine in the second codon position.

Finally, we also show that two most significant factors differ between eukaryotes and prokaryotes are: (a) Eukaryotes have more serine residues and (b) less of codons with a T in position two (T2), which results in fewer hydrophobic residues (FLIVM).

Financial Disclosure

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Acknowledgements

We do thank Lukas Käll, Lars Arvestad, Jens Lagergren and Patrick Bryant for valuable inputs and discussions. We do also thank for valuable discussions with the members of the COST Action BM1405 NGP-net.

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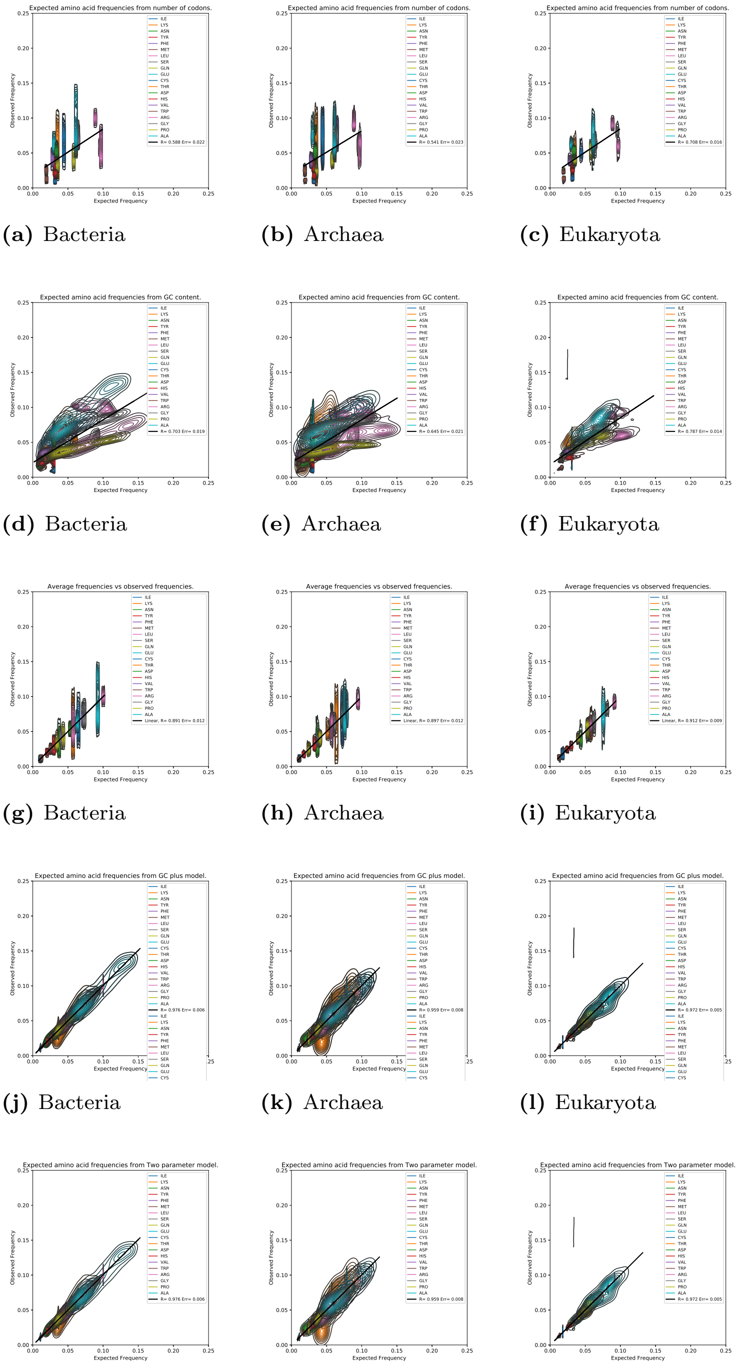
6 Supplementary Material



(a) Bacteria **(b)** Archaea **(c)** Eukaryota

(d) Bacteria **(e)** Archaea **(f)** Eukaryota

(g) Bacteria **(h)** Archaea **(i)** Eukaryota

(j) Bacteria **(k)** Archaea **(l)** Eukaryota

(m) Bacteria **(n)** Archaea **(o)** Eukaryota

Figure S1. Correlation between expected and observed amino acid frequency for all amino acids over all genomes.

