

Molecular adaptation reflects taxon-specific mutational biases

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Abstract

A fundamental question in molecular evolution is the extent to which patterns of adaptive change are shaped by mutational biases that make some variants more likely than others to arise. Past studies provide support for important effects of mutation bias on adaptive change, but leave open the empirical question of how strongly and how broadly evolutionary patterns depend on taxon-specific mutational tendencies. To characterize this effect quantitatively, we aggregated frequency spectra of adaptive amino acid changes from 14 species, comprising over 5000 total adaptive events, primarily from adaptive laboratory evolution studies. We then paired each species-specific spectrum of adaptive changes with independent measurements of the mutation spectrum of that same species. Across the 14 species, we find considerable heterogeneity in the relative frequencies of the six possible types of single-nucleotide changes, for both the mutational and adaptive spectra. Comparing these spectra across species, we find that, for any given mutation type, the stronger the bias toward or against that type in a species' mutation spectrum, the more enriched or depleted it tends to be in that species' adaptive spectrum. We conclude that, for the adaptation of proteins via amino acid changes, taxon-specific evolutionary preferences are strongly responsive to taxon-specific mutational preferences over their observed range.

Significance statement

The rates at which different types of mutation occur vary widely, both for different types of mutations within a single species and across species. Historically, a strong concordance between patterns of sequence evolution and mutational biases was used to support the thesis that most sequence evolution is caused by variants that do not affect fitness. However, more recent evidence shows that mutational biases can also influence adaptive evolution. Using data from 14 species, we find a strong relationship between the rate at which different kinds of nucleotide mutations emerge and their frequency among adaptive amino acid changes during protein evolution. These results suggest that processes that bias the production of variation may have a strong impact even during adaptive evolution.

Introduction

Broad patterns of gene and genome evolution reflect what is mutationally likely, an observation that has historically been taken as evidence for neutral evolution [1]. Yet population-genetic theory also suggests conditions under which such biases in mutation shape the course of adaptation, since the tendency of mutationally favored alleles to appear earlier than other alleles, i.e., arrival bias, makes them more likely to contribute to adaptation [2, 3]. The notion that mutational or other variational biases are a key factor in explaining evolutionary patterns, such as parallelisms and taxon-specific evolutionary tendencies, has persisted as a minority view for over a century [4, 5, 6, 3], despite a lack of robust evidence that such biases actually shape the course of adaptation. However, recent work has addressed this lack of evidence, establishing that, for a given species or taxonomic group, mutational biases often have a substantial effect on patterns of adaptive change [7, 8, 9, 10, 11, 12]. Nucleotide transitions and changes at CpG sites, for example, both occur at higher rates and contribute more to parallel adaptation [13, 14, 15, 16]. In another line of evidence, direct manipulation of the mutation spectrum during experimental evolution has a strong effect on which mutations serve as the drivers of adaptation, both when the spectrum is manipulated genome-wide [8] or altered locally by modulating the strength of a mutational hotspot [11] (for a review, see [17, 18] or Ch. 9 of [3]).

Importantly, the underlying theory for arrival bias predicts not merely an excess of likelier mutation types but systematically graduated effects. In other words, the greater the mutational bias, the greater the enrichment for that type of mutation amongst evolved adaptive changes, other things being equal [2, 17, 12]. Although prior empirical work by Cano, *et al.* [12] comparing patterns of adaptation and mutation in three species suggests that the spectrum of adaptive changes within a species quantitatively reflects the mutation spectrum for that species, we do not currently know how broadly and how strongly evolutionary preferences respond (statistically) to species-specific mutational preferences.

Here we address this question using data from 14 species for which published studies provide both (1) a nucleotide mutation spectrum and (2) sufficient data on adaptive changes to support a statistical analysis. We gathered empirical measurements of the mutation spectrum for each species, relying primarily on independent mutation-accumulation studies, together with with adaptive substitutions data, the latter consisting of a total of 5,488 single-nucleotide mutations that resulted in evolved adaptive amino acid changes. Both the mutation spectra and the spectra of adaptive changes show substantial taxonomic heterogeneity; for example, transition-transversion bias varies approximately 10-fold across our 14 species. Across all species and datasets, we found a strong and systematic pattern of correlation in which mutation types with higher rates contribute more to adaptation, and mutation types with lower rates contribute less. This pattern applies to the heterogeneity of rates across the six nucleotide mutation classes, the heterogeneity of specific rates across species, and heterogeneity across both species and mutation classes. We conclude that, for the adaptation of proteins via amino acid changes, evolutionary preferences are strongly responsive to taxon-specific preferences for nucleotide mutation, over their observed range.

