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Genomic estimates of mutation and substitution rates contradict the evolutionary speed hypothesis of the latitudinal diversity gradient

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The latitudinal diversity gradient (LDG) refers to a decrease in biodiversity from the equator to the poles. The evolutionary speed hypothesis, backed by the metabolic theory of ecology, asserts that nucleotide mutation and substitution rates per site per year are higher and thereby speciation rates are higher at higher temperatures, generating the LDG. However, prior empirical investigations of the relationship between the temperature and mutation or substitution rate were based on a few genes and the results were mixed. We here revisit this relationship using genomic data. No significant correlation between the temperature and mutation rate is found in 13 prokaryotes or in 107 eukaryotes. An analysis of 234 diverse trios of bacterial taxa indicates that the synonymous substitution rate is not significantly associated with the growth temperature. The same data, however, reveal a significant negative association between the nonsynonymous substitution rate and temperature, which is explainable by a larger fraction of detrimental nonsynonymous mutations at higher temperatures due to a stronger demand for protein stability. We conclude that the evolutionary speed hypothesis of the LDG is unsupported by genomic data and advise that future mechanistic studies of the LDG should focus on other hypotheses.

1. Introduction

One of the most striking patterns of biodiversity is the latitudinal diversity gradient (LDG)—a decline in the number of species and higher taxa with latitude. Although not universal, the LDG is remarkably general across taxa [1], including bacteria [2]. Numerous hypotheses have been proposed to explain the origin of LDG from the angles of different rates of speciation, extinction and/or immigration at different latitudes [3,4]. Of particular interest is the evolutionary speed hypothesis that links higher environmental temperatures of lower latitudes with faster biological processes such as mutagenesis [5]. The hypothesis further posits that an increase in mutation rate per nucleotide site per year will (i) increase the speed of genetic differentiation between populations and thereby accelerate speciation and (ii) increase the nucleotide substitution rate and thereby accelerate the genetic divergence between species, promoting the generation of higher taxa. In short, the evolutionary speed hypothesis explains the LDG by higher mutation and substitution rates at higher temperatures.

Why should mutation and substitution rates be higher at higher temperatures? Martin and Palumbi suggested that mutagenic oxygen radicals, which are byproducts of aerobic respiration, could connect high temperature to high mutagenesis [6]. More broadly, the metabolic theory of ecology proposes that mutations are caused by free radical damage, replication errors, and other processes that are all ultimately consequences of metabolism [7]. Based on this idea, Gillooly *et al.* derived a formula in which $\ln(\mu M^{1/4})$ is a linear decreasing

function of $1/(kT)$, where μ is the mutation rate per site per year, M is body mass, T is absolute temperature and k is Boltzmann's constant [7]. Under the neutral theory of molecular evolution [8], Gillooly *et al.* then derived a formula on the substitution rate (v) per site per year from $v = \mu f$, where f is the fraction of mutations that are neutral and is assumed constant across taxa (or at least independent of T and M). They subsequently derived a formula showing that ln(speciation rate) is also a linear decreasing function of $1/(kT)$ [9]. These results provided a theoretical foundation for the evolutionary speed hypothesis of the LDG.

