Developmental Selection and the Perception of Mutation Bias

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Abstract

The notion that mutations are random relative to their fitness effects is central to the Neo-Darwinian view of evolution. However, a recent interpretation of the patterns of mutation accumulation in the genome of *Arabidopsis thaliana* has challenged this notion, arguing for the presence of a targeted DNA repair mechanism that causes a nonrandom association of mutation rates and fitness effects. Specifically, this mechanism was suggested to cause a reduction in the rates of mutations on essential genes, thus lowering the rates of deleterious mutations. Central to this argument were attempts to rule out selection at the population level. Here, we offer an alternative and parsimonious interpretation of the patterns of mutation accumulation previously attributed to mutation bias, showing how they can instead or additionally be caused by developmental selection, that is selection occurring at the cellular level during the development of a multicellular organism. Thus, the depletion of deleterious mutations in *A. thaliana* may indeed be the result of a selective process, rather than a bias in mutation. More broadly, our work highlights the importance of considering development in the interpretation of population-genetic analyses of multicellular organisms, and it emphasizes that efforts to identify mechanisms involved in mutational biases should explicitly account for developmental selection.

Key words: **developmental selection, mutation bias, evolutionary theory, cellular fitness, genetic diversity.**

Introduction

Genetic mutations are not "strongly random" events (Merlin 2016). They tend to have certain propensities (Nuño de la Rosa and Villegas 2022), which means that properties of the mutational process can complement natural selection in causing directional trends in adaptive evolution (Yampolsky and Stoltzfus 2001). For example, biases in the available genetic variation in a population might arise from a higher likelihood of certain mutation types (Payne et al. 2019), from structural properties of specific DNA sequences (Xie et al. 2019), from different rates of mutations across different regions of the genome (Hodgkinson and Eyre-Walker 2011) or the presence of mutator or antimutator mechanisms (Wielgoss et al. 2012). Although such molecular biases exist, mutations have nevertheless been traditionally regarded as random relative to their fitness effects (Wright 1932; Lenski and Mittler 1993). Recently, however, Monroe et al. (2022) reported a mutational bias in *Arabidopsis thaliana* that not only reflects molecular propensities of mutagenesis but also the importance of the biological functions of genes. Specifically, they found lower levels of genetic diversity in genomic regions with essential functions relative to nonessential functions and attributed this to a localized decrease in mutation rates caused by epigenome-guided DNA repair mechanisms. Molecular mechanisms that can direct or fine-tune mutation rates have been identified in several lineages (e.g., Odegard and Schatz 2006, Li, 2007; Schatz and Swanson 2011; Wielgoss et al. 2012), but what makes the mechanism proposed for *A. thaliana* stand out is the generalized suggestion that genes "subject to stronger purifying selection have a lower mutation rate" (Monroe et al. 2022). This claim, which we will refer to as the mutation bias hypothesis, has major implications for how the directionality of evolutionary trajectories is understood, because it implies that mutagenesis is not blind relative to its fitness outcome, at least in some lineages.

A major confounding factor of the mutation bias hypothesis is the effect of purifying selection. The reason is mutations may appear less often in essential relative to nonessential genes not because they occur at lower rates, but rather because they have more deleterious fitness effects. Indeed, selective purging causes deleterious mutations to be underrepresented in mutation accumulation experiments (Barrick and Lenski 2013). Whereas attempts were made to rule out purifying selection as a confounder at the population level (Monroe et al. 2022), we reasoned that developmental selection at the intra-organismal level offers an alternative and parsimonious explanation to the mutation bias hypothesis. As has been recognized since

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early in the history of evolutionary theory (Roux 1881; Buchholz 1922), cells can compete with their sisters in developmental contexts for resources such as nutrients, signals, and space (Bowling et al. 2019). Mutations affecting cellular fitness can therefore lead to sibling cell competition between mutant and ancestral wild-type genotypes during development (Morata and Ripoll 1975; Simpson and Morata 1981). The selection at this level results in a biased representation of certain genotypes within multicellular organisms, which can simultaneously bias the evolutionary process at the population level (Hastings 1989; Otto and Orive 1995; Otto and Hastings 1998; Majic et al. 2022). Developmental selection taking place during the formation of the germline can lead to an overrepresentation of genotypes that increase cellular fitness in the gametes, and to an underrepresentation of those that decrease cellular fitness, which can result in biases in the existing heritable genetic variation in a population's gene pool, as has been shown by both theoretical models (Hastings 1989; Otto and Orive 1995; Otto and Hastings 1998) and empirical data (Extavour and García-Bellido 2001; Cruzan et al. 2022; Tseng et al. 2022).