Results

In this study, we sought to compare *de novo* mutation rates with adaptive outcomes. Using a codon-based model of single-nucleotide mutation enables us to specify an expected distribution of adaptive outcomes based on mutation rates and the codon composition of protein-coding regions. We begin with the genome-wide mutation spectra for multiple species, representing the distribution of frequencies with which each of the six types of single-nucleotide mutation occurs, corrected for species-specific genome nucleotide composition. Mutation spectra are obtained from published mutation-accumulation experiments for most species, or from patterns of segregating neutral variation in the case of *Mycobacterium tuberculosis* and *Toxoplasma gondii*, and exhibit considerable taxonomic heterogeneity, consistent with prior studies [19]. By correcting the genome-wide mutation spectra using weights assigned by the species-specific frequencies of each codon (see Materials and Methods), we estimated the spectra of *de novo* mutations that result in an amino acid change (Fig. 1A and Fig. S1A). In particular, this spectrum specifies the fraction of all new missense mutations, m_i , that belong to each mutation class i , which will hereafter be referred to as the mutation spectrum.

Next, we assembled a novel, multi-taxon dataset in which each of the mutation spectra is paired with an independently sourced dataset of adaptive amino acid substitutions (Fig. 1B). To ensure that the adaptive data consist of *bona fide* adaptations, with minimal contamination by non-adaptive mutations, we restricted the dataset to include only mutational events that meet multiple distinct criteria of evidence for having an adaptive effect (see Materials and Methods for details). In most cases, these data were obtained from experimental evolution studies, in which organisms were exposed to a selection pressure, such as a xenobiotic stressor or a novel niche, followed by genetic sequencing to identify mutations conferring

the observed adaptive phenotype. The resulting data covers 14 species and includes, for each species, a set of adaptive amino acid changes (median 37 adaptive events) and a nucleotide mutation spectrum obtained from independent sources (Table S1; see Materials and Methods). Consistent with Cano, *et al.* [12], comparing the two reveals positive intra-specific correlations between mutational bias and bias in adaptive outcomes for 13 of the 14 species (Fig. S2 and Table S2). Thus, with the enlarged multi-species dataset used here, we extend the finding of Cano, *et al.* [12] to roughly four-fold more species.

Transition bias of adaptive substitutions reflects species-specific *de novo* transition bias

A distinct issue from the one addressed in Cano *et al.* [12], and our main focus here, is how interspecific differences in mutation bias shape interspecific differences in adaptive evolutionary outcomes. In other words, when we look across species, does the strength of mutation bias predict the bias in adaptive outcomes? To address this question, we begin by considering transition bias, defined as the ratio of transitions (purine-to-purine or pyrimidine-to-pyrimidine) to transversions (purine-to-pyrimidine or vice versa). Although transitions tend to be overrepresented among adaptive changes [20, 21], the cause for this remains a topic of debate, with some studies attributing transition-biased adaptation to selection against transversions [22]. To more systematically investigate the effect of biases in mutation, we fit a linear regression by least squares on the log-transformed data,

$$\log\left(\frac{a}{1-a}\right) = \beta \log\left(\frac{m}{1-m}\right) + \beta_0, \quad (1)$$

where m is the frequency of transitions among missense mutations (so that $m/(1-m)$ is the transition:transversion ratio for the mutational spectrum), a is the frequency of transitions among adaptive amino acid changes (so that $a/(1-a)$ is the transition:transversion ratio among adaptive changes), β_0 is the intercept, and β is a statistic that captures the influence of mutation bias on adaptive outcome, such that $\beta = 1$ indicates a directly proportional relationship between mutation bias and bias in adaptive outcome. Thus, the question that we are asking is whether the transition:transversion ratio among missense mutations for a species predicts the transition:transversion ratio among adaptive amino acid substitutions. We observe a strong positive effect of mutation bias ($\beta=0.82$, 95% bootstrap confidence interval 0.44 to 1.24; Fig. 2, bootstrap fits given by fine gray lines). Moreover, this effect of transition bias on adaptive outcome is highly significant when compared against the regression on simulated data under the null model in which transition bias has no effect on the adaptive outcome ($p=0.0003$; see also Table S3). We conclude that transitions contribute more to adaptive change in the species with a more strongly transition-biased mutation spectrum.

Mutation bias predicts bias in adaptive outcomes across taxa and mutational classes

If our results so far represent a generalizable effect of mutation bias, rather than a peculiar feature of transitions, then we expect a similar relationship between mutation bias and adaptive outcome regardless of mutation type. To explore this possibility, we re-expressed the mutational data as six separate biases for each species (one bias per class of possible single-nucleotide mutation), so that if a mutation class occurs at frequency m in the mutation spectrum and at frequency a in the adaptive spectrum then we calculate the corresponding biases as $m/(1-m)$ and $a/(1-a)$. Pooling data over all mutational classes and all species and estimating the coefficient β in Eq. 1 as before, we again found a strong positive relationship between the strength of the bias in the mutation spectrum and the strength of the bias in the adaptive spectrum ($\beta=0.71$, 95% bootstrap confidence interval 0.63 to 0.83, $p<0.0001$ under a null model where biases in mutation rate have no effect on the adaptive outcome; Fig. 3 and Table S3).

Isolating the effect of interspecific differences in mutation bias

The above analysis combines intra- and inter-species variation in mutational and adaptive spectra into a single regression. In order to conduct a regression where our coefficient β captures only the interspecific component of the signal, we fit an additional model with a separate intercept for each mutational class,

$$\log\left(\frac{a}{1-a}\right) = \beta \log\left(\frac{m}{1-m}\right) + \beta_{0,i}, \quad (2)$$

where these class-specific intercepts $\beta_{0,i}$ capture the taxon-independent bias among adaptive substitutions for or against each mutational class. As a result, in this model β captures the extent to which relative differences in mutational bias between species translate to differences in adaptive substitution bias.