Is there empirical evidence for higher mutation rates at higher temperatures? Barely. Mutation accumulation in the near absence of selection followed by whole-genome sequencing (MA + WGS) showed that the *Escherichia coli* mutation rate is higher at 37°C than at 28°C, which is in turn similar to that at 25°C [10]. However, the authors used a mutator line with a disrupted allele of *mutS* that is involved in mismatch DNA repair, so the observation may not represent wild-type *E. coli*. A recent MA + WGS study of the insect *Chironomus riparius* found a U-shaped relationship between mutation rate and temperature, with the lowest mutation rate at an intermediate temperature (17°C) and higher mutation rates at both lower and higher temperatures [11], similar to a finding in yeast in 1960 from a reporter-based assay [12]. Mutation rate is an evolvable trait that is subject to mutation, drift and selection [13,14]. Hence, the mutation rate plasticity at different temperatures revealed in the above studies may not represent the variation of the optimal mutation rate across temperatures, especially because phenotypic plasticity is often maladaptive [15] and because even mild stresses can increase the mutation rate plastically [16]. That mutation rate is evolvable and subject to selection also casts double on the mutation rate predictions of the metabolic theory of ecology. In fact, Drake reported that mutation rate per genome per generation tends to be lower in thermophiles than in mesophiles [17]. He explained this observation by a stronger second-order selection for lower mutation rates at higher temperatures because the fraction of detrimental mutations is expected to be higher as a result of a stronger demand for protein stability at higher temperatures [18,19]. However, Drake's finding was based on only two thermophiles and seven mesophiles (three if viruses are excluded). Additionally, the mutation rate estimates were acquired from different reporter genes in different species so may not be comparable. It is also unclear whether Drake's conclusion holds when mutation rates are measured per site per year. In the nematode *Caenorhabditis elegans*, the germline mutation rate was inferred to be similar between a wild-type strain and a mutant strain with elevated steady-state oxidative stress, suggesting that increased free radical production does not necessarily lead to a substantial increase in mutation rate [20,21]. In summary, the current literature does not provide clear evidence for or against a positive impact of temperature on the (optimal) mutation rate per site per year.

Is there empirical evidence for higher substitution rates at higher temperatures (or lower latitudes)? Yes and no. This trend has been observed in a large number of organisms such as protists [9], plants [22] and various groups of animals including even endotherms [23–27], but it is absent in squamates (snakes, lizards and worm lizards) [28] and in birds as a whole [29]. Note that although the environmental temperature should have no direct relevance to the metabolic rate of endotherms, it has been suggested that indirect factors such as interactions with ectotherms could render the environmental temperature relevant to even endotherms [30]. Nonetheless, the nucleotide substitution rate was not found to be significantly positively correlated with the mass-specific metabolic rate across animals [31], bringing into question the metabolic theory of ecology. Furthermore, an analysis of the gene encoding cytochrome c oxidase subunit I in 8037 animal lineage pairs found only 51.6% of the cases to support faster molecular evolution of the lineage with a lower latitude [32], suggesting limited applicability of the evolutionary speed hypothesis. A major caveat of all of the above studies is that substitution rates were estimated from only one to several genes such as cytochrome b, rRNA, and mitochondrial genes [27]; it is thus unclear whether the reported impact of temperature on the substitution rate is a general feature of the nuclear genome, which is predicted by the metabolic theory of ecology and required for a successful evolutionary speed hypothesis of the LDG.

We therefore respectively revisited the relationship between temperature and mutation rate and the relationship between temperature and substitution rate using genomic data. Specifically, we first analysed mutation rates estimated by MA + WGS (including direct parent–offspring genomic comparison) at or near the natural growth temperatures of 120 species. We then compared the genome-wide nucleotide substitution rates between closely related bacterial taxa with at least a 5°C difference in growth temperature for 234 diverse taxon pairs.

2. Results

(a) Mutation rate is not significantly positively correlated with temperature

We collected from the literature mutation rates per site per generation of 13 prokaryotes (11 eubacteria and two archaea) and 107 eukaryotes (nine unicellular eukaryotes, eight plants, ten invertebrates and 80 vertebrates) estimated by MA + WGS (electronic supplementary material, data S1). When needed, mutation rates per site per year were then computed using generation time information from the literature (see Material and methods). Because the species studied here are evolutionarily related, we used phylogenetically independent contrasts (PICs) [33,34] in the following correlation analysis (see Material and methods).

We found no significant correlation between the growth temperature and mutation rate per site per year among the 13 prokaryotes (Pearson's $r = -0.19$, $p = 0.55$; figure 1*a*). While the metabolic theory of ecology predicts a negative linear correlation between $\ln(\mu M^{1/4})$ and $1/(kT)$ [7], the opposite was found across the 13 prokaryotes ($r = 0.86$, $p = 0.0004$; figure 1*b*). The statistical significance in the above correlation disappeared after the removal of an apparent outlier ($r = 0.061$, $p = 0.86$; figure 1*b*). At any rate, our findings do not support the prediction of the metabolic theory of ecology. Drake [17] proposed that the mutation rate per (functional) genome per generation is lower at higher temperatures. We used the total coding sequence length as a proxy for the functional genome size but found no significant correlation between the mutation rate per functional genome per generation and temperature in the 13 prokaryotes ($r = -0.44$, $p = 0.15$; figure 1*c*).

