The rate and molecular spectrum of mutation are selectively maintained in yeast

Haoxuan Liu¹ & Jianzhi Zhang

What determines the rate (μ) and molecular spectrum of mutation is a fundamental question. The prevailing hypothesis asserts that natural selection against deleterious mutations has pushed μ to the minimum achievable in the presence of genetic drift, or the drift barrier. Here we show that, contrasting this hypothesis, μ substantially exceeds the drift barrier in diverse organisms. Random mutation accumulation (MA) in yeast frequently reduces μ, and deleting the newly discovered mutator gene PSP2 nearly halves μ. These results, along with a comparison between the MA and natural yeast strains, demonstrate that μ is maintained above the drift barrier by stabilizing selection. Similar comparisons show that the mutation spectrum such as the universal AT mutational bias is not intrinsic but has been selectively preserved. These findings blur the separation of mutation from selection as distinct evolutionary forces but open the door to alleviating mutagenesis in various organisms by genome editing.
Mutation is the ultimate source of all genetic variations, including those driving adaptations and those causing hereditary diseases. Therefore, the mutation rate per nucleotide per generation ($\mu$) and its evolution are of broad relevance and interest. Because the vast majority of mutations are deleterious, Sturtevant famously asked in 1937 why $\mu$ has not been reduced by natural selection to zero. While he sighed that "no answer seems possible at present," much progress has been made in the intervening years. It is now recognized that an organism’s $\mu$ is jointly determined by its genotype and environment and is subject to natural selection, and that the selection can arise from three factors: deleterious mutations, beneficial mutations, and the cost of fidelity. Deleterious mutations reduce organismal fitness, leading to the selective fixations of mutation rate modifiers that lower $\mu$ and a decrease of $\mu$ (Fig. 1a). By contrast, beneficial mutations raise organismal fitness, leading to the selective fixations of mutation rate modifiers that increase $\mu$ and an elevation of $\mu$ (Fig. 1a). In these two selections, the fitness effect of the modifier lies entirely in the mutations created and linked with the modifier, so the modifier is subject only to the so-called second-order selection. The cost of fidelity refers to the fitness cost due to the energy and time spent on proofreading, repair, and other biological processes that reduce $\mu$. Hence, the cost of fidelity creates a first-order selection for mutation rate modifiers that increase $\mu$, resulting in an uplift of $\mu$ (Fig. 1a). Therefore, a nonzero $\mu$ can result from a balance between the respective selections for lower and higher $\mu$. We will refer to this answer to Sturtevant’s question as the conventional model.

In the last decade, however, an alternative model termed the drift–barrier hypothesis (DBH) has emerged as the prevailing hypothesis of mutation rate evolution. The DBH considers only the first of the three selections aforementioned and regards the selections for higher $\mu$ negligible. The DBH posits that, as $\mu$ diminishes, the selective benefit of a given fractional reduction of $\mu$ also diminishes; eventually, the benefit becomes so weak relative to genetic drift that $\mu$ can no longer descend, especially when mutations are biased toward creating modifiers that increase $\mu$. 

---

Fig. 1 Theoretical framework of mutation rate evolution and the study design. a Three selective forces (arrows) potentially drive the evolution of the mutation rate $\mu$. 1° and 2° represent first-order and second-order selections, respectively. Beneficial mutations and cost of fidelity induce selections for higher $\mu$, while deleterious mutations induce a selection for lower $\mu$. If the selections raising $\mu$ are negligible, $\mu$ is pushed to the red dotted line that represents the drift barrier; otherwise, $\mu$ is at the green dotted line that is well above the drift barrier. Predicted relationships in $\mu$ among the drift barrier (red line), progenitor for mutation accumulation (MA) (orange line), and multiple resultant MA lines (gray circles), under the drift barrier hypothesis (b) or the conventional model in which a balance between opposing selections maintains $\mu$ well above the drift barrier (c). d Study design. WGS, whole-genome sequencing.
The minimal $\mu$ achievable by selection against deleterious mutations in the presence of drift is known as the drift barrier.

Which of these competing hypotheses provides the right answer to Sturtevant’s question? While a key prediction of the DBH has been validated (see “Discussion”), it is unknown whether the selections for higher $\mu$, which have received empirical support (see “Discussion”), are so inconsequential that $\mu$ approaches the drift barrier. Because $\mu$ would be subject to stabilizing selection under the conventional model but directional selection under the DBH, it is possible to distinguish between them by evaluating the type of selection acting on $\mu$. Another key aspect of mutation is its molecular spectrum, defined by the relative rates of different mutation types. Mutation spectrum could affect the severity of mutations and influence adaptation\(^\text{17}\), but whether the mutation spectrum itself is subject to natural selection is unknown.

In this work, we use the budding yeast Saccharomyces cerevisiae as a model to assess selections in the evolution of mutation rate and spectrum. We show that yeast’s mutation rate is maintained well above the drift barrier by stabilizing selection and that its mutation spectrum has also been shaped by selection.

**Results**

**Mutagenesis frequently reduces $\mu$.** The type of selection acting on a trait can be inferred from its phenotypic change upon the removal of the selection. If $\mu$ has been selectively minimized to the drift barrier, evolution in the absence of selection should generally cause a rise in $\mu$, although occasional small reductions in $\mu$ cannot be excluded\(^\text{18}\) (Fig. 1b). By contrast, if the mutation rate is selectively maintained well above the drift barrier, upon the removal of selection, the probabilities for $\mu$ to go up and go down are both substantial (Fig. 1c). Following this logic, we initiated 96 mutation accumulation (MA) lines from a commonly used laboratory yeast strain, in which the mismatch repair gene $\text{MSH2}$ had been deleted to speed up the accumulation of mutations (see “Methods”). Each MA line went through on average 1511 mitotic generations in the presence of drift is known as the drift barrier.

