Methylation-associated mutagenesis underlies variation in the mutation spectrum across eukaryotes.

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Abstract

Mutation spectra vary across genetic and environmental contexts, leading to differences between and within species. Most research on mutation spectrum has focused on the trinucleotide (3-mer) mutation types in mammals, limiting the breadth and depth of variation surveyed. In this study, we use whole-genome resequencing data across 108 eukaryotic species – including mammals, fish, plants, and invertebrates – to characterize pentanucleotide (5-mer) non-coding mutation spectra using a Bayesian approach. Our findings reveal cytosine transition mutability at CpG and (among plants) at CHG sites as the main drivers of variation in mutation spectra across eukaryotes, correlating strongly with genomic CpG and CHG depletion. However, despite the influence of methylation on CpG mutability, genome-wide average CpG methylation levels do not predict CpG transition rates across species and CHG methylation does not predict CHG transition rate, indicating unknown genetic or environmental factors influencing mutation rates at methylated cytosines. Together, our results illustrate the pivotal role of mutagenesis in shaping genome composition across eukaryotes and highlight a gap in knowledge about the mechanisms governing mutation rates.

Introduction

Mutation is a random process, but it is not uniform. DNA sequence context influences where, what type, and how frequently mutations occur[1,2]. One example is the hypermutability of methylated cytosines at CpG dinucleotides in vertebrate genomes, thought to result from spontaneous deamination of 5-methylcytosine to thymine[3,4]. Recognizing the importance of sequence context, recent work has focused on inference of context-specific mutation spectra, which quantify relative mutability based on the flanking nucleotides of a mutated base[5–9]. Most studies of mutation spectra have focused on trinucleotide (3-mer) contexts, where the mutation of interest is analyzed along with one adjacent nucleotide on each side. Consideration of this context window size has facilitated the identification of mutational signatures in cancer genomes as well as characterization of heterogeneity in polymorphism spectrum across human populations[5–7,10–13]. However, extending the analysis to longer sequence contexts (e.g., 5-mer) provides a higher-resolution view and better captures differences in mutation processes[9,14,15]. We recently developed *Baymer*[8], a Bayesian hierarchical tree approach, enabling accurate and robust inference of mutation spectra from polymorphism data. The number of events observed in polymorphism data is much larger (~100M) relative to *de novo* (~0.1M) datasets[16], justifying the use of polymorphism data to infer the mutational spectrum, at least in non-coding regions where the effect of selection is expected to be minimal and not context-specific.

Mutation rates differ across species due to genetic and environmental factors, yet the evolutionary forces shaping context-specific mutation rates remain largely unexplored outside of humans, primates and a few vertebrates[6,17–19]. With the expansion of large-scale sequencing initiatives and conservation genomics efforts, population-level polymorphism data are now available for diverse eukaryotic taxa beyond mammals[20–22]. These resources provide an opportunity to investigate mutation spectra across a wider phylogenetic range. For example, while cytosine methylation is almost exclusive to CpG contexts in vertebrates, it is found extensively in non-CpG contexts in plants and other species where we expect to discover distinct mutational spectra. To this end, we leverage whole-genome resequencing data from 108 eukaryotic species, including mammals, birds, fish, plants, and invertebrates, to characterize variation in mutation spectra in extended pentanucleotide (5-mer) sequence context. Using *Baymer*[8], we infer context-dependent mutation rates from non-coding polymorphisms and assess the biological and evolutionary factors driving mutation rate variation.

Results

A catalog of polymorphisms in the non-coding genomes of 108 eukaryotic species

The availability of population polymorphism data in diverse species allows us to study the evolution of mutational mechanisms across many previously unexplored lineages[20,21]. We collected publicly available polymorphism data from whole-genome shotgun sequencing for 113 eukaryotic species[23–122], including 35 mammals, 5 birds, 15 fish, 33 plants, and 12 invertebrates (**Fig. 1A, Supplemental Table 1**). To reduce issues related to genome assembly quality, variant callability and the effects of selection on coding regions, we masked repetitive elements, low complexity regions, and exons from each species assembly, with the resulting regions subsequently referred to as the accessible non-coding genome. This resulted in polymorphism datasets with 10^5 – 10^9 SNPs per species (**Fig. 1B**).