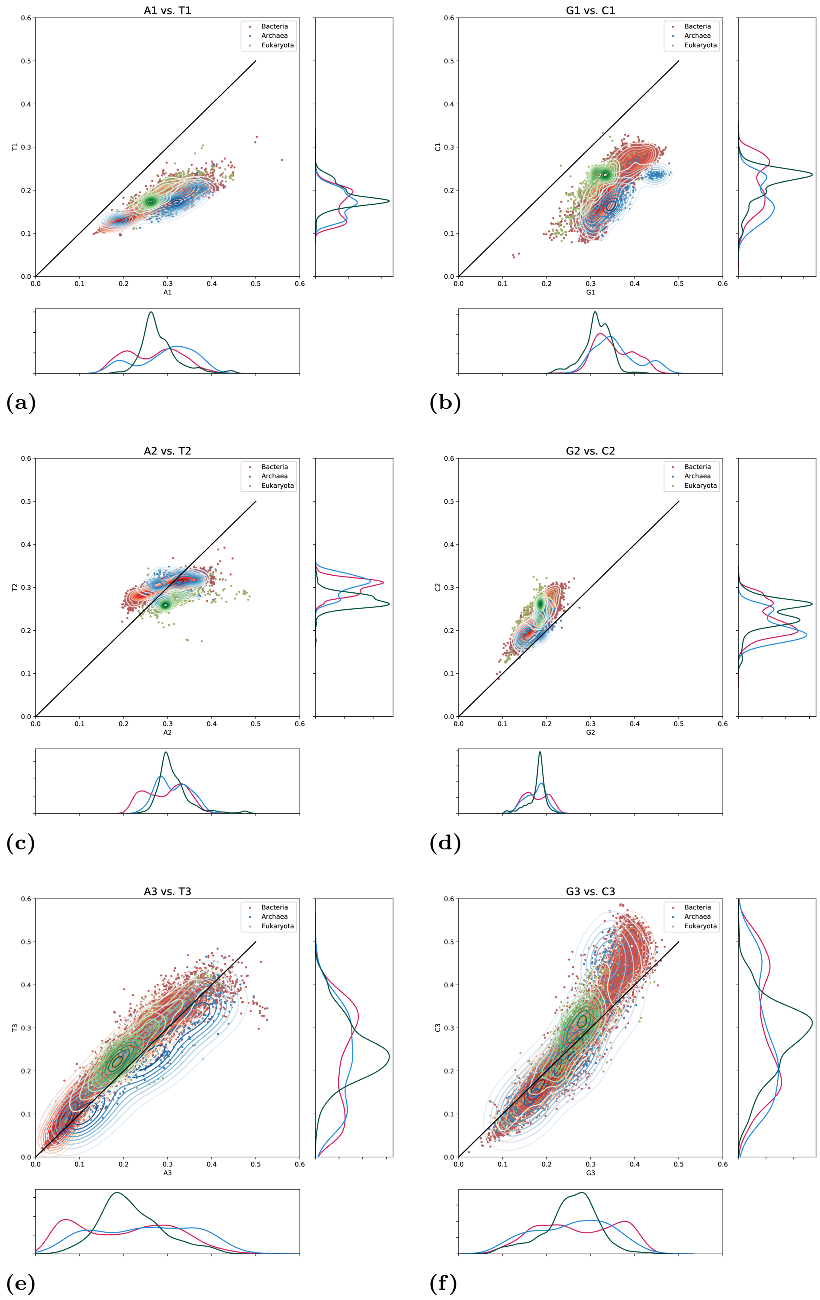
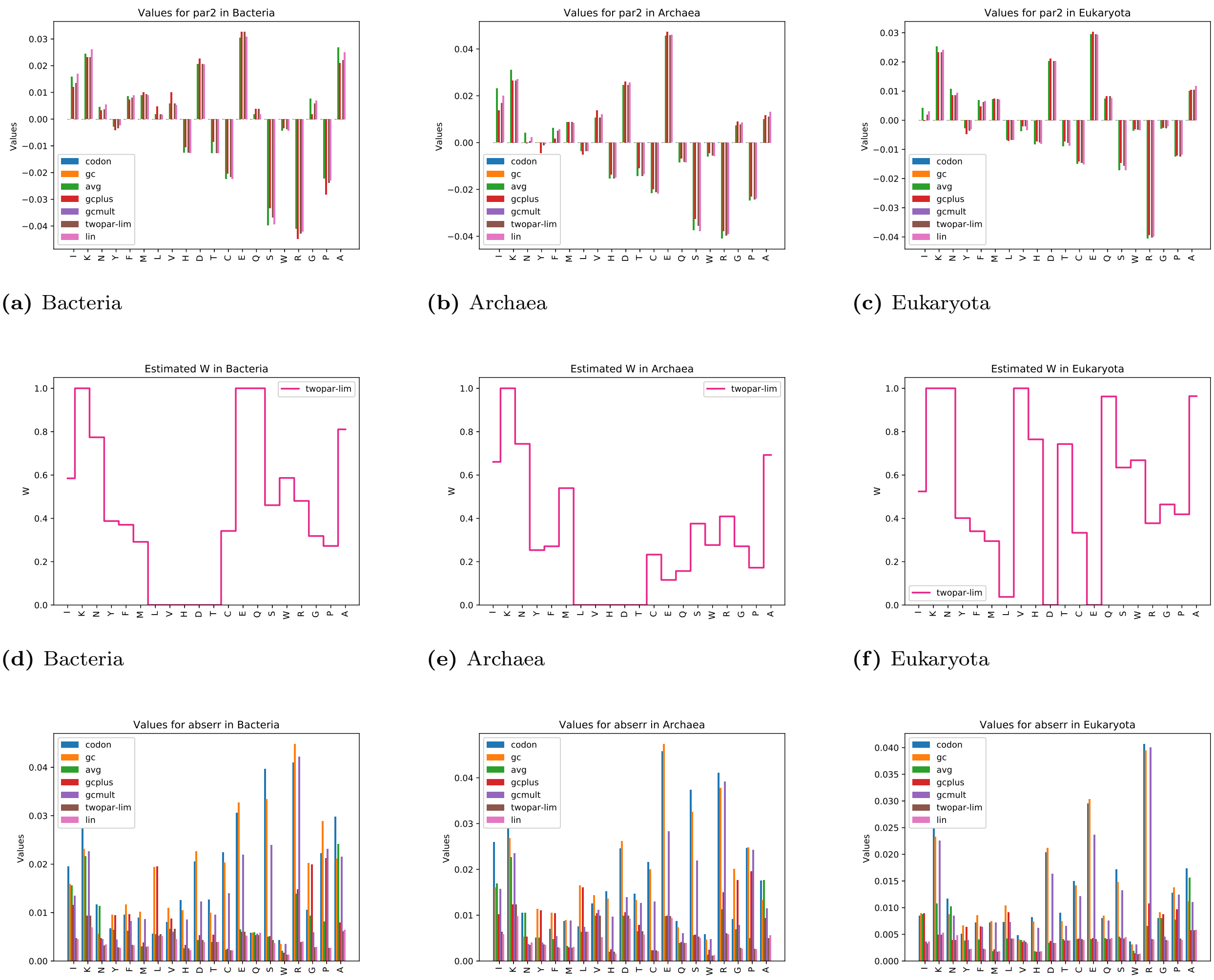


Figure S2. In position three perfect correlation, i.e. GC determines everything



(a) Bacteria (b) Archaea (c) Eukaryota

(d) Bacteria (e) Archaea (f) Eukaryota

(g) Bacteria (h) Archaea (i) Eukaryota

Figure S3. Combined plots from fitting parameters and absolute errors.

Table S1. Summary table of all features in the three kingdoms. Average, standard deviation, max and min values are printed as well as the correlation coefficient with GCcoding.