We hypothesize that both sources of bias, that is mutation bias and developmental selection, could have identical outcomes in terms of the overall genetic diversity that is available at the population level, and that, consequently, population-genetic parameters such as Tajima's D and dN/dS are insufficient to dismiss selection as the cause of the reduced accumulation of mutations in essential genes. Here, we test this hypothesis using a simple simulation-based model. Our results add to the ongoing discussion about the mutation bias interpretation of Monroe et al. (Monroe et al. 2022), which has met criticism from methodological points of view (Liu and Zhang 2022; Charlesworth and Jensen 2023; Wang et al. 2023). We here focus on conceptual issues concerning the interpretation of the trends in genetic diversity reported to support the mutation bias hypothesis, showing that even under the assumption of methodological correctness, selection cannot be ruled out as their cause, not only because of the presence of neutral sites on essential genes and background selection (Charlesworth and Jensen 2023) but also because of the fitness effects of mutations on the lineages of cells in which they arise.

The Effect of Developmental Selection and Mutation Bias on Tajima's *D*

In their study, Monroe et al. (2022) produced a model to predict the accumulation of mutations across the genome of *A. thaliana* based on patterns of epigenetic marks. They verified their model's predictions using genetic variation data from mutation accumulation experiments and from polymorphisms from 1,135 genomes, in which they observed enrichment of mutations both immediately upstream and immediately downstream of gene bodies (Fig. 2 in [Monroe et al. 2022]). To assess whether these trends were caused by a lower mutation rate, rather than by purifying selection, they studied the site frequency spectrum using Tajima's D statistic (Tajima 1989). Tajima's D measures the difference between the number of segregating sites in a population and the pairwise difference in the sites between the genotypes in a sample, so that

$$D = \frac{\hat{k} - \frac{S}{\sum_{i=1}^{n-1}\frac{1}{i}}}{\sqrt{\hat{V}\left(\hat{k} - \frac{S}{\sum_{i=1}^{n-1}\frac{1}{i}}\right)}}, \quad (1)$$

where S is the number of polymorphic sites in a population, n is the total number of DNA sequences in a sample and $\hat{k}$ is the average number of polymorphisms found in comparisons of every possible pair of sequences within a sample. This statistic can be used to assess the strength of purifying selection relative to neutral evolution because whereas S might be strongly affected by the existence of deleterious mutants (even if a single individual has a mutation in a given site, that site counts as polymorphic), $\hat{k}$ is robust to the presence of deleterious mutants of low frequency in the population. Thus, given equation (1), a lower D implies an enrichment of rare variants within a population, because purifying selection will make deleterious mutations less likely to increase in frequency (S increases, but not $\hat{k}$). On the other hand, a higher D is consistent with a reduced mutation rate because a "lower mutation rate causes a depletion of rare alleles" (Monroe et al. 2022) (S does not increase). In considering the natural variation in *A. thaliana*'s genomes, Tajima's D is higher in gene bodies than in intergenic regions, suggesting a lower mutation rate in gene bodies (Monroe et al. 2022). This is one of the strongest arguments for the claim that "evolution around genes in *Arabidopsis* appears to be explained by mutation bias to a greater extent than by selection" (Monroe et al. 2022). However, it was recently pointed out that this is a premature conclusion because Tajima's D can be affected by how sites under purifying selection are distributed across genes, as well as by the population structure of *A. thaliana* (Charlesworth and Jensen 2023). In addition, we argue that selection cannot be ruled out as the factor explaining the trends in Tajima's D, because this is a statistic that is based on polymorphisms at the level of populations. At this level, it is challenging to distinguish whether a mutation is not observed due to a decrease in the mutation rate or because it is eliminated by selection during the development of an organism. Therefore, we hypothesize that mutation bias and developmental selection will have the same effect on Tajima's D for a given sample because both will lead to a depletion of rare alleles in the population.

To test this hypothesis, we simulated a mutation accumulation experiment with a population of developing

multicellular organisms (Materials and Methods). Specifically, we modeled multicellular selfing diploid organisms with a binary genome of L base pairs. During the development of these organisms, which we represented as a branching process resulting in a binary tree (fig. 1A), any cell could mutate in any locus with probability μ. Each cell could then reproduce with a probability proportional to its fitness f_{cell} based on the cell's genome and a per-site selection coefficient s_i. At the end of development, two localized subpopulations of cells could produce isogametes, which combined into a single-celled seed from which the individuals of the next generation then developed. Analogous to the up/downstream versus gene body comparison carried out by Monroe et al., we assumed that the first half of the L-bp genome represents a nonessential sequence and the second half represents an essential sequence. We ran two versions of our model, one in which the essential part of the genome had a lower mutation rate based on a mutational bias b_i, but mutations were all selectively neutral ($\mu_{i\leq L/2}=\mu$, $\mu_{i>L/2}=\frac{\mu}{b_i}$, and $s_i=0$), and another in which the mutation rate was the same across the whole sequence, but mutations on the essential portion could cause a proliferative disadvantage s_i to the cell carrying the mutations ($\mu_i=\mu, s_{i\leq L/2}=0$ and $s_{i>L/2}>0$). In this way, we were able to distinguish between two scenarios: 1) how Tajima's D is affected by a lower mutation rate in essential gene bodies, as argued to support the mutation bias hypothesis (Monroe et al. 2022), and 2) how Tajima's D is affected by purifying selection on mutations in essential gene bodies during development.