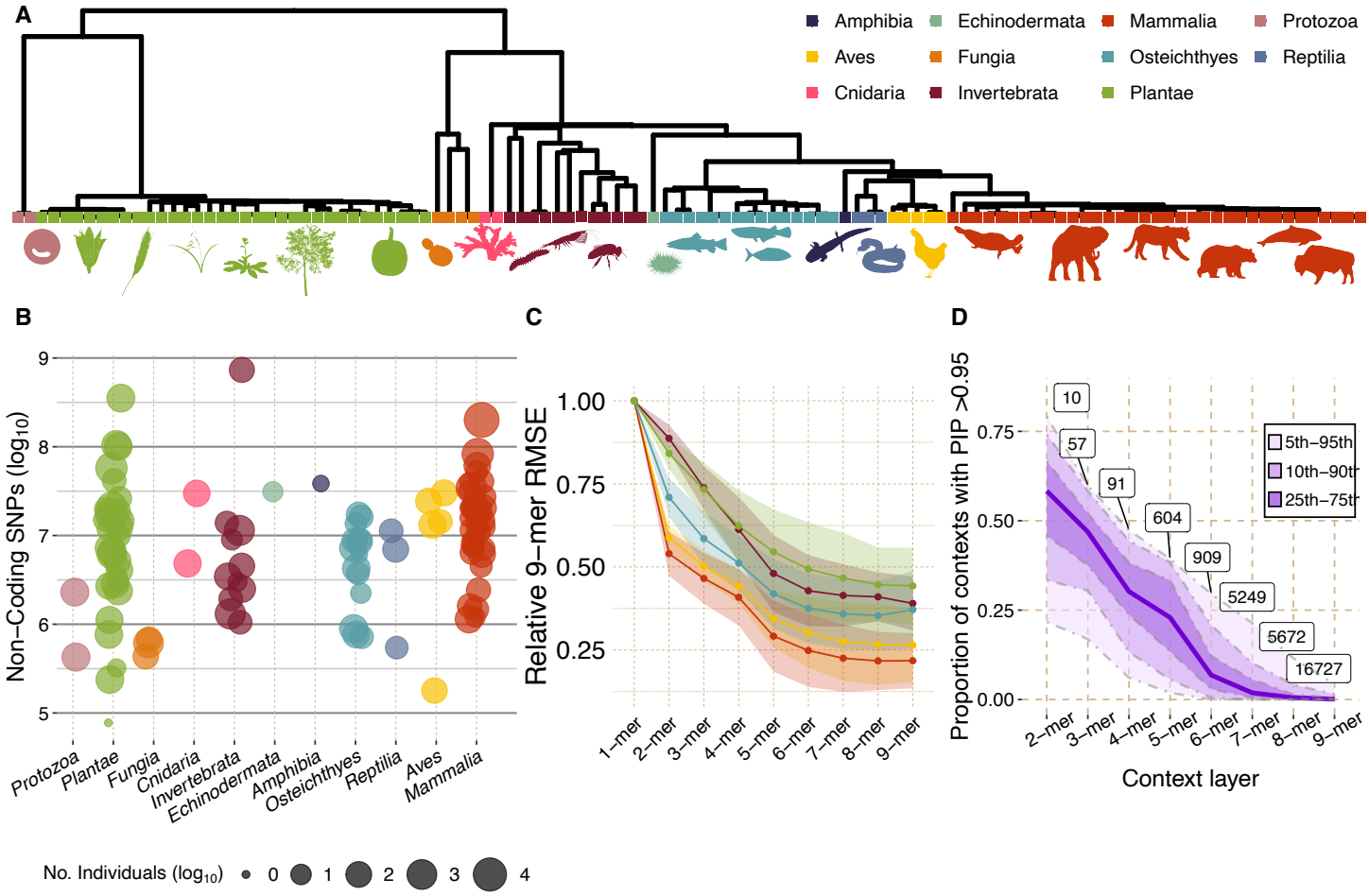

Fig. 1 | Data summary. **A**) Phylogenetic tree of collected species, generated with TimeTree, with assigned clades labeled at tips. **B**) Filtered single-nucleotide polymorphism (SNP) count ($\log_{10}$) per species is shown as individual points, colored by clade and sized by number of individuals ($\log_{10}$) in polymorphism dataset. **C**) Root mean square error (RMSE) between a k-mer mutation model trained on odd-numbered basepairs versus a 9-mer mutation model trained on even-numbered basepairs. Points show mean RMSE across species grouped by clade (only clades with n >=5 are shown). Shaded areas show one standard deviation from the mean. **D**) Proportions of contexts with posterior inclusion probability (PIP) greater than 0.95 in each context layer, for every species. Dark line represents the median proportion across species; shaded areas represent labeled quantiles. Number of contexts in 95th percentile is labeled for each layer.

Baymer captures variation in mutation spectra across species

We used *Baymer*[8] to infer sequence context windows (up to 4 flanking nucleotides, i.e., '9-mers') separately in each of the 113 species. *Baymer* infers the probability of polymorphism–an approximation to the relative mutation rate of each mutation type–by fitting a hierarchical Bayesian model to the number of observed polymorphisms and the corresponding frequency of the mutable context. A spike-and-slab prior allows contexts to have zero effect on mutation rate, preventing overfitting and allowing to model to adapt to species with very different numbers of observations. We use the default prior and MCMC settings as described in Ref. 8.

To evaluate the performance of our mutation models within species, we computed the root mean squared error (RMSE) between the mutation models generated from odd- and even-basepairs of the non-coding regions (**Fig. S1**). A higher RMSE suggests higher uncertainty in estimated rates due to technical noise or data sparsity. There is a large drop in RMSE between 1-mer and 2-mer models for mammals and birds due to the inclusion of CpG contexts. For all species, RMSE decreases for larger contexts, but the decrease is substantially slower above 5-mer contexts (**Fig. 1C**). This reflects the fact that the average posterior inclusion probabilities (PIP)–the proportion of contexts with a non-zero effect–drops from 25% at the 5-mer level to 2% at the 7-mer level (**Fig. 1D**). We noted five species with near zero PIPs for every mutation context across every context layer (**Fig. S3**)–a pattern is expected in random noise or sparse data that are not informative of mutational processes. We therefore excluded these five species from further analysis and focused on 5-mer contexts for downstream analyses in the 108 remaining species.

Cytosine transitions at methylation target sites explain most of the variation in mutation spectra

To investigate the variation of context-specific mutation rates in eukaryotes, we performed principal component analysis (PCA) on the fitted 5-mer context-specific mutation spectra (**Fig. 2A**), noting that PCA applied to 7-mer (but not 3-mer) contexts produced qualitatively similar results (**Fig. S4**). We found that the first two principal components cluster species by clade and capture 84% (PC1: 79.5%, PC2: 4.56%) of the variance in 5-mer mutation spectra across eukaryotes. Two clines emerge from the first two principal components, with the first cline reflecting variation within vertebrates and the second one reflecting variation among plants, with invertebrates and protozoans at the intersection of the two clines. PC1 is characterized by variation in NNCGN > NNTGN mutation rate and PC2 by variation in NNCHG > NNTHG mutation rate (where N is any nucleotide and H is any nucleotide other than guanine; **Fig. 2B**). Mammals, birds, and reptiles have the highest weights in PC1, consistent with higher CpG mutation rates. Plants have

higher weights in PC2, suggesting higher CHG mutation rates, consistent with variable rates of cytosine methylation at CHG contexts sites across plant species[5]/28/25 10:29:00 AM. The correlation between PCA results and context-specificity of cytosine methylation indicate that most of the variation in context-specific mutation rates across eukaryotes is driven by variation in the rate of cytosine transitions at methylation target sites.