	Feature	Kingdom	Average	Stdev	Max	Min	Cc-to-GC
0	GC (genomic)	All	0.4974	0.1242	0.7590	0.1350	0.9834
1	GC (genomic)	Bacteria	0.5085	0.1256	0.7590	0.1350	0.9978
2	GC (genomic)	Archaea	0.4758	0.1131	0.7010	0.2430	0.9984
3	GC (genomic)	Eukaryota	0.4384	0.0844	0.6750	0.1880	0.8907
4	GC1	All	0.5584	0.0932	0.7699	0.1701	0.9840
5	GC1	Bacteria	0.5649	0.0947	0.7699	0.1701	0.9871
6	GC1	Archaea	0.5349	0.0890	0.7236	0.3359	0.9720
7	GC1	Eukaryota	0.5309	0.0648	0.7063	0.3164	0.9785
8	GC2	All	0.4028	0.0611	0.5830	0.1788	0.9644
9	GC2	Bacteria	0.4027	0.0621	0.5830	0.1788	0.9766
10	GC2	Archaea	0.3824	0.0479	0.5052	0.2595	0.9603
11	GC2	Eukaryota	0.4174	0.0489	0.5298	0.2323	0.9299
12	GC3	All	0.5562	0.2172	0.9737	0.0347	0.9933
13	GC3	Bacteria	0.5649	0.2236	0.9737	0.0347	0.9942
14	GC3	Archaea	0.5270	0.2085	0.9259	0.1312	0.9912
15	GC3	Eukaryota	0.5291	0.1397	0.9065	0.1334	0.9789
16	GC (coding)	All	0.5058	0.1221	0.7581	0.1301	1.0000
17	GC (coding)	Bacteria	0.5108	0.1255	0.7581	0.1301	1.0000
18	GC (coding)	Archaea	0.4814	0.1130	0.7061	0.2422	1.0000
19	GC (coding)	Eukaryota	0.4925	0.0819	0.6957	0.2435	1.0000
20	T	All	0.2343	0.0520	0.3913	0.1264	-0.9853
21	T	Bacteria	0.2333	0.0536	0.3913	0.1264	-0.9881
22	T	Archaea	0.2326	0.0475	0.3307	0.1384	-0.9838
23	T	Eukaryota	0.2365	0.0357	0.3370	0.1418	-0.9651
24	C	All	0.2418	0.0712	0.4007	0.0513	0.9926
25	C	Bacteria	0.2438	0.0733	0.4007	0.0513	0.9949
26	C	Archaea	0.2181	0.0658	0.3454	0.0877	0.9869
27	C	Eukaryota	0.2435	0.0482	0.3736	0.1007	0.9836
28	A	All	0.2599	0.0715	0.5062	0.1155	-0.9923
29	A	Bacteria	0.2559	0.0730	0.5062	0.1155	-0.9936
30	A	Archaea	0.2858	0.0669	0.4325	0.1551	-0.9918
31	A	Eukaryota	0.2707	0.0483	0.4417	0.1553	-0.9808
32	G	All	0.2640	0.0522	0.3805	0.0788	0.9861
33	G	Bacteria	0.2670	0.0531	0.3805	0.0788	0.9903
34	G	Archaea	0.2634	0.0492	0.3629	0.1545	0.9765
35	G	Eukaryota	0.2490	0.0356	0.3642	0.1415	0.9697
36	T1	All	0.1702	0.0336	0.3237	0.0953	-0.9476
37	T1	Bacteria	0.1674	0.0339	0.3237	0.0953	-0.9618
38	T1	Archaea	0.1681	0.0281	0.2588	0.1128	-0.9402
39	T1	Eukaryota	0.1876	0.0250	0.3038	0.1103	-0.9214
40	T2	All	0.2974	0.0205	0.3923	0.1749	-0.7388
41	T2	Bacteria	0.3007	0.0175	0.3923	0.2206	-0.9043

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Table S1. Summary table of all features in the three kingdoms. Average, standard deviation, max and min values are printed as well as the correlation coefficient with GCcoding.

	Feature	Kingdom	Average	Stdev	Max	Min	Cc-to-GC
42	T2	Archaea	0.3047	0.0218	0.3476	0.2468	-0.7019
43	T2	Eukaryota	0.2691	0.0170	0.3439	0.1749	-0.7678
44	T3	All	0.2353	0.1092	0.4842	0.0126	-0.9766
45	T3	Bacteria	0.2317	0.1127	0.4842	0.0126	-0.9798
46	T3	Archaea	0.2251	0.1012	0.4654	0.0324	-0.9742
47	T3	Eukaryota	0.2529	0.0707	0.4530	0.0493	-0.9520
48	C1	All	0.2109	0.0530	0.3332	0.0442	0.9495
49	C1	Bacteria	0.2126	0.0540	0.3288	0.0442	0.9573
50	C1	Archaea	0.1774	0.0437	0.2778	0.0791	0.9395
51	C1	Eukaryota	0.2166	0.0373	0.3332	0.0826	0.9253
52	C2	All	0.2266	0.0363	0.3241	0.0875	0.9211
53	C2	Bacteria	0.2263	0.0363	0.3207	0.0875	0.9459
54	C2	Archaea	0.2075	0.0298	0.2820	0.1449	0.8877
55	C2	Eukaryota	0.2396	0.0321	0.3241	0.1264	0.8650
56	C3	All	0.2879	0.1304	0.6298	0.0112	0.9845
57	C3	Bacteria	0.2925	0.1346	0.6298	0.0112	0.9863
58	C3	Archaea	0.2693	0.1291	0.5298	0.0390	0.9870
59	C3	Eukaryota	0.2743	0.0836	0.5339	0.0532	0.9546
60	A1	All	0.2713	0.0618	0.5597	0.1317	-0.9677
61	A1	Bacteria	0.2677	0.0627	0.5597	0.1317	-0.9705
62	A1	Archaea	0.2969	0.0633	0.4053	0.1527	-0.9491
63	A1	Eukaryota	0.2812	0.0427	0.4487	0.1777	-0.9449
64	A2	All	0.2998	0.0459	0.4859	0.1964	-0.9550
65	A2	Bacteria	0.2965	0.0464	0.4472	0.1964	-0.9666
66	A2	Archaea	0.3128	0.0358	0.4026	0.2371	-0.8606
67	A2	Eukaryota	0.3132	0.0369	0.4859	0.2337	-0.8766
68	A3	All	0.2084	0.1105	0.5263	0.0137	-0.9873
69	A3	Bacteria	0.2034	0.1132	0.5263	0.0137	-0.9886
70	A3	Archaea	0.2478	0.1098	0.4951	0.0376	-0.9851
71	A3	Eukaryota	0.2177	0.0712	0.4307	0.0442	-0.9738
72	G1	All	0.3475	0.0452	0.4824	0.1241	0.9160
73	G1	Bacteria	0.3523	0.0442	0.4784	0.1241	0.9445
74	G1	Archaea	0.3575	0.0493	0.4824	0.2568	0.9198
75	G1	Eukaryota	0.3143	0.0312	0.4218	0.2140	0.9256
76	G2	All	0.1762	0.0270	0.2746	0.0870	0.9464
77	G2	Bacteria	0.1765	0.0276	0.2746	0.0870	0.9568
78	G2	Archaea	0.1749	0.0225	0.2341	0.1146	0.8717
79	G2	Eukaryota	0.1779	0.0204	0.2397	0.1059	0.8670
80	G3	All	0.2683	0.0897	0.4679	0.0235	0.9734
81	G3	Bacteria	0.2723	0.0917	0.4679	0.0235	0.9769
82	G3	Archaea	0.2577	0.0849	0.4497	0.0700	0.9334
83	G3	Eukaryota	0.2548	0.0609	0.4446	0.0735	0.9354

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Table S1. Summary table of all features in the three kingdoms. Average, standard deviation, max and min values are printed as well as the correlation coefficient with GCcoding.