**Discussion**

MSH2-excluded them (marked with stars in Fig. 2a) from all the mismatch repair gene.

Furthermore, 10 of the 13 lines with significantly decreased $\mu$ had $\mu$ reduced by at least 50%, while the remaining three lines had $\mu$ reduced by 40% to 43% (Fig. 2a). That over 40% of MA lines with significantly altered $\mu$ exhibit such drastic reductions in $\mu$ is consistent with the DBH, because when $\mu$ is near the drift barrier, mutations are expected to be strongly biased toward increasing $\mu$ and are not expected to cause such large reductions of $\mu$ so frequently (Fig. 1b). Our finding suggests that the progenitor’s $\mu$ is well above the drift barrier (Fig. 1c). We found a significant positive correlation between $\mu$ and the number of mutations accumulated during the MA, but $\mu$ was not significantly correlated with the growth rate of the MA line (Supplementary Fig. 2).

Because the above estimation of $\mu$ was based on loss-of-function mutations in one gene, we attempted to verify these results by performing another round of MA followed by WGS in 16 of the above 49 MA lines as well as the progenitor (and a diploid version of the progenitor), all with an intact $\text{MSH2}$ (Fig. 1d). 4–20 parallel lines were established from each strain, and on average $8.94 \times 10^5$ generations of MA were performed in the medium similar to that used in the fluctuation test (Supplementary Table 2, Supplementary Data 1, see “Methods”). Four of the 16 MA lines were apparently diploid, because the majority of the mutations observed in MA + WGS were in heterozygous state. Diploids should not produce mutant colonies in the fluctuation test. To be conservative in inferring mutation rate reductions in the first round of MA, we additionally regarded a line that was not subject to MA + WGS but had only two mutant colonies in the fluctuation test as putatively diploid (right most line marked with a star in Fig. 2a). We excluded these five diploid lines from all CAN1-based analyses. Because haploid and diploid progenitors showed similar mutation rates (Fig. 2b, c), all 16 lines with MA + WGS were included in analyses based solely on MA + WGS. The MA + WGS results were generally consistent with those from the fluctuation test. For instance, compared with the progenitor, all eight lines with higher CAN1-based $\mu$ exhibited higher MA + WGS-based SNV (Fig. 2b) or indel (Fig. 2c) rates. Among the four lines with lower CAN1-based $\mu$, three exhibited significantly lower MA + WGS-based SNV or indel rates (Fig. 2a). CAN1-based $\mu$ is significantly correlated with both the MA + WGS-based SNV rate ($r = 0.88$, $P = 8.9 \times 10^{-5}$; Fig. 2d) and the MA + WGS-based indel rate ($r = 0.78$, $P = 1.6 \times 10^{-3}$; Fig. 2e), although the latter correlation is weaker than the former. This observation is not unexpected given that most loss-of-function mutations in CAN1 are SNVs instead of indels\(^\text{22}\).

**Stabilizing selection of $\mu$.** The above analysis strongly suggests that $\mu$ is not selectively minimized to the drift barrier in the progenitor. To assess the selective forces acting on $\mu$, we took advantage of published CAN1-based $\mu$ estimates from seven natural yeast strains of diverse origins\(^\text{14}\) (Supplementary Table 3, Supplementary Fig. 3). For a neutrally evolving trait, the ratio of its genetic variance among natural strains of a species ($V_g$) to the mutational variance generated by mutations per generation ($V_m$) is expected to equal 4$N_e$ in primarily asexual diploids such as $S.\ ceresiviae$\(^\text{23}\). By contrast, stabilizing selection would reduce $V_g$ and render $V_g/V_m$ smaller than 4$N_e$. Because $\mu$ is not normally distributed among the MA lines, we first log$_{10}$-transformed $\mu$ (Fig. 2a) before computing $V_m$ and $V_g$ although the results are not qualitatively different without the transformation (Supplementary Table 4). We estimated $V_g$ of $\mu$ from the seven natural strains. To estimate $V_m$ that is comparable with $V_g$, we used the CAN1-based $\mu$ estimates from the 44 haploid MA lines, but corrected for the increased mutagenesis in the MA induced by deleting $\text{MSH2}$. We employed three corrections by respectively assuming that deleting $\text{MSH2}$ caused the same fold change in the rate of each mutation type as the observed fold change of the total rate of
SNVs and indels ($V_{\text{m1}}$), as that of indels ($V_{\text{m2}}$), and as that of SNVs ($V_{\text{m3}}$). Because deleting MSH2 increased the indel rate much more than increasing the SNV rate (see “Methods”), among the three $V_m$ values, $V_{\text{m1}}$ is probably the closest to the truth, while $V_{\text{m2}}$ is underestimated and $V_{\text{m3}}$ is overestimated. Hence, $V_{\text{m3}}$ and $V_{\text{m2}}$ allow determining the lower and upper bounds of $V_{\text{m}}/V_m$ respectively.

We found $V_{\text{m}}/V_m$ to be at least 540 times lower than the neutral expectation of $4N_c \approx 4 \times 10^7$ (see “Methods”), regardless of the particular $V_m$ used (Table 1), indicating strong stabilizing selection of $\mu$. This signal of stabilizing selection is not an artifact of the physical limits of $\mu$, because the range of $\mu$ among the natural strains is even smaller than that of the MA lines (Fig. 2a).