The next two principal components together explain ~5% (PC3: 2.98%, PC4: 2.25%) of the variance in 5-mer mutation rates and are characterized by differences in C>T rates in different contexts (**Fig. S5A**). Principal component loadings correspond broadly to differences in mutability between cytosine methylation target sites flanked by strong (CG) versus weak (AT) bases ($P < 10^{-7}$ for CpG contexts and $P < 10^{-2}$ for CHG contexts, **Fig. S5B&C**). Overall, these results demonstrate that transitions at cytosine methylation target sites are the main driver of context-specific mutation rate variation across eukaryotes, capturing 90% of the variation.

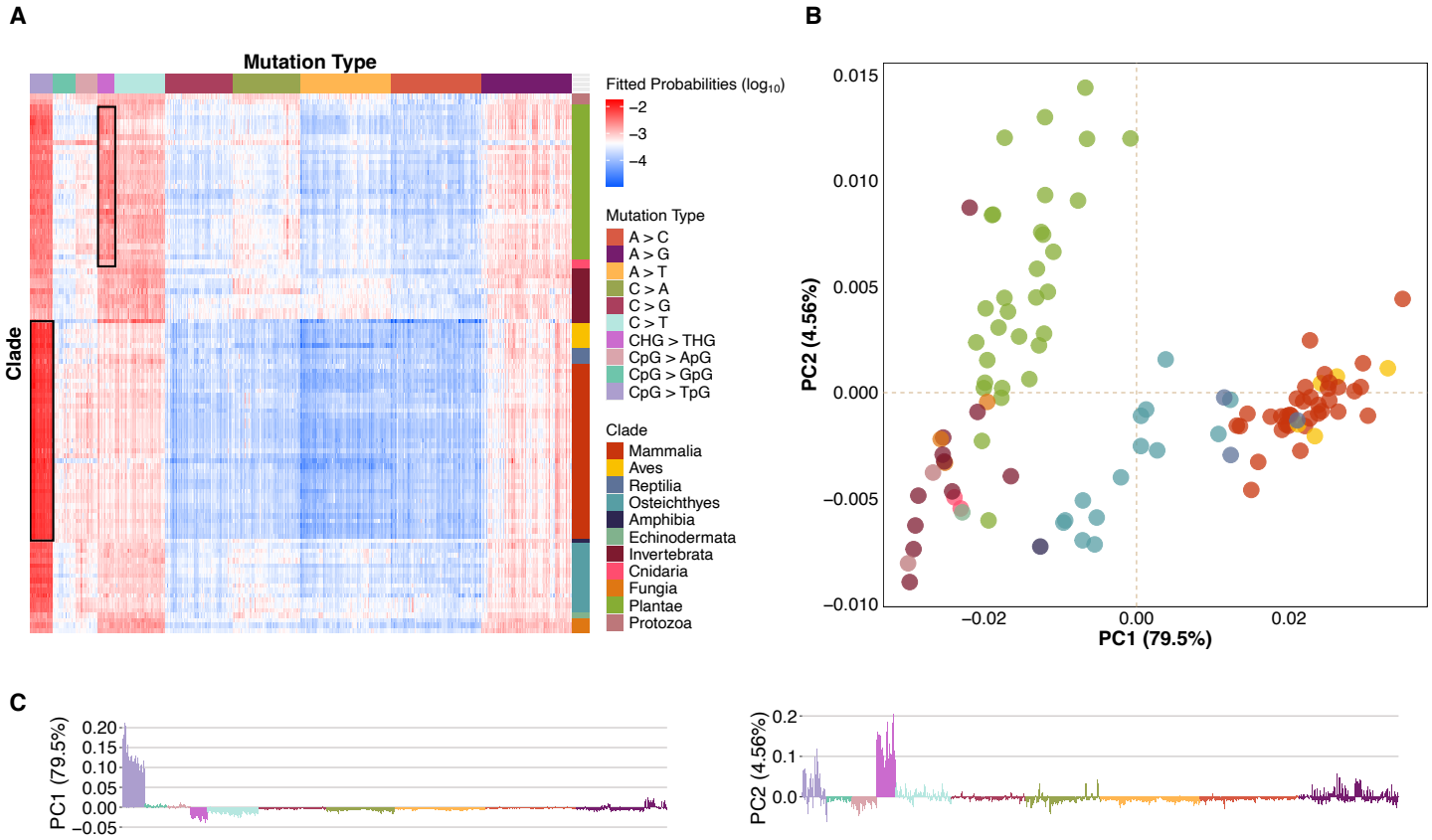

Fig. 2 | Principal component analysis (PCA) of fitted 5-mer polymorphism probabilities. **A**) Heatmap of 5-mer context fitted probabilities. **B**) First two principal components for each species, colored by clade. **C**) First two principal component loadings, showing individual bars for each mutation type, colored by mutation type.