	Feature	Kingdom	Average	Stdev	Max	Min	Cc-to-GC
84	ATA	All	0.0129	0.0130	0.1024	0.0000	-0.7756
85	ATA	Bacteria	0.0124	0.0130	0.1024	0.0000	-0.7825
86	ATA	Archaea	0.0255	0.0163	0.0792	0.0003	-0.7921
87	ATA	Eukaryota	0.0118	0.0087	0.0637	0.0004	-0.8819
88	ATC	All	0.0258	0.0102	0.0619	0.0011	0.8142
89	ATC	Bacteria	0.0268	0.0104	0.0619	0.0011	0.8218
90	ATC	Archaea	0.0232	0.0106	0.0542	0.0037	0.7551
91	ATC	Eukaryota	0.0212	0.0058	0.0352	0.0042	0.7561
92	ATT	All	0.0247	0.0163	0.0803	0.0001	-0.9341
93	ATT	Bacteria	0.0251	0.0167	0.0802	0.0001	-0.9479
94	ATT	Archaea	0.0229	0.0152	0.0639	0.0007	-0.9375
95	ATT	Eukaryota	0.0202	0.0089	0.0594	0.0023	-0.9383
96	ATG	All	0.0241	0.0038	0.0418	0.0118	-0.3016
97	ATG	Bacteria	0.0242	0.0039	0.0370	0.0118	-0.3334
98	ATG	Archaea	0.0236	0.0041	0.0367	0.0123	-0.4212
99	ATG	Eukaryota	0.0235	0.0025	0.0418	0.0147	-0.1893
100	ACA	All	0.0114	0.0072	0.0381	0.0002	-0.8749
101	ACA	Bacteria	0.0107	0.0073	0.0381	0.0002	-0.8870
102	ACA	Archaea	0.0134	0.0071	0.0365	0.0013	-0.8521
103	ACA	Eukaryota	0.0150	0.0045	0.0309	0.0019	-0.8072
104	ACC	All	0.0188	0.0093	0.0494	0.0002	0.8779
105	ACC	Bacteria	0.0196	0.0096	0.0494	0.0002	0.8831
106	ACC	Archaea	0.0144	0.0073	0.0332	0.0016	0.8975
107	ACC	Eukaryota	0.0157	0.0051	0.0327	0.0026	0.8085
108	ACG	All	0.0128	0.0067	0.0387	0.0001	0.7768
109	ACG	Bacteria	0.0130	0.0066	0.0386	0.0001	0.7733
110	ACG	Archaea	0.0126	0.0093	0.0350	0.0002	0.9189
111	ACG	Eukaryota	0.0119	0.0058	0.0387	0.0007	0.7186
112	ACT	All	0.0099	0.0063	0.0319	0.0000	-0.8768
113	ACT	Bacteria	0.0093	0.0063	0.0314	0.0000	-0.9005
114	ACT	Archaea	0.0108	0.0056	0.0263	0.0008	-0.8633
115	ACT	Eukaryota	0.0137	0.0043	0.0295	0.0020	-0.7121
116	AAC	All	0.0172	0.0040	0.0405	0.0032	0.2815
117	AAC	Bacteria	0.0166	0.0037	0.0356	0.0032	0.3181
118	AAC	Archaea	0.0179	0.0039	0.0290	0.0062	0.4956
119	AAC	Eukaryota	0.0210	0.0041	0.0405	0.0075	0.3179
120	AAT	All	0.0207	0.0150	0.1207	0.0001	-0.9373
121	AAT	Bacteria	0.0202	0.0145	0.1207	0.0001	-0.9553
122	AAT	Archaea	0.0188	0.0146	0.0679	0.0006	-0.9261
123	AAT	Eukaryota	0.0223	0.0163	0.1106	0.0009	-0.9209
124	AAA	All	0.0332	0.0247	0.1837	0.0000	-0.9370
125	AAA	Bacteria	0.0335	0.0250	0.1837	0.0000	-0.9462

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Table S1. Summary table of all features in the three kingdoms. Average, standard deviation, max and min values are printed as well as the correlation coefficient with GCcoding.

	Feature	Kingdom	Average	Stdev	Max	Min	Cc-to-GC
126	AAA	Archaea	0.0348	0.0250	0.0997	0.0019	-0.9195
127	AAA	Eukaryota	0.0272	0.0173	0.1018	0.0019	-0.9426
128	AAG	All	0.0240	0.0076	0.0697	0.0000	0.0986
129	AAG	Bacteria	0.0229	0.0069	0.0540	0.0000	0.0986
130	AAG	Archaea	0.0287	0.0119	0.0645	0.0086	-0.1085
131	AAG	Eukaryota	0.0307	0.0062	0.0574	0.0107	0.5236
132	AGC	All	0.0128	0.0045	0.0508	0.0000	0.6831
133	AGC	Bacteria	0.0127	0.0043	0.0350	0.0000	0.6970
134	AGC	Archaea	0.0119	0.0045	0.0274	0.0013	0.6555
135	AGC	Eukaryota	0.0146	0.0052	0.0508	0.0016	0.7529
136	AGT	All	0.0084	0.0051	0.0381	0.0002	-0.8843
137	AGT	Bacteria	0.0079	0.0051	0.0381	0.0002	-0.8985
138	AGT	Archaea	0.0084	0.0041	0.0210	0.0009	-0.8203
139	AGT	Eukaryota	0.0113	0.0038	0.0249	0.0015	-0.9058
140	AGA	All	0.0086	0.0072	0.0416	0.0002	-0.8149
141	AGA	Bacteria	0.0077	0.0070	0.0370	0.0002	-0.8308
142	AGA	Archaea	0.0151	0.0088	0.0416	0.0007	-0.8538
143	AGA	Eukaryota	0.0119	0.0056	0.0299	0.0008	-0.8089
144	AGG	All	0.0058	0.0050	0.0641	0.0000	-0.0994
145	AGG	Bacteria	0.0050	0.0037	0.0522	0.0000	-0.1556
146	AGG	Archaea	0.0149	0.0127	0.0641	0.0011	0.0322
147	AGG	Eukaryota	0.0090	0.0037	0.0290	0.0008	0.2373
148	CTA	All	0.0061	0.0045	0.0343	0.0001	-0.6955
149	CTA	Bacteria	0.0056	0.0045	0.0343	0.0001	-0.7280
150	CTA	Archaea	0.0095	0.0060	0.0289	0.0008	-0.5104
151	CTA	Eukaryota	0.0082	0.0025	0.0223	0.0009	-0.5047
152	CTC	All	0.0183	0.0132	0.0762	0.0000	0.8157
153	CTC	Bacteria	0.0181	0.0134	0.0731	0.0000	0.8163
154	CTC	Archaea	0.0235	0.0167	0.0762	0.0008	0.9445
155	CTC	Eukaryota	0.0191	0.0091	0.0656	0.0014	0.8598
156	CTG	All	0.0287	0.0206	0.0937	0.0000	0.8511
157	CTG	Bacteria	0.0306	0.0215	0.0937	0.0000	0.8682
158	CTG	Archaea	0.0178	0.0102	0.0634	0.0010	0.8179
159	CTG	Eukaryota	0.0206	0.0111	0.0713	0.0008	0.6816
160	CTT	All	0.0150	0.0079	0.0556	0.0003	-0.6754
161	CTT	Bacteria	0.0149	0.0083	0.0556	0.0003	-0.7004
162	CTT	Archaea	0.0162	0.0074	0.0352	0.0013	-0.6886
163	CTT	Eukaryota	0.0150	0.0042	0.0289	0.0037	-0.3661
164	CCA	All	0.0084	0.0050	0.0319	0.0000	-0.6836
165	CCA	Bacteria	0.0074	0.0044	0.0244	0.0000	-0.7743
166	CCA	Archaea	0.0103	0.0050	0.0222	0.0011	-0.8020
167	CCA	Eukaryota	0.0149	0.0037	0.0319	0.0025	-0.4671

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Table S1. Summary table of all features in the three kingdoms. Average, standard deviation, max and min values are printed as well as the correlation coefficient with GCcoding.