Fitting this new model, we find that the mutational bias still has a strong positive association with bias in adaptive outcomes ($\beta=0.71$, 95% bootstrap confidence interval 0.53 to 0.91, $p<0.0001$ against a null model where biases in mutation rate have no effect on adaptive outcome; Fig. 4 and Table S3). Similarly, if we fit a separate regression for each mutation class individually, we again still observe a positive effect relative to the distribution of β expected under the null model (Fig. S3 and Table S3). In conclusion, when a given mutation type occurs with higher relative frequency in one species than in other species, it also tends to make a larger contribution toward adaptive protein evolution within that species.

Mutation bias predicts both sites of adaptive changes and the identity of the mutant base

Biases in the rates of different types of mutations can be influenced by two distinct aspects of the mutation process. Some nucleotide bases may tend to be more mutationally volatile than others, a phenomenon we call site bias. At the same time, mutations starting from a given base may tend to prefer certain alternate bases, which we refer to as alternate-base bias. Does the spectrum of adaptive substitutions reflect site bias, alternate-base bias, or both?

To address this question, we repeated the regression analyses in a manner that partitions the mutation spectra and adaptive spectra into site bias and alternate-base bias. Under our six-rate spectra, site bias corresponds to whether G:C or A:T sites are more likely to be changed (see Methods). We observe a relatively small (roughly two-fold) range of mutation site biases for most species, with the exception of *L. lactis*, which had a six-fold bias in favor of mutations occurring at G:C sites. Nonetheless, we observe a significant positive effect of site bias on adaptive outcome ($\beta=0.49$, 95% bootstrap confidence interval 0.16 to 0.85, $p=0.0006$ against a null model where biases in mutation rate have no effect on the adaptive outcome; Fig. S4). In contrast, alternate-base bias in mutation often varies by more than an order of magnitude between species, and we observe a strong positive effect on adaptive outcome ($\beta=0.74$, 95% bootstrap confidence interval 0.54 to 0.97, $p<0.0001$ against a six-intercept null model; Fig. S5). Thus, both site bias and alternate-base bias in mutation influence the adaptive spectrum, but the overall concordance between mutational and adaptive spectra mainly reflects alternate-base bias, due to the larger degree of interspecific variation in alternate-base bias.

Assessing the effect of taxonomic outliers

We sought to follow up on our findings by assessing their robustness to potential sources of error. From the publicly available data, we were able to pair a *de novo* mutation spectrum with a spectrum of adaptive changes for 14 species. Due to the relatively small set of species, we considered the possibility that a single outlier species might be responsible for the apparent effect of mutation bias on outcomes of adaptive evolution.

How might such an outlier affect our analysis? To address this question, we repeated the previously described regression analyses while omitting each of the 14 species one at a time. Upon removal of any given species, the regressions that incorporate information from all six mutation classes always produce positive estimates of β (Fig. S6; Table S4). Likewise, for each of the six mutation-specific regressions, the estimated value of β is consistently positive (with the one exception that $\beta = -0.097$ for A:T>C:G when *E. coli* is omitted; see Fig. S6 and Table S4). The estimated effects of site bias and alternate-base bias are also positive and qualitatively consistent upon omission of any given species. These results indicate that our main conclusions are not being driven by the data from any single species.

Assessing the effect of non-adaptive contamination

Finally, we considered whether contamination of the adaptive spectra by non-adaptive changes could impact our conclusions. Although we applied stringent filtering criteria to remove mutations for which we do not have substantial evidence of an adaptive effect (see Materials and Methods), the resulting dataset may still contain some neutral or deleterious hitchhikers. In the case of thermal adaptation in *E. coli* [23], for example, if we were to remove the criterion that all included changes must be missense mutations then four synonymous changes would have met the same criteria that were used to identify the 282 adaptive missense changes included in our study (see SI Methods). If we assume the synonymous changes to be neutral hitchhikers, their presence at 1.4% frequency (4 of 286) implies that approximately 3.6% of the missense changes are also neutral hitchhikers, given the relative rates of mutation adjusted for codon usage in *E. coli*. In contrast, zero synonymous changes would have met the same criteria that were used to identify the 202 adaptive missense changes in *S. cerevisiae*, suggesting an even lower level of contamination; likewise, in *M. tuberculosis*, all changes included in our dataset are known to confer antibiotic resistance [20].