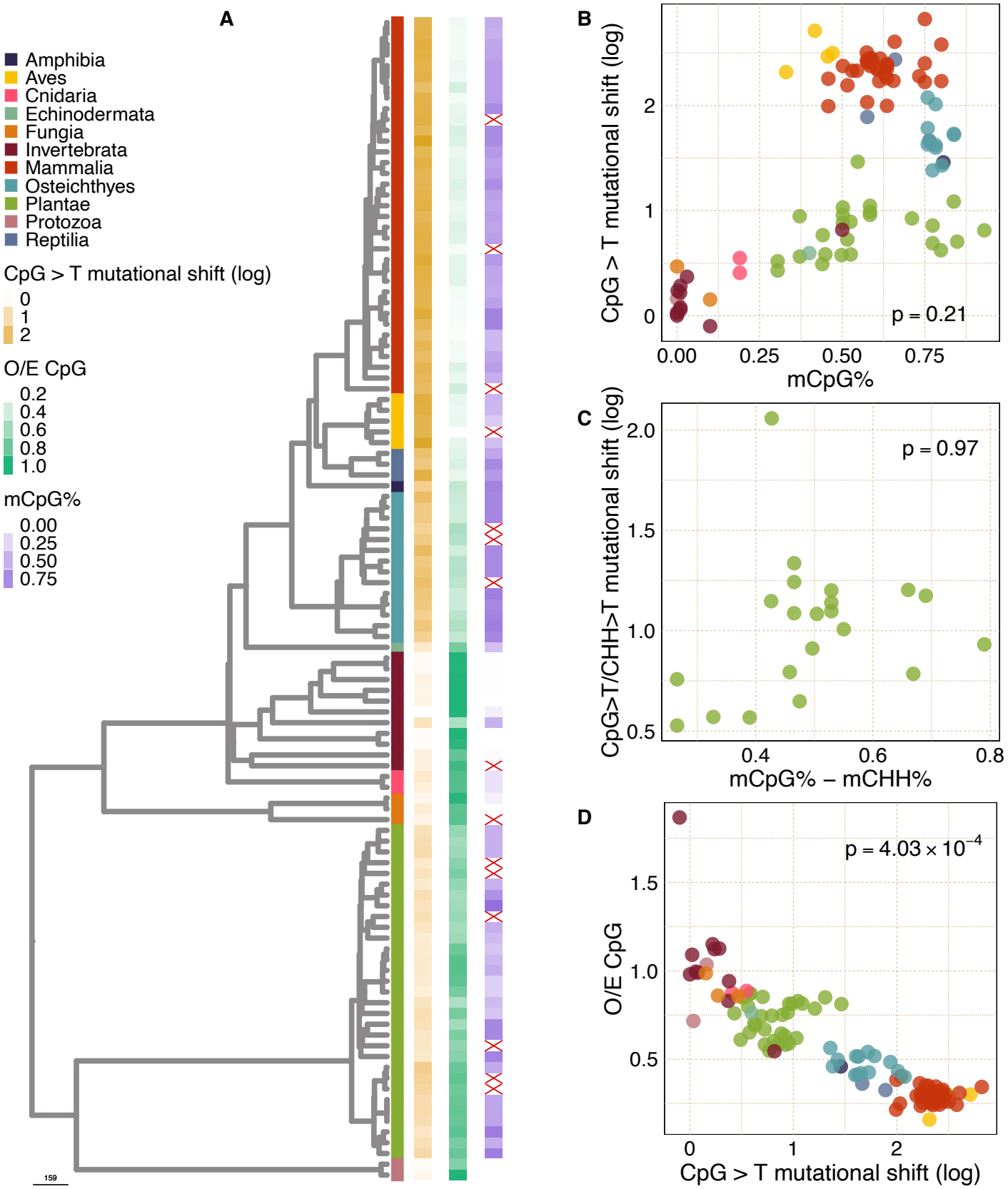

Fig. 3 | Relationship between CpG methylation level, mutation rate, and genome composition. **A**) Phylogenetic tree with CpG-related data shown at tips. **B**) CpG > T mutational shift vs genome-wide average CpG methylation. **C**) As B) but restricted to plants and removing the effect of CHG sites on CpG mutational shift and methylation levels. **D**) Observed/Expected CpG non-coding genome composition vs CpG > T mutational shift. **B-D**) P-values correspond to phylogenetic regression results.

CpG transition rates predict genomic CpG depletion but are not predicted by methylation levels

We used the ratio of CpG>T and CpH>T polymorphism probabilities to measure the change in mutation rate due to the CpG context and refer to this as the CpG>T mutational shift. Consistent with the mutagenic effect of methylation, this shift is largest (5- to 20-fold) in vertebrates, which have high levels of CpG methylation, lower (1.5 to 4.5-fold) in plants and less than 1.5-fold in species without substantial methylation (**Fig. 3A**). However, a closer analysis based on genome-wide CpG methylation levels derived from whole-genome bisulfite sequencing[24,123–133] reveals a more complicated pattern. Phylogenetic linear regression of CpG>T mutational shift on average CpG methylation is not significant (P=0.21; **Fig. 3B**). This lack of relationship is clearly visible among vertebrates, where fish have high methylation levels but low mutational shifts, and birds vice versa. Thus, CpG methylation entails a higher CpG>T mutational shift, but genome average CpG methylation levels do not predict the magnitude of the shift. This indicates that some other genetic or environmental factors shape CpG mutation rates across eukaryotes by modifying deamination, repair, or replication-driven error rates at methylated cytosine. In addition to CpG methylation, many plants have methylation in CHG and CHH contexts. We therefore corrected our analysis of CpG mutability by regressing the CpG>T / CHH>T mutational shift on the difference between CpG and CHH genome-wide average methylation levels in plants (**Fig. 3C**) with consistent results (phylogenetic regression P = 0.97).

We next evaluated the effect of the CpG>T mutational shift on CpG content of the accessible non-coding genome, as measured by the ratio of observed CpG fraction among all dinucleotides and the expected CpG fraction based on GC content (O/E CpG). We found that CpG>T mutational shift strongly predicts the magnitude of CpG depletion (phylogenetic regression P = 4.03×10^{-4}) (**Fig. 3D**), with no depletion in species without significant CpG>T mutational shift. This aligns with the expectation of lower equilibrium genome representation of highly mutable contexts and suggests that CpG content in the non-coding genome is largely determined by the mutation spectrum with minimal influence of selective constraint on CpG content. The one outlier is the honeybee *Apis mellifera*, which has previously been noted to have an exceptionally high genomic CpG content[134].