	Feature	Kingdom	Average	Stdev	Max	Min	Cc-to-GC
168	CCC	All	0.0115	0.0070	0.0469	0.0002	0.8647
169	CCC	Bacteria	0.0115	0.0071	0.0469	0.0002	0.8721
170	CCC	Archaea	0.0101	0.0057	0.0282	0.0010	0.8851
171	CCC	Eukaryota	0.0130	0.0060	0.0306	0.0009	0.8497
172	CCG	All	0.0157	0.0100	0.0451	0.0002	0.9159
173	CCG	Bacteria	0.0166	0.0103	0.0451	0.0002	0.9284
174	CCG	Archaea	0.0114	0.0080	0.0322	0.0002	0.9332
175	CCG	Eukaryota	0.0113	0.0062	0.0379	0.0005	0.8048
176	CCT	All	0.0087	0.0044	0.0266	0.0000	-0.6961
177	CCT	Bacteria	0.0080	0.0040	0.0258	0.0000	-0.8105
178	CCT	Archaea	0.0090	0.0042	0.0186	0.0007	-0.8430
179	CCT	Eukaryota	0.0136	0.0037	0.0266	0.0023	-0.0109
180	CAC	All	0.0103	0.0048	0.0252	0.0000	0.8740
181	CAC	Bacteria	0.0100	0.0049	0.0237	0.0000	0.8934
182	CAC	Archaea	0.0098	0.0048	0.0201	0.0007	0.9468
183	CAC	Eukaryota	0.0125	0.0038	0.0252	0.0021	0.8899
184	CAT	All	0.0103	0.0040	0.0237	0.0003	-0.6729
185	CAT	Bacteria	0.0102	0.0040	0.0237	0.0003	-0.6867
186	CAT	Archaea	0.0078	0.0037	0.0162	0.0005	-0.8436
187	CAT	Eukaryota	0.0120	0.0033	0.0225	0.0020	-0.7703
188	CAA	All	0.0151	0.0096	0.0555	0.0002	-0.7490
189	CAA	Bacteria	0.0148	0.0099	0.0555	0.0002	-0.7573
190	CAA	Archaea	0.0103	0.0062	0.0284	0.0010	-0.7421
191	CAA	Eukaryota	0.0183	0.0064	0.0417	0.0035	-0.7479
192	CAG	All	0.0195	0.0085	0.1377	0.0002	0.7634
193	CAG	Bacteria	0.0197	0.0081	0.0441	0.0002	0.7989
194	CAG	Archaea	0.0141	0.0059	0.0322	0.0016	0.7799
195	CAG	Eukaryota	0.0215	0.0103	0.1377	0.0025	0.6253
196	CGA	All	0.0053	0.0029	0.0225	0.0000	0.0365
197	CGA	Bacteria	0.0049	0.0025	0.0204	0.0000	-0.0196
198	CGA	Archaea	0.0057	0.0043	0.0180	0.0000	0.5272
199	CGA	Eukaryota	0.0085	0.0035	0.0225	0.0005	0.2697
200	CGC	All	0.0186	0.0146	0.0671	0.0000	0.9169
201	CGC	Bacteria	0.0201	0.0151	0.0671	0.0000	0.9359
202	CGC	Archaea	0.0091	0.0094	0.0419	0.0000	0.8668
203	CGC	Eukaryota	0.0120	0.0075	0.0429	0.0001	0.8701
204	CGG	All	0.0106	0.0087	0.0501	0.0000	0.8521
205	CGG	Bacteria	0.0112	0.0091	0.0501	0.0000	0.8565
206	CGG	Archaea	0.0084	0.0088	0.0422	0.0000	0.8379
207	CGG	Eukaryota	0.0078	0.0041	0.0232	0.0001	0.8421
208	CGT	All	0.0087	0.0042	0.0348	0.0000	-0.0177
209	CGT	Bacteria	0.0089	0.0043	0.0348	0.0000	-0.0592

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Table S1. Summary table of all features in the three kingdoms. Average, standard deviation, max and min values are printed as well as the correlation coefficient with GCcoding.

	Feature	Kingdom	Average	Stdev	Max	Min	Cc-to-GC
210	CGT	Archaea	0.0046	0.0024	0.0146	0.0001	0.4049
211	CGT	Eukaryota	0.0085	0.0033	0.0241	0.0004	0.0860
212	GTA	All	0.0113	0.0075	0.0448	0.0000	-0.8571
213	GTA	Bacteria	0.0115	0.0079	0.0448	0.0000	-0.8759
214	GTA	Archaea	0.0140	0.0072	0.0328	0.0012	-0.8481
215	GTA	Eukaryota	0.0091	0.0037	0.0236	0.0010	-0.8328
216	GTC	All	0.0200	0.0131	0.0729	0.0001	0.8849
217	GTC	Bacteria	0.0203	0.0134	0.0729	0.0001	0.8931
218	GTC	Archaea	0.0236	0.0181	0.0685	0.0013	0.9320
219	GTC	Eukaryota	0.0178	0.0074	0.0505	0.0021	0.8415
220	GTG	All	0.0224	0.0112	0.0677	0.0002	0.8786
221	GTG	Bacteria	0.0233	0.0115	0.0677	0.0002	0.8989
222	GTG	Archaea	0.0182	0.0091	0.0636	0.0015	0.7291
223	GTG	Eukaryota	0.0184	0.0071	0.0474	0.0020	0.6287
224	GTT	All	0.0167	0.0091	0.0449	0.0003	-0.8888
225	GTT	Bacteria	0.0164	0.0094	0.0422	0.0003	-0.8984
226	GTT	Archaea	0.0203	0.0094	0.0449	0.0019	-0.8293
227	GTT	Eukaryota	0.0162	0.0053	0.0363	0.0027	-0.7598
228	GCA	All	0.0171	0.0075	0.0957	0.0015	-0.6445
229	GCA	Bacteria	0.0169	0.0076	0.0444	0.0015	-0.6994
230	GCA	Archaea	0.0191	0.0079	0.0396	0.0035	-0.6821
231	GCA	Eukaryota	0.0181	0.0062	0.0957	0.0036	0.0704
232	GCC	All	0.0313	0.0211	0.1020	0.0004	0.9306
233	GCC	Bacteria	0.0333	0.0218	0.1020	0.0004	0.9426
234	GCC	Archaea	0.0215	0.0144	0.0564	0.0015	0.9467
235	GCC	Eukaryota	0.0222	0.0108	0.0779	0.0016	0.8956
236	GCG	All	0.0248	0.0182	0.1028	0.0003	0.8876
237	GCG	Bacteria	0.0266	0.0186	0.1028	0.0003	0.9035
238	GCG	Archaea	0.0191	0.0163	0.0726	0.0003	0.9193
239	GCG	Eukaryota	0.0151	0.0107	0.1003	0.0005	0.7641
240	GCT	All	0.0160	0.0071	0.0486	0.0007	-0.7307
241	GCT	Bacteria	0.0154	0.0073	0.0486	0.0007	-0.7870
242	GCT	Archaea	0.0150	0.0061	0.0333	0.0024	-0.6719
243	GCT	Eukaryota	0.0200	0.0048	0.0405	0.0048	0.0072
244	GAC	All	0.0255	0.0134	0.0844	0.0005	0.9035
245	GAC	Bacteria	0.0255	0.0136	0.0775	0.0005	0.9176
246	GAC	Archaea	0.0292	0.0195	0.0844	0.0027	0.8848
247	GAC	Eukaryota	0.0251	0.0085	0.0605	0.0049	0.8795
248	GAT	All	0.0275	0.0110	0.0581	0.0008	-0.8951
249	GAT	Bacteria	0.0273	0.0112	0.0581	0.0008	-0.8979
250	GAT	Archaea	0.0275	0.0119	0.0561	0.0032	-0.8646
251	GAT	Eukaryota	0.0279	0.0083	0.0529	0.0029	-0.9007