After running our model, we found that genomic regions with lower mutation rates have a higher Tajima's D, as observed by Monroe et al. (Fig. 1B and 1C in Monroe et al. 2022), but we also found that, as we hypothesized, the same patterns are observed when developmental selection acts on cells with equal mutation rates across genomic regions (fig. 1B). This is true for a range of values of mutational bias and proliferative disadvantage that go from mild to strong (fig. 1C). The higher values of Tajima's D on essential regions of the genome in our model are explained by a slower accumulation of mutations, which causes a lag in the increase of the number of polymorphic sites and the average pairwise difference among sequences in a population when mutation bias or developmental selection affect essential genes (see supplementary fig. S1A, Supplementary Material online). Because the difference in Tajima's D between essential and nonessential regions is due to discrepancies in the rate of accumulation of mutations rather than discrepancies in the site frequency spectrum, this difference is transient and it would disappear if populations were to reach equilibrium. Our choice of parameters reflects the dataset used by Monroe et al. in terms of mutation rate and number of generations, because the studied population of *A. thaliana* primarily consists of individuals belonging to a lineage that expanded from a glacial refugium 20,000 years ago (Alonso-Blanco et al. 2016). As such, the slower accumulation of mutations in gene bodies relative to intergenic regions may explain the pattern of Tajima's D observed in the genomes of *A. thaliana* (Monroe et al. 2022). We note that our model is phenomenological and it omits many aspects of the real biology, evolutionary history, and demography of *A. thaliana* that can also influence the genetic diversity within the population and, therefore, the estimations of Tajima's D (see Charlesworth and Jensen 2023). Nonetheless, our results are sufficient to challenge the conclusion that mutation rates are necessarily reduced in functionally important genomic regions due to localized epigenome-guided DNA repair mechanisms because these same patterns are just as easily explained by developmental selection.

Ratios of Nonsynonymous to Synonymous Mutations in Mutation Accumulation Lines

An observation in support of the mutation bias hypothesis comes from the study of the ratio of nonsynonymous to synonymous mutations accumulated in laboratory lines of *A. thaliana* after 25 generations. Specifically, this observation showed that there is no significant difference in the ratio of nonsynonymous to synonymous mutations in two comparisons, one between the mutations accumulated de novo in the *A. thaliana* lines and a null model of random mutations, and another between the mutations accumulated on genes classified as "lethal" or "nonlethal" (Fig. 1B in Monroe et al. 2022). The support of this observation for the mutation bias hypothesis is grounded on the principle that if the observed bias was the result of natural selection, one would expect to have fewer nonsynonymous mutations relative to synonymous mutations in the mutation accumulation lines than in the null model, and on "lethal" genes than on "nonlethal" genes. Several facts need to be considered when evaluating this result. First, although the study of ratios of nonsynonymous to synonymous mutations is a traditional way of quantifying the selective pressure acting on genes, it is known that measurements such as dN/dS are problematic for evaluating selection within populations of related individuals (Kryazhimskiy and Plotkin 2008) and cases of recent accumulation of de novo mutations (Rocha et al. 2006). Differences in the ratio of nonsynonymous to synonymous mutations between distinct sets of genes (e.g., "lethal" vs. "nonlethal" as done in Monroe et al. 2022) will depend on the total number of accumulated mutations and the distribution of fitness effects of mutations on each gene, which makes this ratio unreliable for the study of selection under low mutation burden (Turajlic et al. 2019).

To explore whether the dynamics of dN/dS during mutation accumulation experiments can be used to distinguish between a scenario with developmental selection from a scenario of neutral mutation accumulation, we modified the developmental model described above to include synonymous and nonsynonymous mutations (Materials and Methods). For each generation, we