To investigate whether the stabilizing selection prohibits the evolution of higher $\mu$, lower $\mu$, or both, we separated $V_m$ into two components that respectively measure the variance of $\mu$ created by mutations decreasing $\mu$ ($V_{\text{mL}}$) and increasing $\mu$ ($V_{\text{mH}}$). If there is no selection against a reduction in $\mu$, $V_{\text{g}}$ should be at least as large as $4N_cV_{\text{mL}}$. However, we found $V_{\text{g}}$ to be at least 300 times lower than $4N_cV_{\text{mL}}$ (Table 1), indicating the action of selection prohibiting a reduction of $\mu$ in evolution. Similarly, $V_{\text{g}}$ was at least 230 times lower than $4N_cV_{\text{mH}}$ (Table 1), indicating the action of selection prohibiting a rise of $\mu$ in evolution. In the above tests,
the smallest difference observed between $V_g$ and a neutral expectation was 230 times, based on $V_{m2}$ that corresponds to a conservative test. Therefore, it is exceedingly unlikely that our test results are due to confounding factors such as mutation spectrum differences between wild-type and $MSH2$-lacking strains or the inaccuracies of $V_m$, $V_g$ and $N_e$ estimates (see “Methods”). Together, the above results demonstrate that $\mu$ has been selectively maintained at an intermediate level in $S. cerevisiae$. Note that the selective forces to increase and to suppress $\mu$ are not equally strong, because the mean $\mu$ of the MA lines is higher than the progenitor ($P = 4.3 \times 10^{-3}$ for CAN1-based $\mu$, $t$-test; $P = 5.7 \times 10^{-3}$ for WGS-based SNV rate, $t$-test).

To examine if the above finding extends beyond the species concerned, we examined the evolution of $\mu$ in the divergence between $S. cerevisiae$ and its sister species $S. paradoxus$. $S. paradoxus'$ SNV rate was recently estimated by MA + WGS to be $7.27 \times 10^{-11}$ per site per generation$^{24}$, about one third that of $S. cerevisiae^{15}$. Under neutral evolution, the squared difference in mutation rate between the two species ($D^2$) should equal $V_m$ times the number of generations separating the two species ($T$)$^{25}$, which we have estimated to be $2.89 \times 10^9$ (see “Methods”). We obtained $V_m$, based on the 16 MA lines with MA + WGS-based estimates of SNV rates and corrected the impact of deleting $MSH2$ as in the above analysis. We found $D^2/V_m$ to be at least 4000 times smaller than $T$ (Table 1). We also respectively estimated $V_{ml}$ and $V_{mH}$ using the 16 MA lines with MA + WGS-based estimates of $\mu$. Again, we found $D^2/V_{ml}$ to be at least 400 times smaller and $D^2/V_{mH}$ at least 2400 times smaller than $T$ (Table 1), demonstrating selection against lowering as well as increasing $\mu$ in the divergence of $Saccharomyces$ species. The above conclusion holds irrespective of whether the SNV rates are log$_{10}$-transformed (Table 1) or not (Supplementary Table 4).

$\mu$ is well above the drift barrier in diverse organisms. To directly confirm that $\mu$ is maintained above the drift barrier, we estimated the drift barriers for a diverse set of organisms including $S. cerevisiae$ (see “Methods”). The drift barrier is commonly considered in terms of the number of mutations per functional genome per generation ($U$), which equals $\mu G$, where $G$ is the size of the functional genome or the number of nucleotides where mutations would be subject to selection. Although the drift barrier varies by the parameters assumed, our estimates were based on the best available information that was also used in the formulation of the BDH$^{18}$. In every species examined, $U$ is substantially higher than the drift barrier, often by one to several orders of magnitude (Table 2). For example, $S. cerevisiae$’s $U$ is over 3000 times the estimated drift barrier.

Table 1 Test of stabilizing selection of the mutation rate in yeast.

| Species       | $V_m$             | $V_{ml}$         | $V_{mH}$         |
|---------------|-------------------|------------------|------------------|
| $V_{m1}$      | $4 \times 10^{-6}$| $1.9 \times 10^{-5}$| $1.4 \times 10^{-6}$|
| $V_{m2}$      | $2.2 \times 10^{-5}$| $2.4 \times 10^{-4}$| $2.6 \times 10^{-5}$|
| $V_{m3}$      | $3.4 \times 10^{-7}$| $3.7 \times 10^{-6}$| $3.1 \times 10^{-4}$|

Note that estimating the drift barrier requires knowing $N_e$, which is typically inferred from the synonymous nucleotide diversity under the assumption that synonymous mutations are neutral (see “Methods”). If synonymous mutations are overall slightly deleterious as has been suggested$^{26}$, $N_e$ would have been underestimated and drift barrier overestimated, rendering the true difference between the observed $U$ and the drift barrier even larger than that shown in Table 2. In other words, our conclusion based on Table 2 is conservative.

Table 2 Observed SNV mutation rates per functional genome per generation and the corresponding drift barriers of model organisms.

| Species              | $N_e$ | $D^2$ | $V_g$ | $U$ |
|----------------------|-------|-------|-------|-----|
| Escherichia coli     | $4 \times 10^8$ | $4 \times 10^{-7}$ | $8.3 \times 10^{-4}$ |
| Bacillus subtilis    | $6 \times 10^{-7}$ | $6 \times 10^{-6}$ | $1.2 \times 10^{-3}$ |
| Saccharomyces cerevisiae | $1 \times 10^7$ | $5 \times 10^{-7}$ | $1.7 \times 10^{-3}$ |
| Schizosaccharomyces   | $1 \times 10^7$ | $1 \times 10^{-6}$ | $1.6 \times 10^{-3}$ |
| pombe                | $4 \times 10^7$ | $3.8 \times 10^{-5}$ | $3.8 \times 10^{-2}$ |
| Chlamydomonas reinhardtii | $4 \times 10^5$ | $3.8 \times 10^{-3}$ | $2.9 \times 10^{-1}$ |
| Arabidopsis thaliana | $4 \times 10^5$ | $1 \times 10^{-5}$ | $1.9 \times 10^{-1}$ |
| Drosophila melanogaster | $1 \times 10^5$ | $1.5 \times 10^{-2}$ | $7 \times 10^{-1}$ |
| Mus musculus         | $4 \times 10^7$ | $1.5 \times 10^{-1}$ | $2.9 \times 10^{-1}$ |

See “Methods” for references and drift barrier estimations.
confirmed it by additional replications \( (P = 0.0095, \text{Wilcoxon rank-sum test;} \text{Fig. 3b}) \). That removing PSP2 reduces \( \mu \) by 42\% (Fig. 3b) suggests that it is a major mutator gene. PSP2 (polymerase suppressor 2) was originally discovered from a screening of rescuers of heat-sensitive mutations in \( \text{POL1} \) and \( \text{POL3} \), which encode the catalytic subunit of DNA polymerase I and \( \delta \), respectively.\(^\text{28} \). PSP2 is an RNA-binding protein and promotes P-activity in increasing \( \mu \) (Fig. 3e), suggesting that PSP2 regulates \( \mu \) through RNA binding but not protein interaction.