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Table S1. Summary table of all features in the three kingdoms. Average, standard deviation, max and min values are printed as well as the correlation coefficient with GCcoding.

	Feature	Kingdom	Average	Stdev	Max	Min	Cc-to-GC
252	GAA	All	0.0349	0.0148	0.0775	0.0010	-0.8958
253	GAA	Bacteria	0.0352	0.0150	0.0775	0.0010	-0.9092
254	GAA	Archaea	0.0380	0.0165	0.0757	0.0048	-0.7765
255	GAA	Eukaryota	0.0306	0.0111	0.0686	0.0030	-0.9479
256	GAG	All	0.0283	0.0122	0.0869	0.0000	0.7692
257	GAG	Bacteria	0.0275	0.0117	0.0798	0.0000	0.7972
258	GAG	Archaea	0.0395	0.0174	0.0869	0.0068	0.8494
259	GAG	Eukaryota	0.0314	0.0097	0.0584	0.0055	0.8696
260	GGA	All	0.0152	0.0076	0.0460	0.0000	-0.7375
261	GGA	Bacteria	0.0148	0.0079	0.0460	0.0000	-0.7596
262	GGA	Archaea	0.0208	0.0082	0.0386	0.0053	-0.8021
263	GGA	Eukaryota	0.0162	0.0040	0.0328	0.0029	-0.3754
264	GGC	All	0.0283	0.0172	0.0734	0.0008	0.9331
265	GGC	Bacteria	0.0301	0.0177	0.0734	0.0008	0.9459
266	GGC	Archaea	0.0215	0.0134	0.0534	0.0015	0.9232
267	GGC	Eukaryota	0.0199	0.0103	0.0662	0.0009	0.9007
268	GGG	All	0.0123	0.0057	0.0513	0.0000	0.6654
269	GGG	Bacteria	0.0124	0.0056	0.0513	0.0000	0.6658
270	GGG	Archaea	0.0149	0.0069	0.0398	0.0018	0.7832
271	GGG	Eukaryota	0.0106	0.0045	0.0270	0.0007	0.6273
272	GGT	All	0.0158	0.0060	0.0443	0.0022	-0.6421
273	GGT	Bacteria	0.0158	0.0062	0.0443	0.0022	-0.6691
274	GGT	Archaea	0.0153	0.0060	0.0370	0.0030	-0.5617
275	GGT	Eukaryota	0.0158	0.0044	0.0352	0.0038	-0.3462
276	TCA	All	0.0091	0.0060	0.0422	0.0002	-0.8642
277	TCA	Bacteria	0.0083	0.0056	0.0274	0.0002	-0.9013
278	TCA	Archaea	0.0114	0.0065	0.0328	0.0008	-0.8583
279	TCA	Eukaryota	0.0136	0.0054	0.0422	0.0021	-0.8219
280	TCC	All	0.0108	0.0050	0.0331	0.0000	0.6298
281	TCC	Bacteria	0.0104	0.0049	0.0331	0.0000	0.6501
282	TCC	Archaea	0.0098	0.0040	0.0270	0.0021	0.6529
283	TCC	Eukaryota	0.0144	0.0041	0.0292	0.0018	0.7665
284	TCG	All	0.0108	0.0060	0.0455	0.0001	0.8124
285	TCG	Bacteria	0.0107	0.0059	0.0297	0.0004	0.8339
286	TCG	Archaea	0.0098	0.0068	0.0267	0.0001	0.8967
287	TCG	Eukaryota	0.0122	0.0059	0.0455	0.0007	0.6575
288	TCT	All	0.0094	0.0063	0.0367	0.0001	-0.8528
289	TCT	Bacteria	0.0086	0.0061	0.0364	0.0001	-0.9071
290	TCT	Archaea	0.0097	0.0057	0.0252	0.0006	-0.8939
291	TCT	Eukaryota	0.0148	0.0048	0.0316	0.0023	-0.6446
292	TTC	All	0.0189	0.0077	0.0398	0.0010	0.8507
293	TTC	Bacteria	0.0187	0.0079	0.0398	0.0010	0.8687

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Table S1. Summary table of all features in the three kingdoms. Average, standard deviation, max and min values are printed as well as the correlation coefficient with GCcoding.