Our analysis of the 107 eukaryotic species yielded similar results. No significant correlation was detected between temperature and mutation rate per site per year (figure 1*d*), between

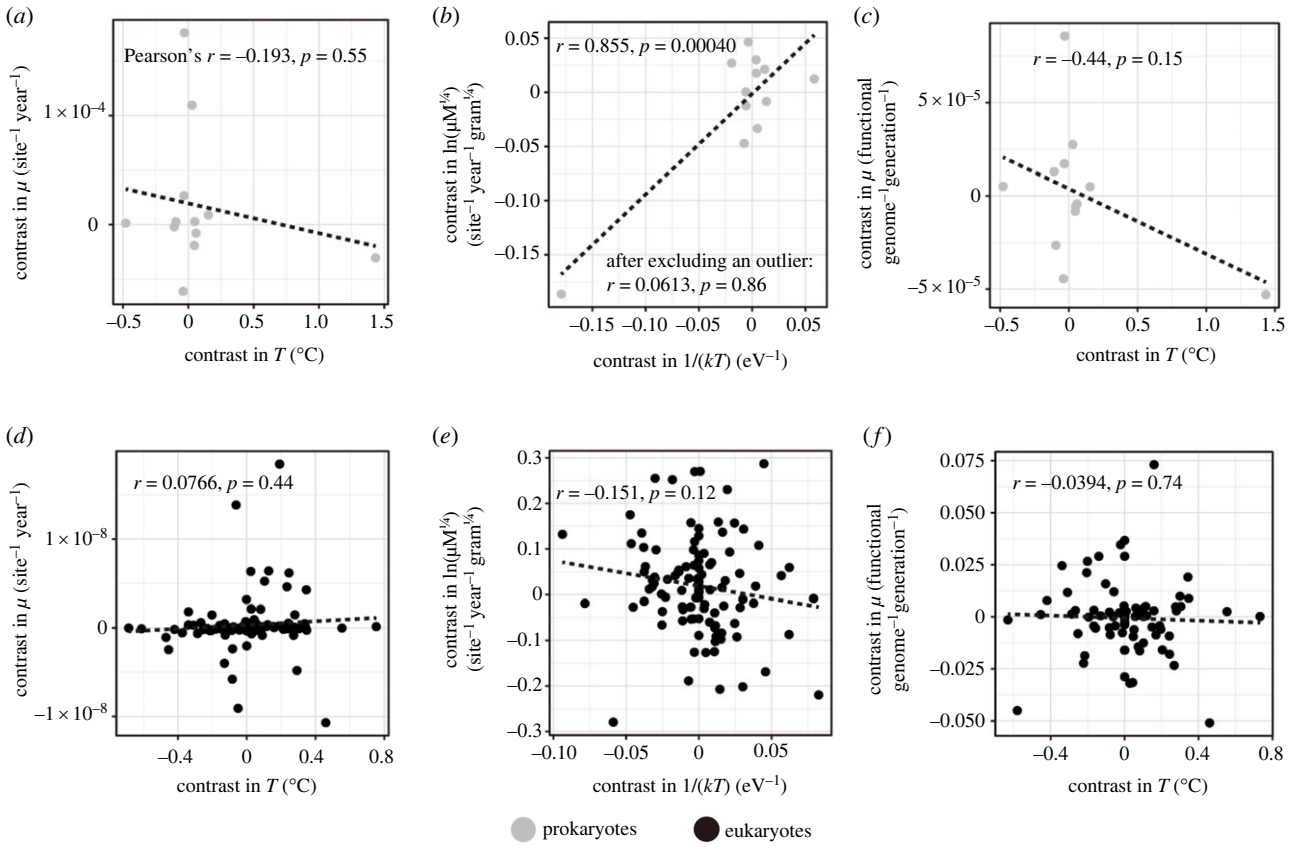

Figure 1. Correlations between mutation rate and growth temperature contrasts. Each dot represents a phylogenetically independent contrast. (*a*) Relationship between mutation rate per site per year and growth temperature across prokaryotes. (*b*) Relationship between $\ln(\mu M^{1/4})$ and $1/(kT)$ across prokaryotes, where μ is mutation rate per site per year, M is body size in grams, k is Boltzmann's constant (8.62×10^{-5} eV K^{-1}) and T is absolute temperature in kelvin. (*c*) Relationship between mutation rate per functional genome per generation and growth temperature across prokaryotes. (*d*) Relationship between mutation rate per site per year and temperature across eukaryotes. (*e*) Relationship between $\ln(\mu M^{1/4})$ and $1/(kT)$ across eukaryotes. (*f*) Relationship between mutation rate per functional genome per generation and temperature across eukaryotes. Growth temperature equals body temperature for endotherms. Total coding sequence length of a genome is used as a proxy for the functional genome size. In panel (*f*), we excluded 30 vertebrates and one unicellular eukaryote of which the functional genome size is unknown due to the lack of annotation of the reference genome. Dotted lines show linear regressions.

$\ln(\mu M^{1/4})$ and $1/(kT)$ (figure 1*e*), or between the mutation rate per functional genome per generation and temperature (figure 1*f*). Furthermore, similar results were obtained when unicellular and multicellular eukaryotes were separately analysed (electronic supplementary material, table S1). The multicellular eukaryotes considered vary greatly in life-history traits such as the generation time. However, such traits need not be controlled in the analysis because the mutation (or substitution) rate prediction of the metabolic theory of ecology is in part based on the theoretical consideration of the impact of metabolism on these traits. For example, according to the metabolic theory of ecology, raising the temperature increases the mutation rate per year in part because of the predicted shortening of the generation time at higher temperatures.