In plants, CHG transition rates predict CHG depletion but are not predicted by methylation levels

To test the relationship between CHG methylation, mutation rate, and genome composition in plants, we extracted genome-wide average CHG methylation levels from whole-genome bisulfite sequencing[123] and computed the CHG>T / CHH>T mutational shift and the O/E CHG content for each species. Analogous to the findings for CpG methylation across eukaryotes, we found that the CHG>T mutational shift is not predicted by CHG methylation (phylogenetic regression P = 0.57, **Fig. 4B**) but does predict the O/E CHG content (phylogenetic regression P = 6.75×10^{-7}, **Fig. S6**). To investigate whether the same, or different, factors underly variation in mutability of CpG and CHG contexts, we regressed species' mutational shifts on their methylation levels separately for CpG and CHG contexts and found that the residuals were highly correlated (Pearson's coefficient = 0.829; p = 3.3×10^{-6}) (**Fig. 4C**). This suggest that the same factors underlying the unexplained variation in CpG transition rates also underlie variation at CHG sites.

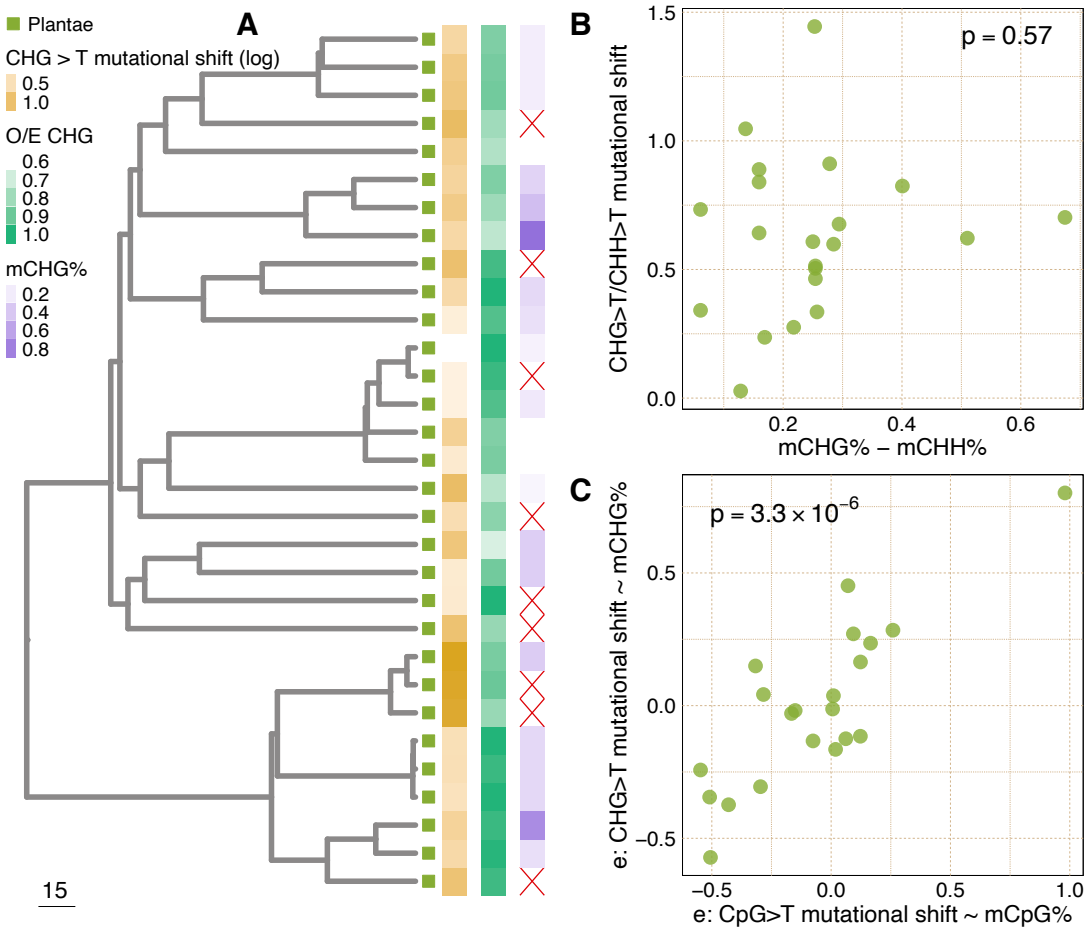

Fig. 4 | Plant CHG methylation and mutation rate. **A**) Phylogenetic tree with CHG data shown on tips. **B**) CHG > T mutational shift vs genome-wide average CHG methylation. **C**) Plant CpG and CHG phylogenetic regression residuals from CpG/CHG>T mutational shift against CpG/CHG methylation. Plant species are shown as individual points (Pearson's r = 0.829).

Mutation signature analysis confirms transitions at methylation target sites as the main driver of mutation spectrum variation in eukaryotes.

To validate our findings from our mutational analysis from Baymer-inferred context-specific mutation rates, we used SigFit[135] to model the observed 5-mer polymorphism spectrum in each species as a linear combination of *k* mutational signatures weighted by differential exposures in each species. Following Beichman et al.[136], we computed the cosine similarity between the observed and reconstructed mutation spectrum vectors to evaluate how well each model fitted the data. After analyzing the reconstruction performance for *k*=2 to 10, we determined that four mutational signatures had optimal performance and interpretability (**Fig. S7**). These four signatures reflect a CpG transition signature, a CpG and CHG transition signature, and two background signatures (**Fig. 5A**). Consistent with our previous observations, the CpG transition signature was most active in mammals, birds, and reptiles while the signature of CpG and CHG methylation was most active but variable in plants (**Fig. 5B**).