	Feature	Kingdom	Average	Stdev	Max	Min	Cc-to-GC
294	TTC	Archaea	0.0208	0.0073	0.0375	0.0048	0.8597
295	TTC	Eukaryota	0.0207	0.0045	0.0324	0.0051	0.7161
296	TTT	All	0.0219	0.0135	0.1003	0.0000	-0.9387
297	TTT	Bacteria	0.0223	0.0140	0.1003	0.0000	-0.9541
298	TTT	Archaea	0.0180	0.0114	0.0471	0.0009	-0.9386
299	TTT	Eukaryota	0.0189	0.0082	0.0559	0.0041	-0.9117
300	TTA	All	0.0153	0.0160	0.0851	0.0000	-0.8746
301	TTA	Bacteria	0.0154	0.0162	0.0851	0.0000	-0.8793
302	TTA	Archaea	0.0151	0.0141	0.0694	0.0001	-0.8743
303	TTA	Eukaryota	0.0114	0.0111	0.0562	0.0002	-0.9236
304	TTG	All	0.0153	0.0073	0.0528	0.0005	-0.4940
305	TTG	Bacteria	0.0152	0.0075	0.0528	0.0005	-0.5200
306	TTG	Archaea	0.0125	0.0059	0.0361	0.0027	-0.5383
307	TTG	Eukaryota	0.0171	0.0058	0.0463	0.0033	-0.4608
308	TAC	All	0.0132	0.0043	0.0374	0.0000	0.5661
309	TAC	Bacteria	0.0127	0.0040	0.0328	0.0000	0.6005
310	TAC	Archaea	0.0171	0.0061	0.0374	0.0045	0.7504
311	TAC	Eukaryota	0.0157	0.0036	0.0263	0.0038	0.6555
312	TAT	All	0.0167	0.0095	0.0601	0.0001	-0.9271
313	TAT	Bacteria	0.0169	0.0096	0.0552	0.0001	-0.9410
314	TAT	Archaea	0.0154	0.0095	0.0601	0.0008	-0.9128
315	TAT	Eukaryota	0.0142	0.0077	0.0504	0.0010	-0.9368
316	TGC	All	0.0066	0.0030	0.0245	0.0000	0.5759
317	TGC	Bacteria	0.0062	0.0027	0.0245	0.0000	0.6517
318	TGC	Archaea	0.0059	0.0027	0.0176	0.0008	0.3239
319	TGC	Eukaryota	0.0094	0.0032	0.0201	0.0014	0.5596
320	TGT	All	0.0048	0.0031	0.0247	0.0001	-0.7338
321	TGT	Bacteria	0.0043	0.0027	0.0247	0.0001	-0.8217
322	TGT	Archaea	0.0054	0.0026	0.0129	0.0006	-0.6477
323	TGT	Eukaryota	0.0083	0.0037	0.0211	0.0009	-0.7869
324	TGG	All	0.0121	0.0027	0.0280	0.0000	0.7476
325	TGG	Bacteria	0.0122	0.0026	0.0243	0.0000	0.7725
326	TGG	Archaea	0.0106	0.0018	0.0165	0.0055	0.4767
327	TGG	Eukaryota	0.0127	0.0025	0.0280	0.0046	0.6963
328	ILE	All	0.0639	0.0186	0.1767	0.0209	-0.9183
329	ILE	Bacteria	0.0647	0.0184	0.1767	0.0209	-0.9504
330	ILE	Archaea	0.0723	0.0205	0.1278	0.0257	-0.9339
331	ILE	Eukaryota	0.0534	0.0126	0.1125	0.0229	-0.9240
332	LYS	All	0.0576	0.0243	0.1894	0.0115	-0.9264
333	LYS	Bacteria	0.0568	0.0248	0.1894	0.0115	-0.9295
334	LYS	Archaea	0.0640	0.0271	0.1227	0.0129	-0.8995
335	LYS	Eukaryota	0.0581	0.0148	0.1179	0.0268	-0.8824

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Table S1. Summary table of all features in the three kingdoms. Average, standard deviation, max and min values are printed as well as the correlation coefficient with GCcoding.

	Feature	Kingdom	Average	Stdev	Max	Min	Cc-to-GC
336	ASN	All	0.0381	0.0141	0.1303	0.0119	-0.9194
337	ASN	Bacteria	0.0371	0.0134	0.1245	0.0119	-0.9438
338	ASN	Archaea	0.0370	0.0128	0.0746	0.0168	-0.9140
339	ASN	Eukaryota	0.0436	0.0155	0.1303	0.0198	-0.8871
340	TYR	All	0.0301	0.0074	0.0650	0.0133	-0.8672
341	TYR	Bacteria	0.0299	0.0075	0.0619	0.0155	-0.8781
342	TYR	Archaea	0.0328	0.0062	0.0650	0.0225	-0.6527
343	TYR	Eukaryota	0.0301	0.0058	0.0578	0.0133	-0.8309
344	PHE	All	0.0410	0.0074	0.1061	0.0125	-0.8306
345	PHE	Bacteria	0.0412	0.0076	0.1061	0.0125	-0.8443
346	PHE	Archaea	0.0392	0.0058	0.0573	0.0226	-0.7546
347	PHE	Eukaryota	0.0397	0.0053	0.0648	0.0183	-0.8018
348	MET	All	0.0251	0.0037	0.0425	0.0135	-0.2492
349	MET	Bacteria	0.0253	0.0037	0.0375	0.0135	-0.2835
350	MET	Archaea	0.0251	0.0041	0.0377	0.0161	-0.3884
351	MET	Eukaryota	0.0236	0.0025	0.0425	0.0148	-0.1917
352	LEU	All	0.0990	0.0075	0.1473	0.0733	0.3244
353	LEU	Bacteria	0.1002	0.0071	0.1473	0.0781	0.3659
354	LEU	Archaea	0.0946	0.0077	0.1171	0.0733	0.0808
355	LEU	Eukaryota	0.0916	0.0053	0.1390	0.0767	-0.0629
356	SER	All	0.0614	0.0096	0.1064	0.0338	-0.4210
357	SER	Bacteria	0.0586	0.0063	0.0921	0.0338	-0.6545
358	SER	Archaea	0.0610	0.0070	0.0805	0.0442	-0.3691
359	SER	Eukaryota	0.0812	0.0061	0.1064	0.0568	-0.0079
360	GLN	All	0.0348	0.0082	0.1804	0.0113	-0.0925
361	GLN	Bacteria	0.0346	0.0075	0.0704	0.0113	-0.1348
362	GLN	Archaea	0.0244	0.0050	0.0420	0.0121	0.0050
363	GLN	Eukaryota	0.0403	0.0097	0.1804	0.0209	0.0844
364	GLU	All	0.0638	0.0085	0.1174	0.0281	-0.4623
365	GLU	Bacteria	0.0633	0.0080	0.0967	0.0281	-0.5361
366	GLU	Archaea	0.0785	0.0121	0.1174	0.0554	0.1546
367	GLU	Eukaryota	0.0622	0.0053	0.0963	0.0388	-0.3936
368	CYS	All	0.0113	0.0040	0.0333	0.0016	-0.1393
369	CYS	Bacteria	0.0104	0.0029	0.0333	0.0016	-0.1450
370	CYS	Archaea	0.0112	0.0027	0.0189	0.0061	-0.2769
371	CYS	Eukaryota	0.0178	0.0046	0.0320	0.0094	-0.2465
372	THR	All	0.0533	0.0054	0.0768	0.0207	0.2728
373	THR	Bacteria	0.0529	0.0051	0.0730	0.0207	0.2720
374	THR	Archaea	0.0514	0.0082	0.0768	0.0353	0.5098
375	THR	Eukaryota	0.0566	0.0050	0.0699	0.0375	0.3193
376	ASP	All	0.0535	0.0059	0.0892	0.0216	0.3904
377	ASP	Bacteria	0.0533	0.0055	0.0888	0.0216	0.4380

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Table S1. Summary table of all features in the three kingdoms. Average, standard deviation, max and min values are printed as well as the correlation coefficient with GCcoding.