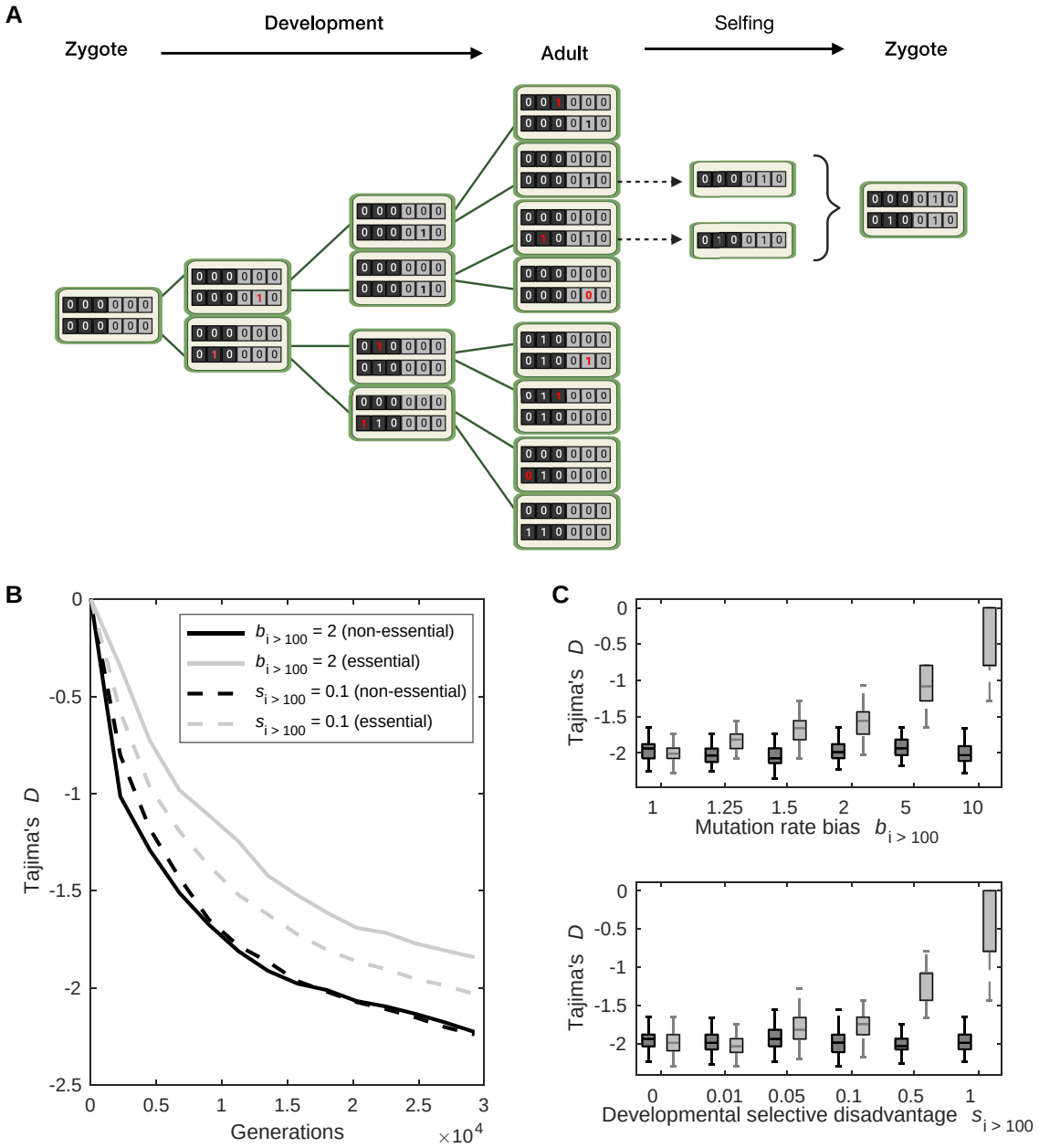

Fig. 1. (*A*) Schematic representation of our developmental model. Each cell of the developing multicellular organism has a diploid genome of length $L = 200$ bp with possible alleles 0 and 1 at each locus, which is shown here as six loci for ease of visualization. Half of each genome represents a nonessential part, shown in black, and an essential part, shown in gray. Mutations, shown in red, could happen after each round of cell division taking place during development. The developing organism reached an adult size whenever it surpassed 10,000 cells. From the total number of cells, two separate sets of 20 cells represented a germline that could give rise to the gametes. Each organism was capable of selfing by the fusion of its gametes to produce the embryo of the next generation. We used two versions of this model, one in which each cell division had a probability of reproducing defined by a fitness proportional to the developmental selective disadvantage s_i for mutations occurring in the essential portion of the genome ($i > 100$), and another in which the essential portion of the genome had a decreased mutation rate determined by a mutation rate bias b_i. In panel (*B*) we show the dynamics of Tajima's *D* in relation to the number of generations elapsed since the beginning of simulations with $b_i = 2$ (which approximates the scenario proposed by Monroe et al.) in solid lines and $s_i = 0.1$ (when a single mutation reduces cellular fitness by roughly 9%, eq. 2) in dashed lines. Gray lines indicate the dynamics of Tajima's D for an essential part of the genome, and black lines represent the nonessential parts of the genome. For all our simulations we used $F = 0.1$ and $\mu = 5 \times 10^{-10}$. Panels in (*C*) show the values for Tajima's *D* in relation to b_i (top) and s_i (bottom) after 20,000 generations. We used a range of values for b_i in which there is no mutation bias ($b_i = 1$) to a case where essential genomic regions have a ten-times lower mutation bias ($b_i = 10$). For s_i we considered a range that goes from $s_i = 0$ where there is essentially no selection, to one in which $s_i = 1$, meaning that a single mutation decreases a cell's fitness to $f_{cell} = F$. For each value of s_i and b_i, we show the Tajima's *D* statistic calculated for the nonessential (black) and essential (gray) parts of the genome. We calculated Tajima's *D* from populations of 1,135 individuals sampled from a pool of 12,000 parallel mutation accumulation simulations. The results shown correspond to 100 different samplings for each parameter combination. Note that the trends in both panels in (*C*) are qualitatively the same.