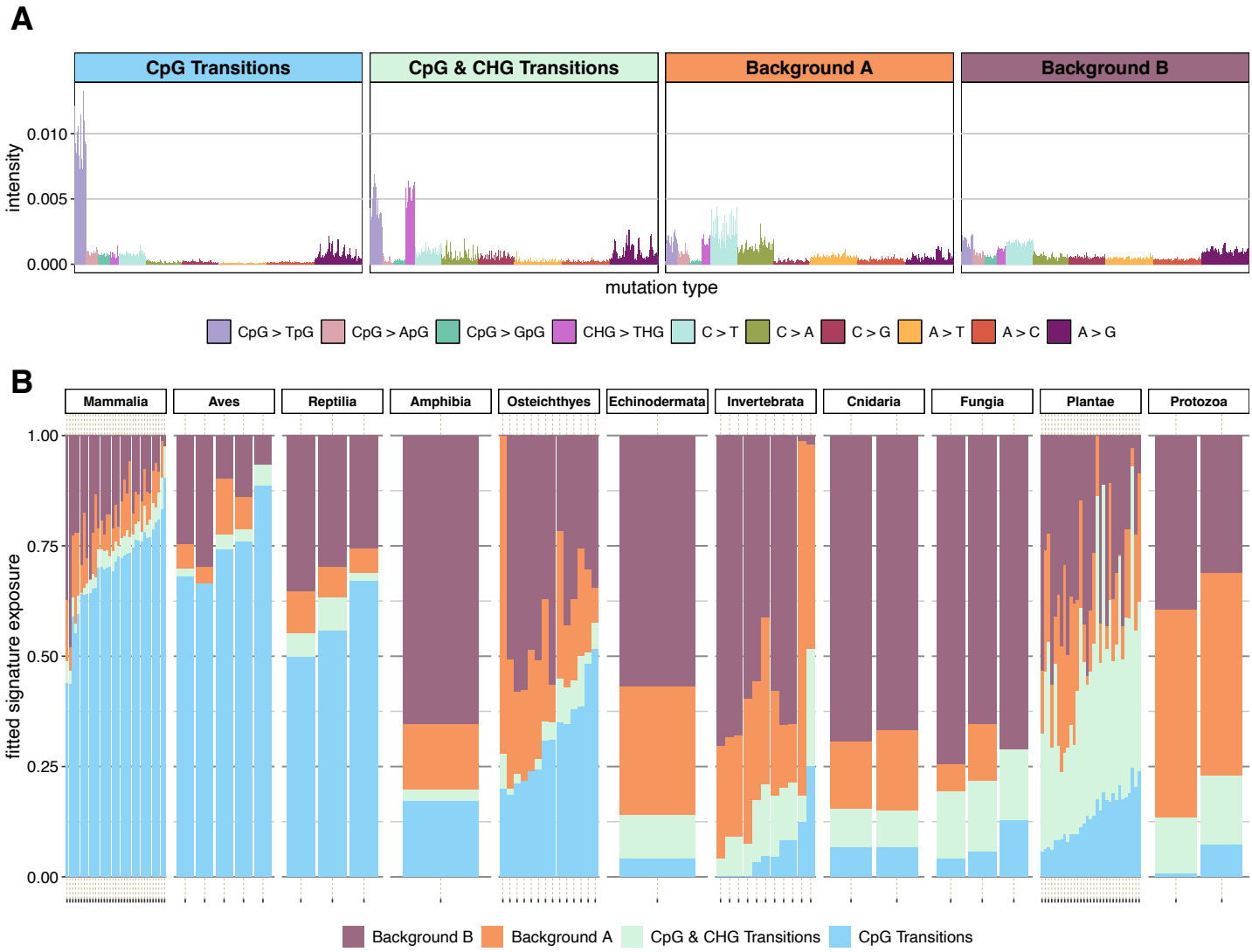

Fig. 5 | Mutational signature analysis in 5-mer polymorphism spectra. **A**) Extracted signature mutation spectra. **B**) Estimated contributions from each inferred signature to each species' polymorphism counts.

Discussion

Our study provides a comprehensive analysis of context-specific mutation spectra across 108 eukaryotic species, leveraging a phylogenetically diverse collection of polymorphism data from whole-genome sequencing. By extending mutation spectrum analysis to 5-mer contexts and looking across a wider range of species, we discover a new axis of mutation spectrum variation, corresponding to transitions at CHG contexts, in addition to the known role of CpG transitions. This is particularly important in plants, where methylation in CHG contexts is common and CHG > T mutations explain a large proportion of mutation spectrum variation.

While our findings reinforce the predominant role of transitions at methylation target sites on mutation spectrum variation across eukaryotic species, they also reveal that methylation is not the only factor driving mutation rates at these sites since genome-wide average methylation levels do not predict mutation rates these sites across species. Therefore, while methylation is necessary for high transition rates at CpG and CHG sites, the rates are strongly influenced by other unknown genetic and environmental factors that differ across species and modify rates of deamination, replication error, or repair. In contrast, we found that genomic CpG and CHG content is highly correlated with cytosine transition rates at CpG and CHG sites, suggesting that these aspects of genomic composition are largely determined by mutational pressures rather than selection, biased gene conversion, or other forces.

In summary, this study advances our understanding of mutation spectrum variation across eukaryotes by integrating 5-mer context mutation spectrum models over a wide range of species. We show that polymorphism data can be used as a reliable proxy for mutation data, although we may miss fine-scale variation due to data artefacts or the lack of polarization of ancestral alleles. We are also unable to address variation in the absolute mutation rate. Future work should attempt to identify genetic or environmental correlates of CpG and CHG transition rates after accounting for methylation, investigate variation across different genomic compartments, and to sample more broadly from clades with extreme mutational spectra or genome compositions.

Methods

Data collection

Polymorphism data: We obtained species polymorphism datasets, in the form of .vcf files, from public repositories or directly from authors. We carried out this process by searching through the literature and limiting our selected species to those with polymorphisms called from whole-genome sequencing data, with at least 2x coverage and five individuals (excluding humans, sample size: median=81; mean=259). We also required that the reference genomes used for variant calling had coding region annotations. The full list of species, along with data sources, can be found in **Supplemental Table 1**.