	Feature	Kingdom	Average	Stdev	Max	Min	Cc-to-GC
378	ASP	Archaea	0.0574	0.0132	0.0892	0.0360	0.5227
379	ASP	Eukaryota	0.0532	0.0044	0.0758	0.0319	-0.0011
380	HIS	All	0.0206	0.0035	0.0357	0.0097	0.4462
381	HIS	Bacteria	0.0203	0.0032	0.0346	0.0104	0.5062
382	HIS	Archaea	0.0176	0.0024	0.0243	0.0097	0.5722
383	HIS	Eukaryota	0.0246	0.0025	0.0357	0.0144	0.3402
384	VAL	All	0.0705	0.0094	0.1103	0.0209	0.7249
385	VAL	Bacteria	0.0715	0.0088	0.1103	0.0209	0.7698
386	VAL	Archaea	0.0763	0.0117	0.1095	0.0512	0.8079
387	VAL	Eukaryota	0.0618	0.0057	0.0790	0.0386	0.6323
388	TRP	All	0.0122	0.0026	0.0282	0.0000	0.7417
389	TRP	Bacteria	0.0122	0.0026	0.0244	0.0000	0.7726
390	TRP	Archaea	0.0105	0.0017	0.0160	0.0056	0.5031
391	TRP	Eukaryota	0.0128	0.0025	0.0282	0.0046	0.6940
392	ARG	All	0.0574	0.0153	0.1212	0.0202	0.9362
393	ARG	Bacteria	0.0576	0.0159	0.1212	0.0202	0.9464
394	ARG	Archaea	0.0573	0.0135	0.0956	0.0298	0.8499
395	ARG	Eukaryota	0.0577	0.0090	0.0854	0.0275	0.8247
396	GLY	All	0.0721	0.0114	0.1046	0.0224	0.9025
397	GLY	Bacteria	0.0735	0.0108	0.1046	0.0224	0.9446
398	GLY	Archaea	0.0729	0.0084	0.0934	0.0500	0.9150
399	GLY	Eukaryota	0.0627	0.0103	0.1028	0.0301	0.8792
400	PRO	All	0.0444	0.0100	0.0807	0.0132	0.8532
401	PRO	Bacteria	0.0435	0.0095	0.0704	0.0132	0.9276
402	PRO	Archaea	0.0409	0.0058	0.0543	0.0279	0.8499
403	PRO	Eukaryota	0.0530	0.0098	0.0807	0.0206	0.8471
404	ALA	All	0.0898	0.0274	0.1653	0.0111	0.9389
405	ALA	Bacteria	0.0928	0.0275	0.1653	0.0111	0.9561
406	ALA	Archaea	0.0756	0.0205	0.1251	0.0362	0.9359
407	ALA	Eukaryota	0.0757	0.0204	0.1621	0.0222	0.9013
408	K+R	All	0.1150	0.0133	0.2123	0.0793	-0.6147
409	K+R	Bacteria	0.1144	0.0133	0.2123	0.0805	-0.6043
410	K+R	Archaea	0.1213	0.0197	0.1632	0.0793	-0.6568
411	K+R	Eukaryota	0.1158	0.0087	0.1549	0.0869	-0.6426
412	K+R+H	All	0.1357	0.0118	0.2227	0.0992	-0.5657
413	K+R+H	Bacteria	0.1347	0.0115	0.2227	0.1003	-0.5581
414	K+R+H	Archaea	0.1389	0.0182	0.1767	0.0992	-0.6310
415	K+R+H	Eukaryota	0.1404	0.0084	0.1754	0.1102	-0.5684
416	D+E	All	0.1173	0.0099	0.1861	0.0534	-0.1645
417	D+E	Bacteria	0.1167	0.0085	0.1707	0.0534	-0.2243
418	D+E	Archaea	0.1359	0.0207	0.1861	0.1037	0.4238
419	D+E	Eukaryota	0.1154	0.0067	0.1424	0.0730	-0.3170

Continued on next page

Table S1. Summary table of all features in the three kingdoms. Average, standard deviation, max and min values are printed as well as the correlation coefficient with GCcoding.

	Feature	Kingdom	Average	Stdev	Max	Min	Cc-to-GC
420	F+L+I+V+M	All	0.2995	0.0205	0.3949	0.1756	-0.7249
421	F+L+I+V+M	Bacteria	0.3030	0.0173	0.3949	0.2497	-0.9022
422	F+L+I+V+M	Archaea	0.3075	0.0215	0.3496	0.2602	-0.6987
423	F+L+I+V+M	Eukaryota	0.2701	0.0171	0.3475	0.1756	-0.7651
424	hydrophobics	All	0.4015	0.0222	0.4625	0.3080	0.5749
425	hydrophobics	Bacteria	0.4080	0.0157	0.4625	0.3408	0.8106
426	hydrophobics	Archaea	0.3936	0.0170	0.4359	0.3302	0.2934
427	hydrophobics	Eukaryota	0.3586	0.0123	0.4255	0.3080	0.5664
428	Ile (ATA+ATT)	All	0.0377	0.0263	0.1746	0.0001	-0.9627
429	Ile (ATA+ATT)	Bacteria	0.0375	0.0268	0.1746	0.0001	-0.9708
430	Ile (ATA+ATT)	Archaea	0.0484	0.0282	0.1234	0.0024	-0.9609
431	Ile (ATA+ATT)	Eukaryota	0.0320	0.0166	0.1035	0.0027	-0.9638
432	Ile (ATC)	All	0.0258	0.0102	0.0619	0.0011	0.8142
433	Ile (ATC)	Bacteria	0.0268	0.0104	0.0619	0.0011	0.8218
434	Ile (ATC)	Archaea	0.0232	0.0106	0.0542	0.0037	0.7551
435	Ile (ATC)	Eukaryota	0.0212	0.0058	0.0352	0.0042	0.7561
436	Ser (TCn)	All	0.0401	0.0080	0.0818	0.0139	-0.3136
437	Ser (TCn)	Bacteria	0.0380	0.0057	0.0655	0.0139	-0.4314
438	Ser (TCn)	Archaea	0.0407	0.0066	0.0573	0.0236	-0.3060
439	Ser (TCn)	Eukaryota	0.0549	0.0069	0.0818	0.0315	-0.0651
440	Ser (AGT+AGC)	All	0.0212	0.0043	0.0574	0.0074	-0.3256
441	Ser (AGT+AGC)	Bacteria	0.0206	0.0040	0.0420	0.0074	-0.3879
442	Ser (AGT+AGC)	Archaea	0.0203	0.0033	0.0312	0.0117	-0.1318
443	Ser (AGT+AGC)	Eukaryota	0.0260	0.0038	0.0574	0.0153	0.1340
444	Arg (CGn)	All	0.0432	0.0223	0.1047	0.0002	0.9354
445	Arg (CGn)	Bacteria	0.0452	0.0227	0.1047	0.0007	0.9515
446	Arg (CGn)	Archaea	0.0277	0.0217	0.0886	0.0002	0.8659
447	Arg (CGn)	Eukaryota	0.0367	0.0135	0.0774	0.0016	0.8265
448	Arg (AGA+AGG)	All	0.0144	0.0102	0.0694	0.0009	-0.6290
449	Arg (AGA+AGG)	Bacteria	0.0127	0.0091	0.0621	0.0009	-0.7029
450	Arg (AGA+AGG)	Archaea	0.0300	0.0162	0.0694	0.0023	-0.4403
451	Arg (AGA+AGG)	Eukaryota	0.0209	0.0071	0.0543	0.0017	-0.5186