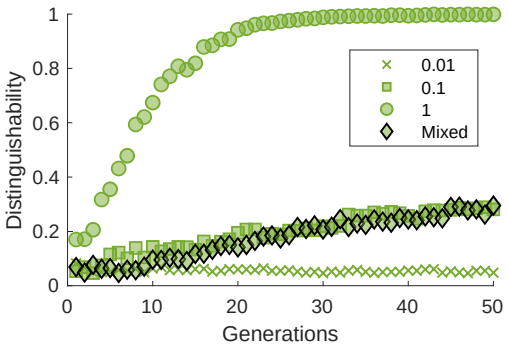

Fig. 2. Distinguishability of the *dN*/*dS* ratio of sequences evolving under neutrality and sequences evolving under developmental selection, shown in relation to the number of generations elapsed since the onset of mutation accumulation. Distinguishability refers to the fraction of the total comparisons that show a significant difference in the accumulation of nonsynonymous mutations according to a χ^2 test. Each point corresponds to a fraction of 1,000 different comparisons. Each individual comparison was based on the mutations accumulated across 100 independent simulations with $\mu = 5 \times 10^{-6}$ and $L = 3{,}200$ (Materials and Methods). The different symbols represent each of the selective regimes: green-edged symbols indicate the distinguishability for the cases in which mutations have a homogenous effect given by a single s_i (see legend), whereas black-edged diamonds indicate the mixed case, in which nonsynonymous mutations on each fourth of the sites on the essential portion of a gene produce a selective disadvantage s_i of 0, 0.01, 0.1, and 1.

estimated the distinguishability of *dN*/*dS* between cases in which mutations were strictly neutral ($s_{i>L/2} = 0$) and cases in which they were caused by a selective handicap to cells ($s_{i>L/2} > 0$). For this, we used a χ^2 test, following Monroe et al., and we defined distinguishability as the fraction of the total number of comparisons that showed a significant difference between the neutral and non-neutral sequences. Our results corroborated that the distinguishability of *dN*/*dS* not only depends on the fitness effects of mutations but also on the number of generations that elapsed since the beginning of the experiment (fig. 2*B*). For genes under stronger selective pressure, the distinguishability between the neutral and non-neutral case is the lowest ($s_{i>L/2} = 1$, circles in fig. 2*B*), which could be used to argue that a comparison of *dN*/*dS* between "lethal" and "nonlethal" genes is appropriate. However, these results come from a model in which all deleterious nonsynonymous changes have the same fitness effect given by a constant s_i. In reality, it is unlikely that nonsynonymous changes on all sites of a gene would have the same fitness effect (e.g., Firnberg et al. 2014, Orengo et al. 2015). This means that, even in the case of lethal genes, in which nonsynonymous mutations on some sites are strongly deleterious, *dN*/*dS* might only reveal a deviation from neutrality late in mutation accumulation lines. This is indeed what we observe when we include a mixture of mildly to strongly deleterious mutations on the essential portion of the genome in our model (Black-edged diamonds in fig. 2*B*, Materials and Methods). Therefore, the heterogeneity of the fitness effects of mutations across a gene can impact whether selection can be ruled out to explain the patterns observed by Monroe et al. (2022). This is not only because such heterogeneity can affect Tajima's *D*, as was recently pointed out (Charlesworth and Jensen 2023), but also because it can affect the ability of the ratio of nonsynonymous to synonymous mutations to distinguish whether a lower accumulation of mutations on essential gene bodies is due to selection or not.

Concerning the relevance of the ratio of nonsynonymous to synonymous mutations as an instrument to dismiss selection, another point to consider is that synonymous mutations are not necessarily neutral (Drummond and Wilke 2008; Lebeuf-Taylor et al. 2019; Sharma et al. 2019): synonymous mutations can affect the fitness of organisms, for example, as a consequence of codon usage biases (Akashi 1994; Frumkin et al. 2018) and their effects on translational accuracy and efficiency (Drummond and Wilke 2008; Jiang et al. 2022; Sun and Zhang 2022), protein folding (Buhr et al. 2016), and splicing disruption (Savisaar and Hurst 2018; Sharma et al. 2019). At least some of these effects are enhanced in highly transcribed genes (Frumkin et al. 2018), which may offer a selection-based explanation for why there is a lower accumulation of synonymous mutations in more highly expressed and more conserved genes in the mutation accumulation lineages (Monroe et al. 2022; Quiroz et al. 2022). Therefore, the lack of a significant difference in the ratio of nonsynonymous to synonymous mutations early in mutation accumulation experiments should not be considered strong support for a lack of selection.

Mutation Bias or Developmental Selection?