**Mutation spectrum has been shaped by selection.** To investigate the potential role of natural selection in shaping yeast’s mutation spectrum, we compared the variance \( (V_g) \) in a component of the mutation spectrum among five divergent natural yeast strains having published MA + WGS data (Supplementary Fig. 3), with the corresponding mutational variance per generation estimated from the 16 MA lines with MA + WGS data. Because haploid and diploid progenitors show similar mutational spectrums (Fig. 4), we analyze the MA lines and natural strains regardless of their ploidy. Even under the most generous calculation, \( V_g/V_m (3.07 \times 10^4) \) of the proportion of mutations that are SNVs is orders of magnitude smaller than the neutral expectation of \( 4 \times 10^7 \) (Supplementary Table 6). In fact, the variance of the proportion of SNVs is much greater than those separating the MA lines even after the correction for the increased mutagenesis of MA lines induced by deleting \( \text{MSH2} \). Similar results were found regarding the proportion of insertions \( (\text{maximal } V_g/V_m = 3.57 \times 10^5) \) and that of deletions \( (\text{maximal } V_g/V_m = 3.27 \times 10^4) \) (Fig. 4a, Supplementary
The second bias, known as the AT mutational bias, refers to the observation that GC → AT mutations outnumber AT → GC mutations. The universality of this bias across all species examined has led to the belief that it arises from the chemical nature of DNA irrespective of variations in replication and repair mechanisms. We found that, in one MA line, the ratio of the number of GC → AT mutations to the number of AT → GC mutations is significantly different from that in the progenitor, and is reversed from >1 to <1 (Fig. 4d). Clearly, the AT mutational bias is subject to genetic control and is not a chemical necessity. Furthermore, \( V_f / V_m \) for the AT mutational bias is at least 120 times lower than the neutral expectation (Supplementary Table 6), indicating that the bias has been maintained by stabilizing selection. Stabilizing selection on mutation spectrum is evident regardless of whether we log10-transform the original trait values (Supplementary Table 7) or not (Supplementary Table 6).

**Discussion**

Consistent with the prediction of the DBH, a strong negative correlation between \( U \) and \( N_e \) was previously observed across diverse organisms, but several considerations suggest that the actual correlation is likely substantially weaker (see "Methods"). Regardless, our finding in yeast that \( \mu \) is selectively maintained well above the drift barrier refutes the DBH and suggests the presence of the first- and/or second-order selection for higher \( \mu \) (Fig. 1a). Indeed, experiments in *Escherichia coli* found that genotypes with relatively high \( \mu \) often outcompete those with relatively low \( \mu \) in 100 generations of evolution despite their lack of difference in fitness prior to
the evolution. Similarly, a S. cerevisiae mutator strain surpassed the wild-type in ~250 generations of evolution in large but not small cultures. In Lenski’s 50,000-generation E. coli experimental evolution in a low-glucose environment, 6 of 12 populations increased in μ and they adapted faster than the other 6 populations. These observations support that modifiers raising μ can be fixed as a result of second-order selection. Furthermore, under certain conditions, the optimal U resulting from the two opposing second-order selections (Fig. 1a) is predicted to decline with Nc in asexualls (Supplementary Fig. 4, see “Methods”), which could partially explain the reported negative correlation between U and Nc. Nonetheless, the predicted optimal U given Nc appears lower than the corresponding observed U (Supplementary Fig. 4). Furthermore, even in asexualls, the second-order selection for higher μ is episodic depending on the environment and the frequency of beneficial mutations, so μ likely fluctuates under its influence. In sexuals, this selection is expected to be ineffective, because the mutation rate modifier becomes quickly unlinked with the beneficial mutation created and loses its selective advantage.

Methods

Strains and genetic manipulations. We knocked out the MSH2 gene from the haploid BY4741 strain of S. cerevisiae, referred to as the progenitor, by homologous recombination with KanMX, followed by selection on YPD (1% yeast extract, 2% peptone, and 2% dextrose) plates with 0.5 μL G418. The knockout of MSH2 was confirmed by Sanger sequencing. This MSH2-lacking strain was used to initiate the first round of MA. Upon the completion of the MA, MSH2 was inserted back to the resultant strains using CRISPR-Cas9 genome editing. Specifically, the wild-type MSH2 from BY4741 was used as the repair fragment, and three different guide RNAs targeting KanMX were used. Transformation was performed in each MA line for up to three times and was confirmed by Sanger sequencing of the reinserted locus. Restoration of MSH2 was successful in only 60 of the 93 MA lines, probably due to reduced transformation efficiencies in the MA lines.

Four candidate mutator genes (RAD5, YDL031W-A, PSP2, and MSH4) and three segments of PSP2 were also respectively removed from the progenitor (with intact MSH2) using CRISPR-Cas9. The start (or stop) codon was left unchanged when we removed the DNA of the N-terminal (or C-terminal) segment of PSP2. Primers used in this study can be found in Supplementary Table 8.