Note that the range of the growth temperature covered here (10 to 42°C) is similar to that (0 to 40°C) in the original work proposing a positive impact of growth temperature on mutation/substitution rate [7], although Drake's study covered the range from 30 to 75°C [17] because of its inclusion of thermophiles that are absent in our analysis due to the lack of MA + WGS data.

(b) Nonsynonymous substitution rate declines with temperature

To study the potential impact of the growth temperature on the nucleotide substitution rate, we focused on eubacteria because of their large range of growth temperature from 3 to 85°C [35] and the availability of many genome sequences. We found that 5602 bacterial species have both available growth temperatures and genome sequences [36] (figure 2*a*). We identified 234 diverse trios of closely related species where each trio comprises two ingroup species whose growth temperatures differ by at least 5°C and an outgroup. Orthologous genes were identified from each trio, followed by sequence alignment and concatenation of the alignments of all orthologous genes. From each trio, we computed the number of synonymous substitutions per synonymous site (d_S), number of nonsynonymous substitutions per nonsynonymous site (d_N), and d_N/d_S for the branch leading to each ingroup species (figure 2*b*; electronic supplementary material, data S2), and then examined whether d_S, d_N or d_N/d_S is higher for the higher-temperature ingroup species than for the lower-temperature ingroup species. Although synonymous mutations are commonly considered neutral, there is increasing evidence that they are often non-neutral, especially in microbes where the efficacy of selection is typically high due to their large effective population sizes [37–39]. Nevertheless, under the assumption that mechanisms rendering synonymous mutations non-neutral similarly render nonsynonymous mutations non-neutral (i.e. an equal fraction of synonymous and nonsynonymous mutations are made non-neutral by the above mechanisms), d_N/d_S can still measure natural selection acting on protein