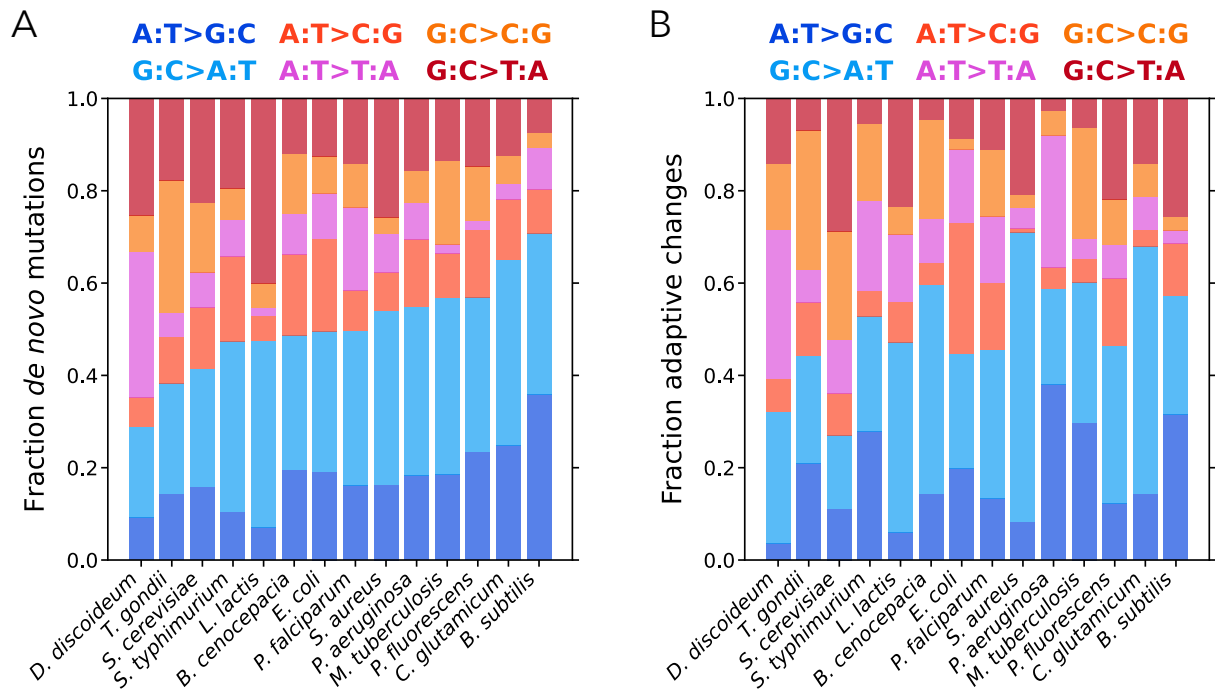


Fig. 1: Mutation spectra and spectra of adaptive substitutions by species. A) Spectra of *de novo* missense mutations by species, arranged in order of increasing ratio of transitions (cool colors) to transversions (warm colors). B) Spectra of adaptive substitutions by species, arranged to preserve the ordering of species in (A).

In order to assess the robustness of our conclusions to even very high levels of non-adaptive contamination, we considered how levels of contamination of 10%, 20%, 30% and 40% would affect our results. For each of these possible levels of contamination, we removed the corresponding percentage of mutations from the adaptive dataset before repeating our analyses. These contaminants were assumed to be drawn from the mutation spectrum, hence the data removed from each mutation class was proportional to the frequency of that class among *de novo* mutations (see Materials and Methods), representing a worst-case scenario in which contamination contributes maximally to the apparent effect of mutation bias on adaptive outcome. For the analyses that jointly considered data from all six mutation classes, our estimates of β were highly consistent and remained significant relative to the null model even under the pessimistic assumption that 40% of the dataset consists of neutral hitchhikers (Fig. S7, top three rows). Although the impact of contamination was greater upon estimating β for each mutation class individually, β remained significant for up to 10% contamination for A:T>C:G, 20% for A:T>T:A, and >40% for A:T>G:C, G:C>C:G, and G:C>T:A (Fig. S7, bottom row). Given the stringency of our data curation and the robustness of our analyses to even implausibly high levels of contamination, we conclude that hitchhiker mutations are unlikely to explain the observed relationship between mutation bias and bias in adaptive outcome.

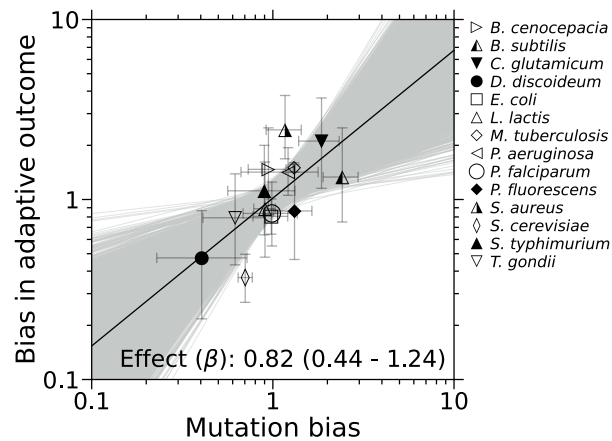


Fig. 2: Transitions contribute more toward adaptive changes in species where transitions occur at a higher *de novo* rate. For each of the 14 species in Fig. 1, the transition-transversion bias among adaptive amino acid substitutions is plotted against the corresponding bias among *de novo* missense mutations. Least-squares linear regression on log-transformed empirical data (solid black line) and 10,000 bootstrap datasets (gray lines). 95% bootstrap confidence interval for mutation bias effect, β , shown in parentheses. Significance assessed by simulating datasets ($n=10,000$) under the null model where biases in mutation rate have no effect on the adaptive outcome, $p=0.0003$.