MA and whole-genome sequencing. The strategy of two rounds of MA was previsualized in animals as a return of the mutation spectrum. In the first round of MA of our study, 96 parallel lines were established from the BY4741 without MSH2. Cells were propagated at 30 °C on YPD plates. A single-cell bottleneck was applied to each line every 48 h, where a randomly picked average-size colony was streaked onto a new plate. Each line went through a total of 80 bottlenecks, and 93 of them survived in the end (the number of generations of each line went through was estimated by the number of generations between bottlenecks multiplied by the number of bottlenecks. We estimated the number of generations between bottlenecks by counting the number of cells in an average-size colony and assuming exponential growth, and took the average of the estimates prior to and after MA. The Nc of an MA line equals the harmonic mean of the number of cells per generation. In our experiment, the average between-bottleneck number of generations is 21, so Nc equals 21/(1/1 + 1/2 + 1/22 + ... + 1/221) ≈ 10. The genomes of the 93 MA lines and the progenitor in the MSH2-lacking background were sequenced. A total of 18 strains, including 16 of the above 93 MA lines, BY4741 (haploid), and BY4742 (diploid), all with intact MSH2, were subjected to MA. After the first round of MA, 96 daughter lines were established from each of the 18 strains. We then performed a second round of MA. Four to 20 replicate lines were established for each strain. Cells were propagated at 30 °C on SC (synthetic complete) plates, similar to that used in the fluctuation test. The total time in the second round of MA for all lines was kept at ~100 days and the number of generations between bottlenecks was kept at ~20. The between-bottleneck duration was different among these 18 strains because of their different generation times. It was 48 h in BY4741, BY4743, MA28, and MA38, 72 h in MA15, MA21, MA23, MA25, MA29, MA33, MA44, MA63 and MA92, and 96 h in MA45, MA51, MA56, MA64, and MA94. The genomes of 209 MA lines at the end of the second round of MA and their 18 ancestral strains were sequenced. The number of generations each MA line went through was estimated in the same way as in the first round of MA.

For each sample to be sequenced, the genomic DNA was extracted using MasterPure Yeast DNA Purification Kit (Lucigen; Cat. No. M9780200). Library was constructed using Nextera DNA Flex Library Prep kit (Illumina; Cat. No. 20018705). Paired-end reads (2 x 150 bases) were generated on Illumina HiSeq 4000 platform by Admera Health (www.admerahealth.com).

Identification of mutations and verification by Sanger sequencing. Sequencing reads from each sample were first mapped to the S. cerevisiae reference genome (version R64-2-1) by Burrows-Wheeler Aligner. Duplicate marking and local realignment around indels were carried out using Genome Analysis Toolkit (GATK). SVs and indels shorter than 30 nucleotides were called by GATK HaplotypeCaller. Variants that differ between each MA line and its ancestral strain (Supplementary Fig. 4) were subject to the following criteria: (i) variant per cell division lower in MSH2 than in MSH2+, which could partially explain the reduced transformation efficiencies in the MA lines.

Twenty shared mutations between MA lines identified in the first round of MA were randomly chosen for verification by Sanger sequencing. For each mutation, Sanger sequencing was performed in both the sample with the mutation and its ancestor, and the mutation was considered confirmed by Sanger sequencing if both results agreed with the results from Illumina sequencing. Polymerase chain reaction and Sanger sequencing were successful in 16 of the 20 cases, and 15 of the 16 mutations were confirmed by Sanger sequencing.

The impact of deleting MSH2 on mutation rates was assessed by comparing the mutations accumulated in the first round of MA in the MSH2-lacking background with those in the second round of MA of the progenitor with intact MSH2. Deleting MSH2 increased the SNV rate per site per generation by 16 times,
in the first round of MA. Because the N_e of the MA lines was about 10, while most mutations are expected to have a fitness effect on the order of 1% or smaller, selection should be infrequent during the MA. These infrequent selections likely concentrated in the one-sixth of yeast genes known as essential genes, because loss-of-function mutations in essential genes cannot accumulate. To confirm the infrequency of selection, we compared the genomic contributions of the mutations in the 93 MA lines with the corresponding contributions of the random expectations. The fraction of SNVs located in genic regions is 73%, slightly but significantly below the random expectation of 74% (P = 0.0046, binomial test). The fraction of coding SNVs that are non synonymous is 70%, also slightly but significantly below the random expectation of 76% (P = 0.001, binomial test). While 16% of all homozygous SNVs were located in genic regions, a slightly lower fraction (a 14%) occurred in genic regions (P = 0.002, chi-squared test). Together, these results confirm that selection was present but infrequent in our MA experiment. The infrequent selection may cause a slight underestimation of V_m (under both the DBH and conventional model), rendering our inference of stabilizing selection more conservative.

Fluctuation test. CAN1-based fluctuation test was performed following Lang48 with a few modifications. CAN1 encodes an arginine transporter; cells must carry loss-of-function mutations in CAN1 to be able to grow in the presence of canavanine, a toxic arginine analog. The strain being tested was precultured in SC-Arg medium. The plate was sealed with an aluminum foil and incubated at 30 °C with shaking for 72 h. Then, 72 100-μl cultures were spot-plated onto the selection plates (SC-Ar g with 60 μg/ml canavanine), while the remaining 24 100-μl cultures were pooled followed by cell counting by a hemocytometer. About 1000 cells from this pool were plated onto a SC-Ar plate (without canavanine) to test plating efficiency. The canavanine plates with cell cultures were first dried in a sterile hood, followed by incubation at 30 °C for 72 h. Finally, the mutant colonies on each plate were manually counted. Because the growth rates of the MA lines were low, the incubation time in this step was longer than the usually used 49 h. We subjected all 60 MA lines with reinserted MS2H to the fluctuation test, but only 49 of them grew in the medium. We also subjected the progenitor (before the deletion of MS2H) to the fluctuation test. CAN1-based μ was estimated by bx-rates49, a web tool that uses an empirical probability generating function to estimate the number of mutations per culture with correction for plating efficiency. The mutation frequency presented is the probability of mutation minus the corresponding rate of production of mutations. The number of generations separating the two species, we used the 95% confidence intervals (CI) of μ estimated by bx-rates49; two strains with non-overlapping 95% CIs were regarded as having significantly different μ.