For *Ornithorhynchus anatinus* (Platypus), we aligned 49 individual genomes (in FASTQ format) and called variants using DeepVariant[137] and its standard protocol (**Supplemental Data**).

Reference genome assembly: Corresponding reference assemblies for each species were downloaded from NCBI or publication repository, along with coding and RepeatMasker annotations. **Supplemental Table 1** contains list of sources for polymorphism data and reference genome assembly ID.

Data preparation

Non-coding genome: For each species, we limited the analysis to the accessible non-coding genome by masking out exons, repetitive elements, and low complexity regions. For coding sequences, we extracted genome coordinates (in the form of .bed files) for exons from coding sequence annotation files (downloaded from NCBI or publication repository in the form of .gtf or .gff files) generated from RNA sequencing, for the vast majority of our species (**Supplementary Table 1)**. Repetitive element coordinates were extracted from RepeatMasker annotations. When RepeatMasker annotation was not available, we used *RepeatMasker-4.1.5* with default parameters and specified clade repeat libraries to generate repetitive element annotations. Low-complexity DNA regions were masked using the NCBI tool, *DustMasker-1.0.0* (with -window = 32; default is 64). Our final genomic region for analysis was defined using *bedtools2-2.30.0 getfasta* function by excluding genome coordinates that overlapped exonic, repetitive, and low-complexity coordinates from the above.

Polymorphism data: We retained only single-nucleotide polymorphisms (SNPs) from the variant call format (vcf) files found in the non-coding genome (defined above). Multiallelic sites were included in our analyses and treated as independent mutations at the same site. Since the ancestral genome assembly is

unavailable for most species, we used reference alleles for polarizing the polymorphisms, assuming the reference allele is the ancestral allele and non-reference allele the mutated allele. Reverse complementary mutation types are combined. Curated polymorphism data are available upon request. To mitigate concerns of high error rate in singletons, we restricted our analysis to SNPs present in at least two individuals within non-coding regions. Because our polymorphism data derive from studies with different sequencing and variant calling strategies, we checked that PCs of the mutation spectrum were uncorrelated with technical features including SNP count and reference genome N50 (**Fig. S8**).

Calculation of CpG and CHG observed/expected (O/E) ratio

The observed-over-expected CpG content ratio ($CpG_{O/E}$) was calculated as:

$$\boldsymbol{CpG_{O/E}} = \frac{[\boldsymbol{CpG}]_f}{[\boldsymbol{C}]_f[\boldsymbol{G}]_f}, \quad (1)$$

where the observed CpG frequency, $[\boldsymbol{CpG}]_f$, was calculated as the fraction of dinucleotide counts that are CpGs and the expected CpG frequency the product of the observed cytosine and guanine single-nucleotide frequencies, $[\boldsymbol{C}]_f$ and $[\boldsymbol{G}]_f$, in the accessible non-coding genome.

The observed-over-expected CHG content ratio ($CHG_{O/E}$) was calculated similarly as:

$$\boldsymbol{CHG_{O/E}} = \frac{[\boldsymbol{CHG}]_f}{[\boldsymbol{C}]_f[\boldsymbol{H}]_f[\boldsymbol{G}]_f}, \quad (2)$$

where the observed CHG (where H is any nucleotide other than guanine) frequency, $[\boldsymbol{CHG}]_f$, was calculated as the fraction of trinucleotide counts that are CHGs and the expected frequency the product of the observed frequencies of cytosine, non-guanine, and guanine single-nucleotide, $[\boldsymbol{C}]_f$, $[\boldsymbol{H}]_f$,and $[\boldsymbol{G}]_f$, in the accessible non-coding genome.

Methylation data collection

<u>Cytosine methylation data</u>: We collected averaged genome-wide cytosine methylation levels at CpG sites in each species and at CHG sites in plants from multiple sources (each species' methylation state and source is indicated in **Supplemental Table 2**) from whole-genome bisulfite sequencing (WGBS) studies[123,124].

Context-specific mutation probability models using Baymer

To construct our mutation probability models, we used Baymer[8], a Bayesian hierarchical tree model approach that estimates context-specific mutation probabilities for increasing context layers, iteratively.

Baymer generates regularized mutation probability estimates and provides a measure of uncertainty for each multiplicative shift in mutation probability leading up to the final k-mer mutation context. The context-specific mutation rates for any given context window are calculated as the product of the multiplicative shifts leading to the final mutation context. To generate context-specific mutation probability models from polymorphism data, Baymer requires a table with DNA context counts and polymorphic sites for the largest context size desired for the models (in our case, 9-mers). For each species, we first generated a table of 9-mer context counts within the non-coding, filtered regions of the genome. Then, we identified and recorded context-specific polymorphic sites in the region, for which the derived allele was observed in at least two individual genotypes to minimize the concern of high false positive rate in singleton variants.

Assessing robustness of the mutation model

We applied Baymer to these inputs to generate three models, each using one of three different partitions of the non-coding genome, which we refer to as ALL, ODD, and EVEN. The ALL model consisted of every context and polymorphism in the non-coding genome, while the ODD and EVEN models were only generated using odd or even genome coordinates in the non-coding genome, respectively. The ODD and EVEN models were generated for cross-validation of model performance in each species. We assessed within-species model robustness by computing the root mean square error (RMSE) between the ODD and EVEN polymorphism probability estimates; calculated as:

$$\boldsymbol{RMSE_{Odd/Even}} = \frac{\mathbf{1}}{\boldsymbol{K}}\sqrt{\sum_{\boldsymbol{i=1}}^{\boldsymbol{K}}(\boldsymbol{log}(\boldsymbol{O_i}) - \boldsymbol{log}(\boldsymbol{E_i}))^{\mathbf{2}}} \tag{3}$$

Where **O** and **E** are the fitted context-specific rate vectors, of length K, from the ODD and EVEN models, respectively. **Fig. S1** shows the $RMSE_{Odd/Even}$ for the 5-mer mutation models. Fig. 1C shows the RMSE between 9-mer Even mutation models versus k-mer (k = 1 to 9) Odd mutation models, normalized by the k=1 RMSE.