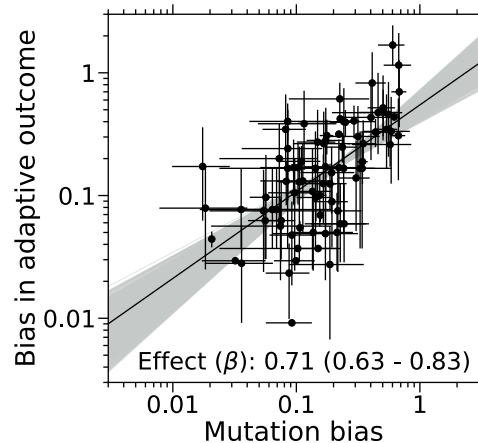


Fig. 3: Mutations that occur at a higher *de novo* rate contribute more frequently toward adaptive outcomes when combining data across taxa and mutational classes. Data points ($n=84$) represent each of the 6 single-nucleotide mutation classes in each of the 14 species from Fig. 1. Values are expressed as odds ratios for each mutation class and species. Least-squares linear regression on log-transformed empirical data (solid black line) and 10,000 bootstrap datasets (gray lines). 95% bootstrap confidence interval for the mutation bias effect, β , shown in parentheses. Significance assessed by simulating datasets ($n=10,000$) under the null model in which biases in mutation rate have no effect on the adaptive outcome, $p<0.0001$.