N_s of S. cerevisiae. The SNV rate of S. cerevisiae was estimated from MA lines to be 1500/10^7 per site per generation in YPD52. A species-wide population genomic survey shows that the nucleotide diversity of the sites is actually lower at non synonymous (0.0014), intronic (0.0027), and intergenic (0.0037) sites than at synonymous sites (0.0091). Under the assumption that synonymous mutations are neutral, N_s was estimated by π_{syn}(μ) = 1.17 × 10^-7. It is possible that n_s is smaller than the neutral nucleotide diversity because of selection at synonymous sites, which renders our estimation of N_s smaller than its true value and our inference of stabilizing selection of mutation rates and spectrum conservative.

Estimation of V_m, V_p, and D^2. V_m of a phenotypic trait such as μ is the variance of μ among MA lines per generation. V_m (or V_m3, V_m2) is the corresponding variance calculated using only MA lines with lower (or higher) μ than that of the progenitor. Because μ is higher in the MS2H-lacking MA lines than in natural strains, we employed three corrections by respectively assuming that deleting MS2H caused the same fold change in the rate of each mutation type as the observed fold change of the total rate of SNVs and indels (V_m), as that of indels (V_m2), and as that of SNVs (V_m3). Specifically, the corrected numbers of generations became 119,850 in estimating V_m, 667,636 in estimating V_m2, and 18,578 in estimating V_m3 respectively. In the top half of Table 1, V_m, V_m2, and V_m3 were estimated using the CAN1-based μ of MA lines from Table 1. V_m was estimated using published CAN1-based μ of seven diverse natural strains of S. cerevisiae41 (Supplementary Table 2, Table 3, Supplementary Fig. 3). In the bottom half of Table 1, V_m and V_m3 were estimated using the MA + WGS-based SNV rates of MA lines from the present study, while D^2 was the squared difference in MA + WGS-based SNV mutation rate between S. cerevisiae41 and S. paradoxus. For D^2 analysis, we employed the above corrected μ, V_m, V_m3, and D^2 (Table 2) or used the original μ values without transformation (Supplementary Table 4).

In Supplementary Table 6, V_m was estimated using the MA + WGS-based mutation rates of MA lines from the present study, while V_p was estimated using published MA + WGS-based mutation rates of the natural strains of S. cerevisiae42,53,54 (Supplementary Fig. 3). We presented results from both log_{10} transformed values (Supplementary Table 7) and untransformed values (Supplementary Table 6).

To test if V_m/V_p is significantly smaller than the neutral expectation, we bootstrapped MA lines as well as natural strains 10,000 times; P-value is the fraction of times when V_m/V_p computed from a bootstrap sample exceeds the neutral expectation. To test if D^2/V_m is significantly smaller than the neutral expectation, we bootstrapped MA lines 10,000 times; P-value is the fraction of times when D^2/V_m computed from a bootstrap sample exceeds the neutral expectation. The V_m and V_m3 calculated are the phenotypic variances, including the genetic component and estimation error, because the environment is fixed. The phenotypic variance caused by estimation error should be similar for natural strains and MA lines because of the use of the same phenotyping method. Because the phenotypic variance is greater for MA lines than for natural strains, the fraction of phenotypic variance contributed by genetics is greater for MA lines than for natural strains. Hence, V_m/V_p is overestimated when computing using phenotypic variance instead of genetic variance, which renders our conclusion that V_m/V_p is smaller than the neutral expectation conservative.

Number of generations separating S. cerevisiae and S. paradoxus. The number of generations separating S. cerevisiae and S. paradoxus was estimated by dividing the nucleotide sequence divergence between the two species per synonymous site (d_s) by the mean SNV mutation rate of S. cerevisiae and S. paradoxus. Here, d_s has been calculated by the reported SNV mutation rates in these two species are 1.95 × 10^-10 and 7.27 × 10^-11 per site per generation, respectively.23,24. Hence, the number of generations separating the two species is 2.89 × 10^9. As noted above, d_s may underestimate the neutral divergence between the two species, which renders our stabilizing selection conclusion conservative.

The mutation rate drift barriers of various species. For (haploid) ascomycetes, the drift barrier (U) is reached when the mutation rate reduction per functional genome per generation by a modifier (ΔU = U_0/U) equals 1/ν_m, where λ is the fractional reduction of the mutation rate and is assumed to be 0.15. Hence, the drift barrier is U_0 = 10/ν_m. For diploid ascomycetes, because ΔU = 2U_0 U/D, let Δm be the per generation rate of mutational production of modifiers decreasing the mutation rate minus the corresponding rate of production of modifiers increasing the mutation rate. When U approaches U_0, Δm will cause an increase in U_18. However, because the magnitude of Δm is expected to be much smaller than U_0, the effect of Δm on U_0 is negligible.

For diploid sexuals at the drift barrier, ΔU = 2U_0 U/D, where s is the mean selective disadvantage of deleterious mutations in the heterogeneous state18 and is assumed to be 0.0121.55, So, U_0 = 1/(4λsD) = 250/ν_m. In females, the effect of Δm on U_0 is amplified by 1/s times, so it is possible that U_0 is increased to some extent due to a negative Δm. However, it is extremely unlikely that the absolute value of Δm = 100/Δm exceeds 5U_0, because it is difficult to imagine that more than 5% of mutations create mutation rate modifiers. Therefore, U_0 should not exceed the times the above estimate, 1500/ν_m.