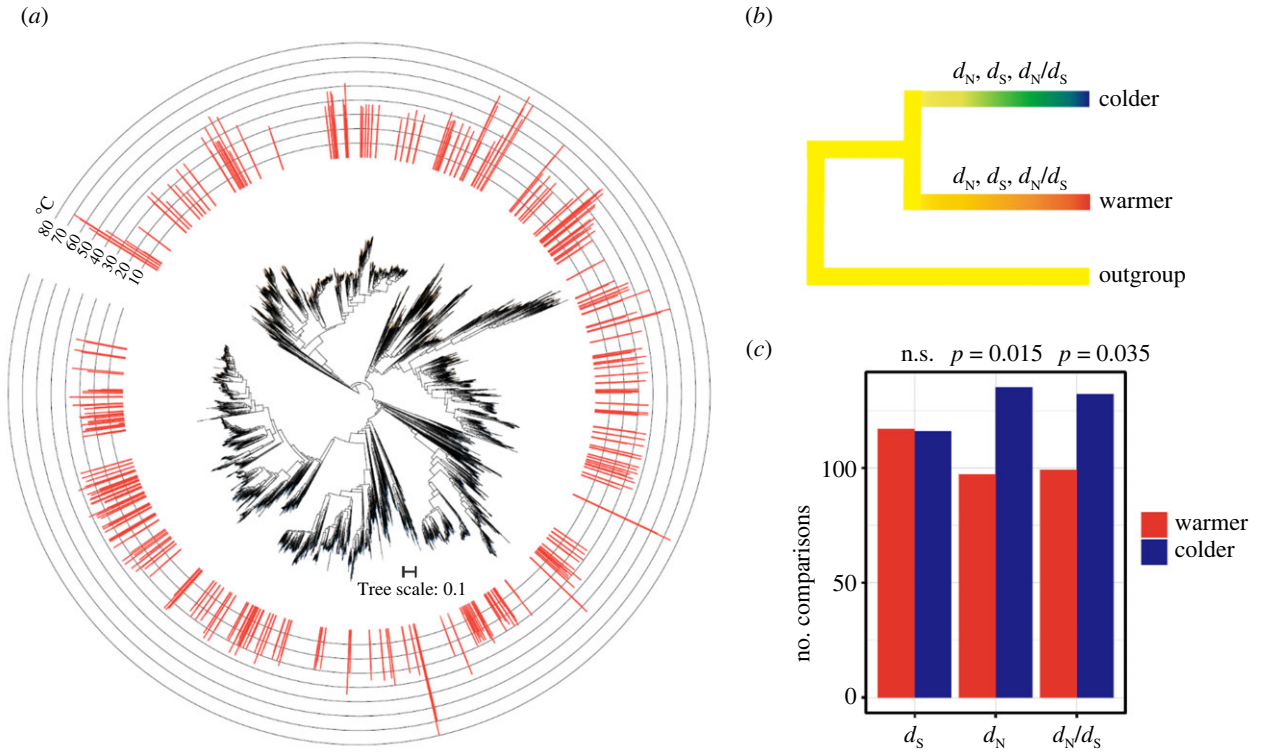

Figure 2. Association between growth temperature and nucleotide substitution rates in bacteria. (*a*) Phylogenetic relationships among the 5602 bacterial species with information on growth temperature. Phylogenetic relationships and tree scale are based on 120 conserved protein sequences (see Material and methods). Red bars represent growth temperatures of the species analysed in this study. (*b*) Schematic illustration of a species trio. (*c*) Association between growth temperature and d_S, d_N or d_N/d_S. A red (or blue) bar shows the number of trios in which the ingroup species with a relatively high (or low) growth temperature has a higher value of d_S, d_N or d_N/d_S than that of the other ingroup species. The *p*-value from a two-tailed binomial test between the adjacent red and blue bars is shown.

sequences, with lower d_N/d_S (when smaller than 1) indicating stronger purifying selection.