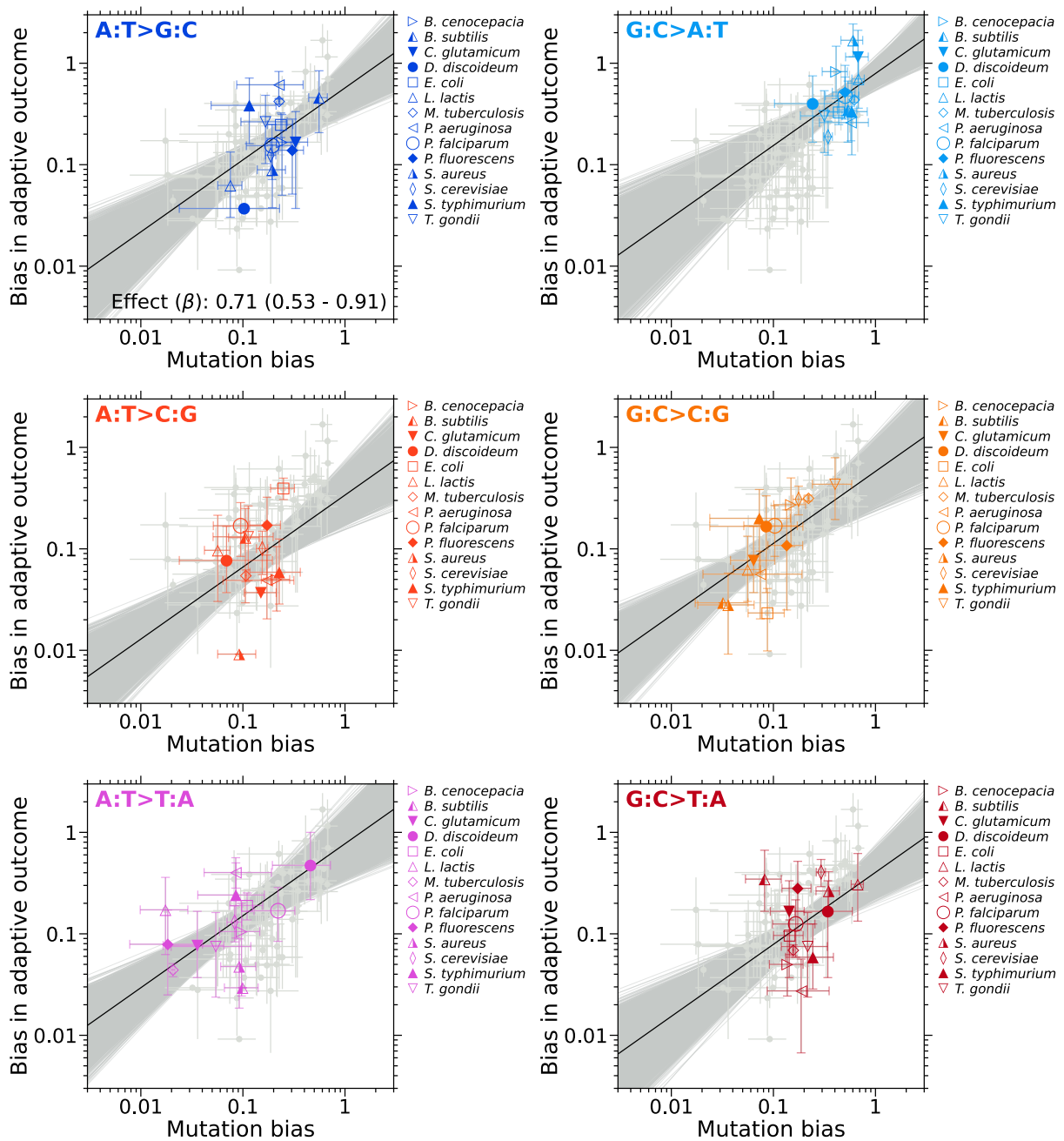


Fig. 4: Variation across species in biases in the spectra of adaptive substitutions reflects interspecific differences in mutational biases. Bias among adaptive substitutions plotted as a function of mutation bias, across 14 species, for each of the six single-nucleotide mutation classes. Regression model with single mutation bias effect parameter, β , and mutation type-specific intercepts, using log-transformed empirical data (solid black line) and 10,000 bootstrap datasets (gray lines). Effect parameter β shown in the plot for A:T>G:C (upper left) with 95% bootstrap confidence interval in parentheses. Significance assessed by simulating datasets ($n=10,000$) under the null model in which biases in mutation rate have no effect on the adaptive outcome, $p<0.0001$.

Discussion

The hypothesis that mutational biases influence the course of adaptation predicts that species with stronger mutational biases will also exhibit stronger biases among adaptive changes. Here, we tested this prediction by compiling a dataset that, for each of 14 species, pairs previously observed adaptive amino acid substitutions with an independently measured nucleotide mutation spectrum. The strength of mutational bias for or against particular types of substitutions differed by roughly an order of magnitude across species, and these differences in mutation bias were strongly predictive of the types of mutations fixed during adaptation, producing a similar type and magnitude of bias among adaptive changes. These results suggest that interspecific differences in mutation spectrum shape adaptive outcomes in a graded manner, wherein mutational biases do not merely enrich for specific mutational classes among adaptive changes, but rather produce a bias in adaptive changes that scales positively with the bias in mutational inputs.

Existing population-genetic theory allows a range of possibilities for the extent to which mutational biases influence adaptive outcomes. Direct proportionality is expected in the limit of low mutational supply [2], but higher levels of mutational supply can produce evolutionary dynamics that show anywhere from no relationship [12] to a strong positive relationship [17], depending on the details of the model (see Appendix A of [18], for example). Under some scenarios, one might even anticipate a negative relationship due to biased depletion of the distribution of fitness effects [24, 25]; that is, to the extent that early steps in an adaptive walk exhaust high-rate mutations, later steps will show a bias toward low-rate mutations. Our results suggest that adaptive evolution in the contexts examined here, specifically laboratory experiments and the natural evolution of drug resistance, mostly occurs in a regime where mutational biases do indeed have a strong influence on adaptive outcomes.

Our results also have implications for long-standing questions about the role of development in evolution [26, 27, 28, 29, 30]. Since the 1980s, it has been common to view development as primarily determining “constraints” that render some types of change inaccessible [31]. The theory of arrival biases [2, 32] suggests that development affects evolution in a different manner, where the process of generating variation (by mutation and altered developmental expression [33]) results in quantitative differences in the probability that different phenotypes are produced, and that these biases combine with biases in survival and reproduction (i.e., selection) to determine the direction of evolutionary change. Viewing mutational biases as the simplest and most easily quantified form of developmental bias, our results showing that several-fold biases in mutation rates can drive several-fold biases in adaptive outcomes suggest that other, more complex forms of developmental bias are likely to have a similar influence.

One limitation of our study is that our dataset, while unprecedented in terms of size and taxonomic breadth, is still not large enough to support analysis of more complex mutational models that include context effects, which are known to be important in work on hotspots [10, 11], cancer mutation signatures [34, 35, 36], and ordinary germline mutation [37, 38]. Another limitation is that our approach is based on correlations, and thus does not directly demonstrate a causal influence of mutational biases. However, the causal influence of mutational biases is already well-established, as has been shown by experimentally manipulating the mutation spectrum and observing the effect on adaptation [8, 11]. In principle, an alternative explanation of our results could be that selective preferences might coincide with mutational preferences, either accidentally or due to some directional process, as argued by Shapiro [39] or Svensson and Berger [40]. Notably, however, where selection coefficients for specific adaptive changes have been measured, they do not align with mutational preferences and are not sufficient to account for the observed adaptive spectra without taking mutational biases into account ([13]; see also the analyses of [41, 15] in [3]). Finally, our source data for adaptive changes is drawn primarily from short-term microbial laboratory evolution experiments, and thus these results have no direct relevance to assessing the degree to which mutational biases shape the evolution of highly polygenic quantitative traits [42, 43].

An important area for future work is the development of additional datasets comprising measurements of both selection coefficients and mutation rates across the same set of adaptive mutations [13, 41, 15]. Knowing the joint distribution of mutation rates and selection coefficients [44] would make it possible to quantitatively partition responsibility for patterns of evolutionary change between selective preferences and mutational biases [32], providing a more granular understanding of how mutational and selective forces combine to determine the genetic basis of adaptation.