Mutational shifts

Our mutation models infer content-specific mutation rates rather than absolute mutation rates. For this reason, we base our mutability analyses on relative differences in context-specific mutation rates, which we refer to as 'mutational shifts'. We estimated these shifts using 5-mer context-specific mutation rates. CpG>T mutational shift was defined for each species as the ratio of the average C>T mutation rate in all 5-mer CpG contexts over that of CpH contexts (H = A,C,T):

$$\boldsymbol{CpG > T\ Mutational\ Shift} = \frac{\frac{\mathbf{1}}{\boldsymbol{K}}\sum_{\boldsymbol{i=1}}^{\boldsymbol{K}}[\boldsymbol{NNCGN>NNTGN}]}{\frac{\mathbf{1}}{\boldsymbol{N}}\sum_{\boldsymbol{j=1}}^{\boldsymbol{N}}[\boldsymbol{NNCHN>NNTHN}]}, \tag{4}$$

where the summation is across all 5-mer contexts matching the specified patterns.

We note that plant species also experience extensive cytosine methylation at non-CpG contexts. Because of this, the CpG>T mutational shift above may be a biased representation of the relative context-specific CpG>T mutation rate in these plants. To account for this, we defined the CpG>T / CHH>T mutational shift for each plant species as the ratio of the average C>T mutation rate in CpG contexts over that of CHH contexts, removing impacts of methylation in CHG contexts:

$$\frac{CpG>T}{CHH>T}\ \boldsymbol{Mutational\ Shift} = \frac{\frac{1}{K}\sum_{i=1}^{K}[NNCGN>NNTGN]}{\frac{1}{N}\sum_{j=1}^{N}[NNCHH>NNTHH]} \tag{5}$$

To analyze the relationship between CHG transition rates, methylation, and genome composition in plants, we defined the CHG>T / CHH>T mutational shift for each plant species as the ratio of the average C>T mutation rate in 5-mer CHG contexts over that of CHH contexts:

$$\frac{CHG>T}{CHH>T}\ \boldsymbol{Mutational\ Shift} = \frac{\frac{1}{K}\sum_{i=1}^{K}[NNCHG>NNTHG]}{\frac{1}{N}\sum_{j=1}^{N}[NNCHH>NNTHH]} \tag{6}$$

Phylogenetic tree

Our phylogenetic tree (in Newick file format) was constructed using estimated divergence times from TimeTree (timetree.org). **Supplemental Table 1** contains the list of species names used for TimeTree.

Principal component analysis (PCA) on fitted mutation probabilities

We performed principal component analysis (PCA) on the 3-mer, 5-mer, and 7-mer context size fitted mutation probability models, separately. We scaled the fitted mutation probabilities for each species so that they added up to one and used the prcomp() *R-4.2* function to perform PCA based on single-value decomposition.

Phylogenetic linear regressions

To perform analysis accounting for the phylogenic tree structure given the species included in our study, we used the *phylolm-2.6.2* R package phylolm()[138] function with Pagel's lambda as a phylogenetic scaling factor and 1000 bootstraps for our regression models. The phylolm() function is an implementation of the phylogenetic generalized least squares regression model:

$$\hat{\boldsymbol{\beta}} = (\boldsymbol{X}^T\boldsymbol{V}(\boldsymbol{\lambda})^{-1}\boldsymbol{X})^{-1}\boldsymbol{X}^T\boldsymbol{V}(\boldsymbol{\lambda})^{-1}\boldsymbol{y} \tag{7}$$

where for n species and k independent variables, $\mathbf{y}$ ($n \times 1$) is the continuous outcome vector, $\boldsymbol{\beta}$ ($n \times 1$) is the vector of coefficients for the variable matrix $\mathbf{X}$ ($n \times k$), and $\mathbf{V}$ ($n \times n$) is the variance-covariance matrix based on the phylogenetic relatedness between the species. Pagel's lambda is then used to transform $\mathbf{V}$ into a new matrix, $\mathbf{V}(\boldsymbol{\lambda})$ ($n \times n$), by scaling the off-diagonal elements of $\mathbf{V}$ using the scalar $\boldsymbol{\lambda}$, which is an estimate of phylogenetic signal for the regressed trait. $\boldsymbol{\lambda}$ ranges between 0 and 1, where 1 indicates that the covariance structure is exactly given by the phylogenetic relatedness structure, and 0 indicates no phylogenetic signal for the regressed trait. We report two-tailed P-values.

Mutational signature analysis with SigFit

We extracted mutational signatures from the observed 5-mer polymorphism spectra using a non-negative matrix factorization approach, implemented in the *SigFit-2.2*[135] R package. SigFit uses a Bayesian multinomial model to fit specified and/or inferred mutational signatures to observed mutation spectra, an approach equivalent to non-negative matrix factorization. For **G** genomes and **M** mutation contexts, SigFit takes as input a mutation counts matrix (**G** by **M**) and a mutation opportunities matrix (**G** by **M**); the mutation opportunities matrix contains the number of mutable contexts for each given 5-mer mutation type in the species' genome. For our SigFit analysis, we used similar mutation counts and context as inputs for Baymer inference. We used the extract_signatures() function from SigFit, with iter = 30000 and nsignatures = k (for k = 2,3,...,10). For each SigFit model, we reconstructed the mutation spectra using the fitted mutational signatures and their estimated activity values in each species. We then computed the cosine similarity between the observed and reconstructed 5-mer mutation spectra vectors to measure each model's performance in each species.

Data and code availability

Code to reproduce the analysis in this paper is available at https://github.com/framos99/MutationSpectraEvolutionAcrossEukaryotes-Project. Fitted mutation spectra for each species are available at https://doi.org/10.5281/zenodo.15464759. Original sources for data are given in Supplementary Table 1.

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