Of the 234 trios, 116 exhibited higher d_S for the higher-temperature ingroup, 117 exhibited the opposite, and one had equal d_S for the two ingroups. Hence, there is no significant impact of growth temperature on d_S ($p = 1$, binomial test; figure 2*c* and electronic supplementary material, figure S1*a*). By contrast, 135 trios showed higher d_N for the lower-temperature ingroup, 97 showed the opposite, and two had the same d_N for the two ingroups, indicating that d_N tends to be higher for lower-temperature than higher-temperature ingroups ($p = 0.015$, binomial test; figure 2*c* and electronic supplementary material, figure S1*b*). Similarly, a higher d_N/d_S is significantly associated with a lower growth temperature ($p = 0.035$, binomial test; figure 2*c* and electronic supplementary material, figure S1*c*). Taken together, these results indicate that nonsynonymous (and hence amino acid) substitutions are slower and purifying selection against nonsynonymous changes are stronger at higher temperatures.

To investigate whether the above patterns are uniform across different ranges of temperature, we divided the 234 trios into four bins based on the average growth temperature of the two ingroup species of each trio (figure 3). From the first to the last bin, the mean growth temperatures of the lower-temperature ingroup species and that of the higher-temperature ingroup species are 10.2 and 24.4, 21 and 29.6, 29 and 37.6, and 49.3 and 60°C, respectively. Interestingly, the impact of raising the growth temperature on d_N is significant in the first two bins, but not significant in the last two bins (figure 3). Because the temperature difference between the two ingroup species is on average similar across the four bins, our results suggest that the impact of raising the temperature on d_N decreases as the temperature increases. The impact of raising temperature on d_S or d_N/d_S is not significant in any bin (figure 3). The trend of d_N/d_S across the four bins resembles that of d_N, so the statistical non-significance in the result on d_N/d_S is likely due to the higher estimation error of d_N/d_S than that of d_N. In theory, for the same amount of temperature increase, the fractional increase in the required protein stability declines with the temperature (see Material and methods), which may render the associated d_N changes less detectable at higher temperatures. Indeed, the fractional difference in d_N between the lower-temperature and higher-temperature ingroup species divided by their growth temperature difference decreases as their mean growth temperature rises (electronic supplementary material, figure S2).

3. Discussion

We found no significant positive correlation between genomic mutation rate per site per year and temperature in prokaryotes or eukaryotes. Our genome-wide estimates of nucleotide substitution rates in 702 diverse bacteria separated into 234 independent trios showed no significant impact of raising the temperature on d_S but a significant, negative impact on d_N and d_N/d_S. Thus, we found no genomic evidence supporting the prediction of the metabolic theory of ecology or the evolutionary speed hypothesis of the LDG. The evolutionary speed hypothesis of the LDG also relies on a positive effect of mutation or substitution rate on the speciation rate, which we did not test due to the difficulty in reliably estimating speciation rates. To our knowledge, there is no empirical evidence for a higher speciation rate