Materials and methods

De novo mutation spectra

For 12 of the 14 species featured in our study, the spectra of *de novo* mutations were obtained from published mutation-accumulation studies. Raw mutation-accumulation data and genomic nucleotide composition are

each obtained from the same study in the case of *B. cenocepacia* [45], *D. discoideum* [46], *L. lactis* [47], *P. fluorescens* [48], *P. falciparum* [49], *S. typhimurium* [50], and *S. aureus* [47], For *P. aeruginosa*, raw mutation-accumulation data [51] was corrected for nucleotide composition data obtained from a separate study [52]. Raw mutation-accumulation data for *B. subtilis* [53], *C. glutamicum* [54], *E. coli* [55, 56], and *S. cerevisiae* [57, 58, 59], were each corrected for nucleotide composition obtained directly from published genome sequences: NCBI Reference Sequence NC_000964.3 (*B. subtilis*), NC_003450.3 (*C. glutamicum*), U00096.3 (*E. coli*), and GCF_000146045.2 (*S. cerevisiae*). For the remaining 2 species, *M. tuberculosis* and *T. gondii*, mutation spectra were estimated from patterns of segregating variation [12, 60], see SI Methods for details.

Raw mutation counts were corrected for genome-wide nucleotide composition to obtain estimates of the relative mutation rates for each mutational type,

$$\mu_i = \frac{N_i / f_i}{\sum_k (N_k / f_k)}, \quad (3)$$

where μ_i , N_i , and f_i are, respectively, the relative mutation rate, the raw number of observed mutations, and the relative mutational target size (the genomic fraction of AT content in the case of mutations at A:T sites or the genomic fraction of GC content in the case of mutations at G:C sites), for mutations of type i .

To obtain the mutation spectra driving protein evolution, the corrected genome-wide mutation rates were re-weighted by codon usage and the accessibility of missense mutational paths,

$$p_i = \frac{\sum_j c_j A_{j,i}}{\sum_i \sum_j c_j A_{j,i}}, \quad (4)$$

where p_i is the fraction of accessible missense paths generated by mutations of type i , c_j is the species-specific fraction of sense codon j (obtained from the Codon Usage Database [61]), and $A_{j,i}$ is the number of available missense mutations of mutation type i from codon j . The final *de novo* mutation spectrum is then given by

$$m_i = \frac{p_i (N\mu_i + 1)}{\sum_k (p_k (N\mu_k + 1))}, \quad (5)$$

where m_i is the fraction of all *de novo* missense mutations that belong to mutation type i , N is the total number of observed mutational events, and 1 is a pseudocount added to the counts for each mutation class, $N\mu_i$, to ensure that the fraction of mutations in each class is positive.

Bootstrap confidence intervals for the mutation spectra were derived by redrawing the class-specific counts $N\mu_i$ from a multinomial distribution with parameters N and m_1, \dots, m_6 and using each draw to produce a bootstrap estimate of m_1, \dots, m_6 using Eq. 5. Confidence intervals were calculated based on 10,000 bootstrap samples.

Spectra of adaptive changes

To reliably examine mutational influences on adaptive protein evolution, we sought to assemble a dataset that maximizes the inclusion of *bona fide* adaptive changes while minimizing the inclusion of neutral or deleterious hitchhikers. In addition, we restricted our dataset to taxa that meet two criteria. First, we only include taxa for which a sufficient number of adaptive substitution events are available to permit the estimation of an adaptive substitution spectrum. Second, we only include taxa for which an independently sourced *de novo* mutation spectrum is publicly available (see Section *De novo* mutation spectra). The resulting dataset consists of 5,488 adaptive amino acid substitutions across 14 species (Table S1). These data represent adaptive substitutions identified in *B. cenocepacia* [62, 63, 64], *B. subtilis* [65, 66, 67], *C. glutamicum* [68, 69, 70, 71], *D. discoideum* [72], *E. coli* [73, 74, 23, 75], *L. lactis* [76, 77, 78, 79], *M. tuberculosis* [12], *P. aeruginosa* [41], *P. falciparum* [80], *P. fluorescens* [81, 82], *S. aureus* [83], *S. cerevisiae* [84, 85], *S. typhimurium* [86], and *T. gondii* [87, 88].

To ensure that we maximized inclusion of adaptive changes while minimizing neutral mutations, we prioritized using data from experimental evolution studies, with the exception of *M. tuberculosis*, for which adaptive substitutions were obtained from published meta-analyses of antibiotic-resistance evolution [12, 20]. Amino acid substitutions identified in experimental evolution studies were evaluated on an individual basis and were included in our dataset depending on the evidence of an adaptive effect. Although the details varied by study and species, substitutions were generally included in our study if they 1) were linked to an adaptive phenotype, and 2) were implicated in the cause of the adaptive phenotype, either in follow-up experiments or due to the causal role of the affected locus, as in the case of mutations that alter the drug target during resistance evolution, for example. See SI Methods for a detailed discussion of the adaptive substitutions dataset.

The spectrum of adaptive changes is the vector of values a_i given by the formula,

$$a_i = \frac{N_i^a + 1}{\sum_k (N_k^a + 1)}, \quad (6)$$

where a_i and N_i^a are the fraction and number, respectively, of observed adaptation events of mutation class i , and the 1 is a pseudocount added to ensure that the frequency of each class of adaptive changes is positive.