Our results challenge the assertion that the reduced accumulation of mutations on essential genes cannot be explained by a selective process and that it is necessarily the result of a bias in mutation rates. This point has also been recently presented by Charlesworth and Jensen (2023), who noted how higher values of Tajima's *D* in essential genes can result from their sequence being a mixture of neutral sites and sites affected by purifying selection. Our work adds another angle of discussion from the perspective of the developmental context of mutations and nonequilibrium dynamics of populations. We argue that 1) a higher Tajima's *D* can be interpreted as either a function-related mutation rate bias or a consequence of intra-organismal selective processes, and that 2) nonsignificant comparisons of ratios of nonsynonymous to synonymous mutations between sets of genes early in mutation accumulation experiments are not necessarily indicative of a lack of selection. Therefore, such population-genetic statistics are insufficient to explain whether fitness-related biases in the allelic diversity of populations are the result of biases in how genetic variation is generated, rather than selective biases in how variation is retained, at least without detailed knowledge of the effects of mutations on cellular fitness. This has the implication that whenever mutation rates at the level of populations are estimated or discussed, it needs to be considered

that differences in mutation rates inferred from population-genetic data are not necessarily reflecting differences in mutation rates at the molecular level, at least in multicellular organisms.

Our developmental model encompasses some essential features of *A. thaliana* biology such as the prevalence of selfing (Shimizu and Purugganan 2005) and the differentiation of the gametes based on positional information (Pagnussat et al. 2009). However, it also contains strong simplifications concerning the mode of growth of the plant. For example, we did not consider growth mediated by a shoot apical meristem, and we also omitted the consideration of a haploid growth phase of the pollen tube, which might be one of the strongest enablers of developmental selection due to the lack of mutational buffering from diploidy (Cruzan et al. 2022). Concerning this last point, our model assumed that a heterozygous cell with a deleterious mutation will have a higher fitness than a cell that is homozygous for that mutation. However, we did not account for allelic dominance, which could allow deleterious mutations to proliferate without affecting the fitness of cells. Our reasoning is that mutations to genes with basic cellular functions are likely to reduce cellular fitness, even if heterozygous because they cause half of the gene's product to be defective. For example, consider the classic case of the *Minute* mutants in *Drosophila*. These are mutations on ribosomal genes, which result in a lower rate of cell division and cause heterozygous cells to be competitively eliminated during development when they are growing as part of an embryonic mosaic together with wild-type cells (Morata and Ripoll 1975; Baker 2020). Our model also simplified molecular details that might affect the strength of developmental selection, as well as the estimation of Tajima's *D* and *dN*/*dS*. For example, our model excludes linked selection, which in the form of background selection can deplete the genetic diversity of essential genes even on neutral sites (Charlesworth and Jensen 2023), and it also omits selection between individuals, which may shape the genetic diversity of populations over thousands of generations and thus the values of Tajima's *D* depending on the mode of selection affecting each gene (e.g., Carlson et al. 2005). More faithful models of plant development could be constructed (e.g., Klekowski and Kazarinova-Fukshansky 1984, Pineda-Krch and LehtilÄ, 2002) and more realistic molecular and demographic details could be considered, however, the simplistic branching tree model we used is sufficient to illustrate our argument that developmental selection can explain the same patterns of genetic diversity that were previously attributed to a bias in mutation rates (Monroe et al. 2022).

We emphasize that our model is not sufficient to rule out the mutation bias hypothesis in favor of the developmental selection hypothesis. However, there are additional lines of evidence that favor the latter hypothesis. Whereas some analyses based on *dN*/*dS* ratios suggest that purifying selection might be weak in somatic evolutionary processes (e.g., in humans [Martincorena et al. 2017]), other studies have argued that this does not apply in the specific case of genes with essential cellular functions (Zapata et al. 2018). Additional evidence that developmental selection purges deleterious mutations come, for example, from self-pollination experiments with a species of *Mimulus*, in which many deleterious mutations are culled during development (Cruzan et al. 2022), and from experiments showing that mutations causing mitochondrial dysfunction are purged early in mouse development (Lima et al. 2021). Considering the mutations accumulated in *A. thaliana*, when Monroe et al. report the associated activities of the genes less likely to contain mutations, they include functions at the most fundamental layers of cellular processes, including translation and mRNA processing (Fig. 3a in Monroe et al. 2022). Mutations on such genes are unlikely to be buffered by zygosity and will likely cause a great handicap to a cell within a developmental context, impeding its proliferation relative to its fitter sister cells. Conversely, genes that are more likely to carry mutations are those that are relevant to ecological interactions of the plant, such as the reaction to chitin or responses to hypoxic conditions (Monroe et al. 2022), which are in principle functions of little relevance to the fitness of individual cells proliferating in a developmental context, and are thus unlikely to be purged by intra-organismal selection. In angiosperm development, shoot apical meristem cells can be replaced whenever they get excluded from their developmental niche (Burian 2021) and, therefore, it is reasonable to think that cells carrying mutations that affect fundamental cellular functions would be impaired from forming the germline—thus being less likely to be present in the gene pool of a population. Besides Tajima's *D* and estimations of ratios of nonsynonymous to synonymous mutations, another observation used by Monroe et al. to support the mutation bias hypothesis over selection is that introns show similar trends in mutation accumulation as coding sequences (Fig. 3C in Monroe et al. 2022). However, it is expected that introns of genes that are essential for basic cellular functions would also be constrained in their developmental clonal expansion if they are disruptive to appropriate protein production. This is especially true in the regions of introns flanking exons, which contain splice sites that tend to be among the most highly conserved noncoding regions of crucifer genomes (Haudry et al. 2013).