We obtained the N_e estimates of E. coli37, Bacillus subtilis32, Schizosaccharomyces pombe36, Chlamydomonas reinhardtii37, Arabidopsis thaliana38, Drosohila melanogaster39, Mus musculus40, and Homo sapiens41 from the literature. The N_e of S. cerevisiae was assumed to be 10^7, as estimated above. S. cerevisiae is considered a diploid asexual because it is normally diploid but undergoes rare sexual reproduction (once per 1000 generations) in the wild18. S. pombe is considered a haploid asexual because it is normally a haploid and very rarely undergoes sexual reproduction (once per 0.6 million generations) in the wild18. Arabidopsis thaliana has become largely selfing since about 1 million years ago40, but we here still consider it outcrossing so that our argument for U that is higher than the drift barrier is more conservative. Chlamydomonas reinhardtii is normally a haploid, but how often it undergoes sexual reproduction in nature is unknown37. To be conservative in our argument, we considered it as a sexual haploid.

The observed U is measured by the number of SNV mutations per functional genome per generation. The functional genome size G is defined as the total number of nucleotides in the genome that are subject to natural selection. To be conservative, G was assumed to equal the total coding sequence length except for D. melanogaster, M. musculus, and H. sapiens. For these three species, G was estimated by the total number of nucleotides in autosomes multiplied by the fraction of sites under purifying selection, which was previously estimated to be 48.7% for D. melanogaster68, 7.2% for M. musculus69, and 8.2% for H. sapiens68, respectively. We obtained the estimates of SNV mutation rate per site per generation of E. coli37, B. subtilis32, S. cerevisiae37, S. pombe36, C. reinhardtii37, A. thaliana38, D. melanogaster70, M. musculus71, and H. sapiens71 from the literature.

On the negative correlation between U and N_e. For three reasons, the cause of the reported negative correlation between U and N_e is uncertain. First, N_e is typically uncharacterized by diploid sexually reproducing organisms G is the total number of nucleotides in 4 diploids for 2 haploids, and the neutral nucleotide diversity is typically approximated by the synonymous nucleotide diversity π_e. Hence, any estimation
error of mut influences the estimates of U and Ns in opposite directions, creating a spurious negative correlation between them. Second, synonymous mutations are not immune to selection. Due to the abundance of negative selection and the increase of selection intensity with Ns, it is possible that the neutral nucleotide diversity is underestimated by ns and that the extent of the underestimation rises with Ns. In other words, the larger the Ns, the greater the underestimation of ns, which increases the apparent effect of Ns on U. Finally, the negative correlation between U and Ns is partially due to the negative correlation between G and Ns. For instance, log(Ns) has a linear correlation coefficient of −0.61 (P = 4.0 × 10−4) with log10(protein size) and −0.77 (P = 1.1 × 10−6) with log10(gene size) when analyzed using published data.16

Optimal U under the two opposing second-order selections in sexes. Raising U increases the number of beneficial mutations as well as that of deleterious mutations. For that found, in the sexes, the optimal U, which is the U value corresponding to the highest speed of adaptation, is the harmonic mean of the coefficient of selection (s > 0) against deleterious mutations. Because deleterious mutations with s smaller than 1/Ns are effectively neutral, the range of s for mutation rate varies between the selected range among Ns, which causes a decrease in the harmonic mean of s and hence the optimal U with Ns. To show this trend numerically, we sampled 100,000 s values from a gamma distribution with mean equal to 0.01 and shape parameter α = 0.1, 0.2, or 0.5. The harmonic mean of s among all sampled s values that are larger than 1/Ns was computed. This harmonic mean is the optimal U. We considered various Ns values and various α values.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The sequencing reads generated have been deposited to NCBI SRA under the accession number PRJNA735524. All other data are presented in the paper and associated supplementary materials. Source data are provided with this paper.

Received: 28 April 2021; Accepted: 10 June 2021; Published online: 30 June 2021

References

1. Sturtevant, A. H. Essays on evolution. I. On the effects of selection on mutation rate. Q. Rev. Biol. 12, 467–477 (1937).
2. Kimura, M. On the evolutionary adjustment of spontaneous mutation rates. Genetics 59, 155–160 (1970).
3. Leigh, E. G. Jr. Natural selection and mutability. Am. Nat. 104, 301–305 (1970).
4. Kondrashov, A. S. Modifiers of mutation-selection balance: general approach and the evolution of mutation rates. Genetics 66, 53–69 (1995).
5. Drake, J. W., Charlesworth, B., Charlesworth, D. & Crow, J. F. Rates of spontaneous mutation. Genetics 148, 1667–1686 (1998).
6. Sniegowski, P. D., Gerrish, P. J., Johnson, T. & Shaver, A. The evolution of mutation rates: separating causes from consequences. Bioessays 22, 1057–1066 (2000).
7. Orr, H. A. The rate of adaptation in asexuals. Genetics 155, 961–968 (2000).
8. Andrè, J. B. & Godelle, B. The evolution of mutation rate in multicellular eukaryotes: causes and consequences. Nat. Rev. Genet. 17, 611–626 (2006).
9. Johnson, T. Benficial mutations, hitchhiking and the evolution of mutation rates in sexual populations. Genetics 151, 1621–1631 (1999).
10. Lynch, M. The cellular, developmental and population-genetic determinants of mutation-rate evolution. Genetics 180, 933–943 (2008).
11. Taddéi, F. et al. Role of mutator alleles in adaptive evolution. Nature 387, 700–702 (1997).
12. Lynch, M. et al. Genetic drift, selection and the evolution of the mutation rate. Nat. Rev. Genet. 17, 704–714 (2016).
13. Baer, C. F., Miyamoto, M. M. & Denver, D. R. Mutation rate variation in multicellular eukaryotes: causes and consequences. Nat. Rev. Genet. 8, 619–631 (2007).
14. Gou, L., Bloom, J. S. & Kruglyak, L. The genetic basis of mutation rate variation in yeast. Genetics 211, 731–740 (2015).
15. Liu, H. & Zhang, Y. Yeast spontaneous mutation rate and spectrum vary with environment. Curr. Biol. 29, 1584–1591 (2019).
16. Zhang, J. Rates of conservative and radical nonsynonymous nucleotide substitutions in mammalian nuclear genes. J. Mol. Evol. 50, 56–68 (2000).
17. Storz, J. F. et al. The role of mutation bias in adaptive molecular evolution: insights from convergent changes in protein function. Philos. Trans. R. Soc. Lond. B Biol. Sci. 374, 20180238 (2019).
18. Lynch, M. The lower bound to the evolution of mutation rates. Genome Biol. Evol. 3, 1107–1118 (2011).
50. Hamon, A. & Ycart, B. Statistics for the Luria-Delbrück distribution. *Electron. J. Stat.* 6, 1219–1272 (2012).