Bootstrap confidence intervals for the adaptive spectra were derived by drawing $N^a = \sum_k N_k^a$ times with replacement from the collection of empirically observed adaptive changes for that species. Each such synthetic dataset was then used to produce a bootstrap estimate of the a_i using Eq. 6. Confidence intervals were calculated based on 10,000 bootstrap samples.

Statistical analysis

Interpreting Eq. 1 as a simple linear model where the log mutational bias and the log bias in adaptive outcomes are, respectively, the independent and dependent variables, we estimated the influence of mutation bias (β) and the intercept (β_0) by least squares. The 6-intercept model (Eq. 2) was interpreted similarly but fit by maximum likelihood assuming zero-mean Gaussian errors on a log bias scale, with a single shared error variance. Uncertainty was assessed via bootstrapping and significance was assessed via comparison against simulated data under the null model in which biases in mutation rate have no effect on the adaptive outcome. To estimate bootstrap confidence intervals for the parameters of these regressions, the set of adaptive changes for each species was resampled by drawing $N^a = \sum_k N_k^a$ times with replacement from the collection of empirically observed adaptive changes for that species. Based on each synthetic dataset, the a_i were then re-estimated using Eq. 6 and the regression coefficients were re-estimated. Bootstrap confidence intervals were based on 10,000 bootstrap samples.

To test the hypothesis that the magnitude of bias among adaptive outcomes depends on the strength of mutation bias, we simulated data under a null model in which biases in mutation rate have no effect on adaptive outcomes ($n=10,000$ simulations). Specifically, under the null model the expected adaptive spectrum in a given species is given by the spectrum of available missense paths; that is, the expected value of a_i is given by p_i (see Eq. 4), which is equivalent to assuming uniform mutation rates in Eq. 5. For each simulation we sampled a new dataset of the same size as our original data and re-estimated the parameters in the corresponding regression model. We define the p-value as the frequency of simulations under the null model that give $\beta_{\text{null}} \geq \beta_{\text{empirical}}$.

Site bias and alternate-base bias

The effect of variation in site bias is assessed using the log odds ratio of the identity of the wild-type base,

$$\log \left(\frac{a_{G:C>H:D}}{a_{A:T>B:V}} \right) = \beta \log \left(\frac{m_{G:C>H:D}}{m_{A:T>B:V}} \right) + \beta_0, \quad (7)$$

where B:V and H:D are the IUPAC representations for any base-pairs other than A:T and G:C, respectively. In other words, $a_{A:T>B:V}$ and $m_{A:T>B:V}$ represent the fractions of all adaptive substitutions and *de novo* missense mutations, respectively, for which the wild-type base-pair is A:T, and $a_{G:C>H:D}$ and $m_{G:C>H:D}$ represent the fractions of all adaptive substitutions and *de novo* missense mutations, respectively, for which the wild-type base-pair is G:C.

The effect of variation in alternate-base bias is assessed using the log odds ratio of each alternate base, conditioned on the identity of the wild-type base:

$$\log \left[\frac{a_i}{\sum_{r \neq i} a_r} \right] = \beta \log \left[\frac{m_i}{\sum_{r \neq i} m_r} \right] + \beta_{0,i}, \quad (8)$$

where r is any mutation class that shares the same wild-type base-pair with mutation class i .

Assessing the effect of neutral contamination in the adaptive substitutions data

For each species, we calculated the spectrum of adaptive substitutions under the assumption that the adaptive substitutions dataset is contaminated by a specified fraction of neutral mutations. The fraction of the adaptive dataset attributed to contamination ranged from 0 to 0.4 in increments of 0.1. Since our intention was to determine the robustness of our conclusions to the possibility of such contamination, we

set the spectrum for the contamination fraction to equal the *de novo* mutation spectrum. This allows us to assess the robustness of our conclusion to a worst-case scenario in which the contamination contributes maximally toward the apparent effect of mutation bias on adaptive outcomes. We specify an expected adaptive spectrum, ϵ , given a certain fraction of contamination, c , which is a mixture of two component spectra, such that,

$$\epsilon_i = m_i c + \alpha_i (1 - c), \quad (9)$$

where m_i is given by Eq. 5 and α_i is the fraction of adaptive substitutions not attributed to contamination that belong to mutation class i . We numerically calculated the spectrum of values α_i as the probability distribution that minimizes the mean squared error between the spectrum of values ϵ_i and the empirical spectrum from Eq. 6. We then repeated the regression analyses and simulations described in the previous sections, using the values α_i as the adaptive spectrum in place of the values a_i , and using $N^a(1 - c)$ as the total number of adaptive substitutions observed.

Data, Materials, and Software Availability

Model fitting, bootstrapping, and maximum-likelihood inference were conducted using a custom Python script. Code and source data are available at <https://github.com/bgitschlag/mbamta>.

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Data availability

The data and code used in this work are available from a GitHub repository (<https://github.com/bgitschlag/mbamta>).

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