We further emphasize that although our results and interpretation offer an alternative explanation to the mutation bias hypothesis, we are not implying that the targeted reduction of mutation rates at specific loci is untenable. Indeed, there are candidate mechanisms that could be responsible for such reductions (Belfield et al. 2018; Quiroz et al. 2022). However, we do argue that such mechanisms would likely complement the filtering of deleterious mutations during development in defining the effective mutation rates at the level of populations. Because of this, we speculate that molecularly directed biases in mutation rates would preferentially evolve to affect genes that are essential for the organism, but not necessarily for

individual cells. Our reasoning is that the selective process taking place during development would suffice to impede the proliferation of deleterious mutations on genes with fundamental functions for cell physiology and reproduction, which would make any evolutionary maintenance of pathways to repair such genes redundant. For example, it may be less important to reduce mutation rates on genes involved in translation or control of cell division because these will be filtered out during development anyway, whereas it may be important to reduce the mutation rates in genes involved in signaling for leaf development, which might jeopardize organismal functioning without being filtered by selection during development. In other words, developmental selection would decrease the selective advantage of evolving and maintaining repair mechanisms targeting genes affecting cellular fitness, thus bringing such mechanisms closer to the so-called "drift barrier" (Lynch 2010; Lynch et al. 2016). We, therefore, suggest that a more detailed study of the mutational process in developing organisms is required to determine whether deleterious mutations actually occur at lower rates, whether they are filtered out by developmental selection, or whether they are affected by both mechanisms.

Overall, our argument highlights the importance of development in defining evolutionary trajectories. Its importance not only stems from phenotypic biases and plasticity (Maynard Smith et al. 1985; Pigliucci et al. 2006; Laland et al. 2015; Uller et al. 2018), but also from the multilevel nature of evolutionary and developmental processes, which help define the genotypic diversity existing in a population. Although developmental selection and biased mutagenesis would have the same consequences for population-genetic statistics such as Tajima's D, the source of variation has fundamentally different implications for the workings of evolution. We thus emphasize that, just like it is important to consider population dynamics of bacterial populations to estimate their mutation rates properly (Frenoy and Bonhoeffer 2018), it is essential to consider development in population-genetic analyses that aim to infer mutation rates and patterns of natural selection of multicellular organisms.

Materials and Methods

Our model simulated parallel mutation accumulation experiments of selfing diploid organisms with multicellular development. In each generation, organisms developed from a single cell containing two sets of chromosomes of length $L = 200$ loci that in our baseline model could take the allelic values 0 or 1. Simulating size-dependent cessation of growth (Hariharan 2015), we stopped the development of each organism when it surpassed a final size threshold of 10^4 cells, which typically occurred after 14 developmental steps ($2^{14} = 16{,}384$ cells). At each developmental step, mutations occurred with probability $\mu = 5 \times 10^{-10}$ per cell per locus. These events represent all sources of point mutations, such as those associated with replication infidelity or DNA damage. After 14 developmental steps, this yielded an expected generational mutation rate of approximately 7×10^{-9} per locus, which is close to empirical estimations for *A. thaliana* (Weng et al. 2019; Belfield et al. 2021). At each developmental step, each cell could also divide with a probability equal to its fitness f_{cell}, which we calculated as:

$$f_{cell} = F + (1 - F)\left(\prod_{i=1}^{L}(1 - s_i g_i)\right), \qquad (2)$$

where g_i is the binary genotypic value of the i^{th} locus (0 or 1), and s_i is the selective disadvantage for the cell with a mutation on the i^{th} site. We initialized each simulation with a wild-type genome represented as a vector in which all sites have allele 0. Therefore, acquiring a 1 allele represents a mutation that arose during the development of an organism in the mutation accumulation experiment. We only considered mutations to be either deleterious or neutral for a cell based on values of s_i between 0 (neutral) and 1 (strongly deleterious so that a single mutation leads to $f_{\text{cell}} = F$). We ran two versions of our model, one in which loci $i > 100$ had a lower mutation rate based on a mutational bias b_i and mutations were all selectively neutral, such that $\mu_{i \leq 100} = \mu$, $\mu_{i>100} = \frac{\mu}{b_i}$, and $s_i = 0$, and another in which the mutation rate was the same across the whole sequence, but mutations on loci $i > 100$ decreases cellular fitness, meaning that $\mu_i = \mu$, $s_{i \leq 100} = 0$, and $s_{i>100} > 0$. In a single adult organism, two specific subsets of 20 cells represented the cells capable of forming complementary isogametes. Defining the germline based on the specific location of cells in the body of the organism captures the mode by which gamete differentiation is defined in *A. thaliana* based on positional cues (Pagnussat et al. 2009). To produce the zygotic genome for the next generation, we combined two randomly chosen L-bp long genomes from single randomly chosen male and female gametes from the same individual. Note that in our model there is no selection above the cellular level. That is because all organisms can develop to adulthood and reproduce, due to the first term in equation (2), F. Having $F > 0$ guarantees a minimum probability of dividing for each cell, even if a cell harbors multiple deleterious mutations. This scenario is motivated by cases such as mutations on genes coding for γ-tubulin complex proteins that impair cell division but still allow for viable, albeit smaller, individuals in *Arabidopsis* (Miao et al. 2019). There is therefore no selection at the level of populations in our simulations. Each simulated mutation accumulation experiment consisted of 30,000 generations, and we ran 12,000 replicates for every parameter combination. We calculated Tajima's D using equation (1) by randomly sampling 1,135 of the final zygotic genomes from those 12,000 replicates. Because we initialized each replicate simulation with the same genotype, our model reflects an expansion from an entirely homozygous population with a small effective population size.

To study the dynamics of dN/dS during mutation accumulation experiments we adapted our model to

also account for synonymous and nonsynonymous mutations. This version of the model had four possible allelic values, 0, 1, 2, and 3. We also included a translation step during the calculation of f_{cell}, such that a given genome g encoded a peptide p. At this step, when $g_i = 0$ or 1, $p_i = 0$, and when $g_i = 3$ or 4, $p_i = 1$. To calculate f_{cell}, we simply replaced g_i for p_i in equation (2). We also adjusted the parameterization of our model to more closely resemble the dN/dS analysis performed by Monroe et al., who calculated dN/dS using mutations accumulated after 25 generations across 2,700 "lethal" genes, which had an average length of ~1600 bp. As our model only includes a single gene, we increased the mutation rate 2,000-fold to $\mu = 1 \times 10^{-6}$ per-locus per-cell division and increased the number of loci to $L = 3{,}200$ (1,600 nonessential, 1,600 essential), running our simulation for 50 generations. Starting also from an initial genome composed entirely of the 0 allele, at each generation, we counted the number of synonymous ($g_i = 0 \rightarrow g_i = 1$) and nonsynonymous ($g_i = 0 \rightarrow g_i = 2$ or 3) changes. We ran a total of 5,000 replicates for each parameter combination. For each generation, we estimated dN/dS by considering the zygotic genome resulting from each generation of R randomly selected replicates such that

$$dN/dS = \frac{\sum_{r=1}^{R} N_{t,r}}{n \sum_{r=1}^{R} S_{t,r}}, \quad (3)$$

where $N_{t,r}$ and $S_{t,r}$ are the total of nonsynonymous and synonymous mutations, respectively, accumulated up until generation t in replicate r. The variable n captures the number of possible nonsynonymous changes relative to the possible synonymous changes ($n = 2$ for our model). For this adapted model we only considered the case in which the essential portion of the genome was affected by developmental selection ($\mu_i = \mu$, $s_{i \leq 1600} = 0$, and $s_{i>1600} > 0$). We ran simulations in which the entire length of the essential portion of the genome was affected by a constant s_i, with values 0.01, 0.1, and 1, and simulations in which the fitness effect of deleterious nonsynonymous mutations was mixed, meaning that each quarter of the sites had a s_i equal to 0, 0.01, 0.1, or 1. We performed 1,000 calculations of dN/dS, each of which used $R = 100$ (for each calculation we subsampled 100 from the total 5,000 simulations, to correspond with the analysis of Monroe et al. (2022), who calculated the ratios of nonsynonymous to synonymous mutations accumulated across 100 replicate lines). In each calculation, recurrent mutations are possible and, in such cases, each mutation was considered separately for the estimations of $N_{t,r}$ and $S_{t,r}$. Because there is no phylogenetic relationship between different replicates, considering recurrent mutations independently is appropriate for describing how synonymous and nonsynonymous mutations accumulate on a sequence, which is the aim of our analysis. To explore to what extent the neutral and non-neutral cases can be distinguished based on dN/dS in each of these 1,000 calculations, we used a χ^2 test to compare the accumulation of nonsynonymous mutations in sequences under non-neutrality ($s_{i>1600}$) and sequences under neutrality ($s_{i \leq 1600}$) at each generation of our simulations. We measured the distinguishability of the neutral and non-neutral cases as the fraction of those comparisons that yielded a significant P value ($P < 0.05$).

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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Data Availability

Code for this article will be available from Zenodo (https://doi.org/10.5281/zenodo.6538447).

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