51. Maclean, C. J. et al. Deciphering the genic basis of yeast fitness variation by simultaneous forward and reverse genetics. *Mol. Biol. Evol.* 34, 2486–2502 (2017).

52. Zhu, Y. O., Siegal, M. L., Hall, D. W. & Petrov, D. A. Precise estimates of mutation rate and spectrum in yeast. *Proc. Natl Acad. Sci. USA* 111, E1353–E1361 (2014).

53. Sharp, N. P., Sandell, L., James, C. G. & Otto, S. P. The genome-wide rate and spectrum of spontaneous mutations differ between haploid and diploid yeast. *Proc. Natl Acad. Sci. USA* 115, ES046–ES055 (2018).

54. Elyashiv, E. et al. Shifts in the intensity of purifying selection: an analysis of genome-wide polymorphism data from two closely related yeast species. *Genome Res.* 20, 1558–1573 (2010).

55. Bobay, L. M. & Ochman, H. Factors driving effective population size and pan-genome evolution in bacteria. *BMC Evol. Biol.* 18, 153 (2018).

56. Fawcett, J. A. et al. Population genomics of the fission yeast Schizosaccharomyces pombe. *PLoS One* 9, e104241 (2014).

57. Smith, D. R. & Lee, R. W. Nucleotide diversity in the mitochondrial and nuclear compartments of Chlamydomonas reinhardtii: investigating the origins of genome architecture. *BMC Evol. Biol.* 8, 156 (2008).

58. Nordborg, M. et al. The pattern of polymorphism in Arabidopsis thaliana. *PLoS Biol.* 3, e196 (2005).

59. Shapiro, J. A. et al. Adaptive genic evolution in the Drosophila genomes. *Proc. Natl Acad. Sci. USA* 104, 2271–2276 (2007).

60. Phifer-Rixey, M. et al. Adaptive evolution and effective population size in wild house mice. *Mol. Biol. Evol.* 29, 2949–2955 (2012).

61. Li, H. & Durbin, R. Inference of human population history from individual whole-genome sequences. *Nature* 475, 493–496 (2011).

62. Nieuwenhuis, B. P. & James, T. Y. The frequency of sex in fungi. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 371, 20150540 (2016).

63. Tang, C. et al. The evolution of selfing in Arabidopsis thaliana. *Science* 317, 1070–1072 (2007).

64. Sasso, S., Stibor, H., Mittag, M. & Grossman, A. R. From molecular manipulation of domesticated Chlamydomonas reinhardtii to survival in nature. *eLife* 7, e39233 (2018).

65. Meader, S., Ponting, C. P. & Lunter, G. Massive turnover of functional sequence in human and other mammalian genomes. *Genome Res.* 20, 1335–1343 (2010).

66. Rands, C. M., Meader, S., Ponting, C. P. & Lunter, G. 8.2% of the Human genome is constrained: variation in rates of turnover across functional element classes in the human lineage. *PLoS Genet.* 10, e1004525 (2014).

67. Lee, H., Popodl, E., Tang, H. & Foster, P. L. Rate and molecular spectrum of spontaneous mutations in the bacterium Escherichia coli as determined by whole-genome sequencing. *Proc. Natl Acad. Sci. USA* 109, E2774–E2783 (2012).

68. Sung, W. et al. Asymmetric context-dependent mutation patterns revealed through mutation-accumulation experiments. *Mol. Biol. Evol.* 32, 1672–1683 (2015).

69. Farlow, A. et al. The spontaneous mutation rate in the fission yeast Schizosaccharomyces pombe. *Genetics* 201, 737–744 (2015).

70. Ness, R. W., Morgan, A. D., Vasanthakrishnan, R. B., Colegrave, N. & Keightley, P. D. Extensive de novo mutation rate variation between individuals and across the genome of Chlamydomonas reinhardtii. *Genome Res.* 25, 1739–1749 (2015).

71. Osowski, S. et al. The rate and molecular spectrum of spontaneous mutations in Arabidopsis thaliana. *Science* 327, 92–94 (2010).

72. Keightley, P. D. et al. Analysis of the genome sequences of three Drosophila melanogaster spontaneous mutation accumulation lines. *Genome Res.* 19, 1195–1201 (2009).

**Acknowledgements**

We thank Lianghe Gou and Leonid Kruglyak for providing the raw replicate mutation rate estimates of seven natural strains of yeast and Alex Kondrashov, Wenfeng Qian, and members of the Zhang laboratory for valuable comments. This work was supported by the U.S. National Institutes of Health research grant R35GM139484 to J.Z.

**Author contributions**

H.L. and J.Z. designed the study and wrote the paper. H.L. performed the research and analyzed the data.

**Competing interests**

The authors declare no competing interests.

**Additional information**

**Supplementary information** The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-24364-6.

**Correspondence** and requests for materials should be addressed to J.Z.

**Peer review information** *Nature Communications* thanks the anonymous reviewers for their contribution to the peer review of this work. Peer reviewer reports are available.

**Reprints and permission information** is available at http://www.nature.com/reprints

**Publisher’s note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s) 2021, corrected publication 2023