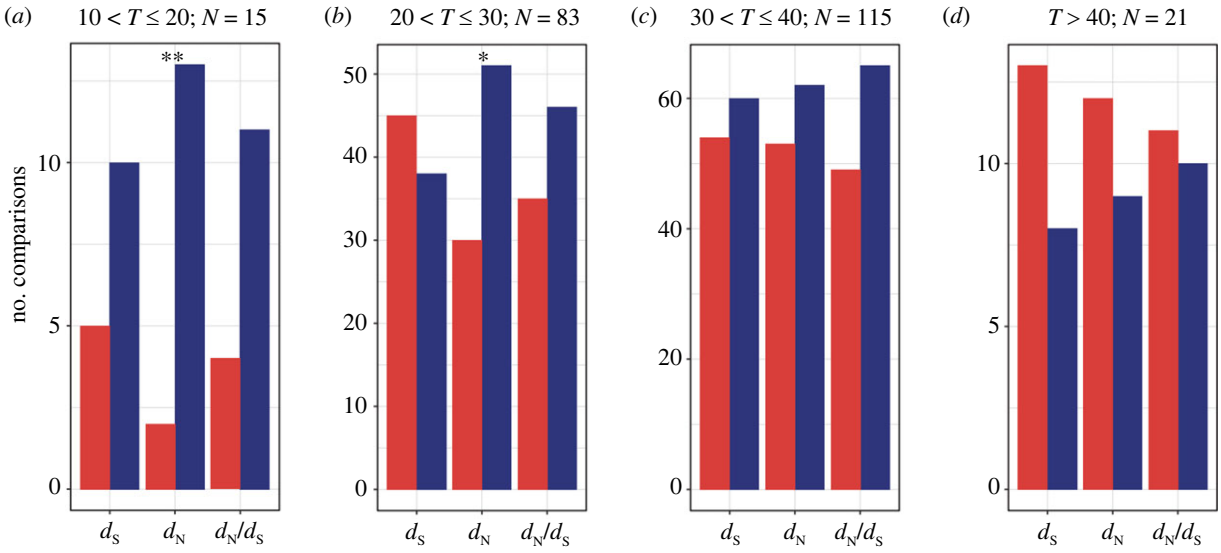

Figure 3. Association between bacterial growth temperature and d_S, d_N, or d_N/d_S in four temperature bins. A red (or blue) bar shows the number of trios in which the ingroup species with a relatively high (or low) growth temperature has a relatively high d_S, d_N, or d_N/d_S. *$p < 0.05$; **$p < 0.01$ from a two-tailed binomial test between the adjacent red and blue bars. Temperatures (T) in ℃ are shown. N, number of trios.

resulting from a higher mutation or substitution rate. Furthermore, because the underlying cause of temperature's potential impact on the speciation rate is its potential impact on mutation and substitution rates [9], our results imply no positive impact of temperature on the speciation rate. We conclude that the evolutionary speed hypothesis of the LDG has no merit and advise that future studies of the cause of the LDG focus on other hypotheses.

If anything, our results on d_S, d_N, and d_N/d_S suggest that, as the temperature rises, nonsynonymous substitutions are slower due to stronger purifying selection. Because the efficacy of natural selection is determined by the product of selection coefficient and effective population size, our observation could occur because (i) the effective population size tends to be larger in species with higher growth temperatures and/or (ii) nonsynonymous mutations are more likely to be deleterious or are more deleterious with the rise of temperature. While the relationship between temperature and effective population size has not been extensively studied, the available data do not suggest the effective population size to be generally larger or smaller for thermophiles than mesophiles (e.g. [40]). Furthermore, d_S is similar between ingroup species that differ in growth temperatures (figure 2*c*). Hence, the above second cause is more likely. Several other observations are consistent with this inference. First, heat-sensitive mutants are more prevalent than cold-sensitive mutants [41]. For example, in the budding yeast *Saccharomyces cerevisiae*, over 1000 heat-sensitive mutants [42,43] but only dozens of cold-sensitive mutants [44] are known. Second, in the seed beetle *Callosobruchus maculatus*, mutations induced by gamma radiation are more harmful at high but not low temperatures when compared with those at normal temperatures [45]. Third, mutations in the water flea *Daphnia pulex* generate a larger variance of reproductive traits at an elevated temperature compared with that at the normal temperature, suggesting that fitness effects of mutations tend to be larger at higher temperatures [46]. The underlying mechanism of all of these phenomena is probably a stronger demand for protein stability at higher temperatures [18]; consequently, nonsynonymous mutations are more likely to be deleterious and are more deleterious at higher temperatures. Consistently, proteins from thermophilic organisms are found to be more stable than their mesophilic orthologues at both normal and elevated temperatures [47,48]. A large-scale analysis found a substantial positive correlation between the growth temperature of an organism and the temperature at which a protein from the organism denatures [19]. Further, proteins from thermophilic organisms have a similar amino acid composition as highly expressed and slowly evolving proteins, which also show increased stability [49].

Because our substitution rate analysis is limited to bacteria, it would be interesting to examine whether other organisms follow the same pattern when a sufficiently large number of suitable genome sequences become available.

4. Material and methods

Mutation rates per site per generation were acquired from the literature (electronic supplementary material, data S1). To calculate the mutation rates per site per year, we acquired the generation time data from either the studies that quantified the mutation rates or other references with growth conditions similar to those in the studies that quantified the mutation rates. In analysing the relationship between the mutation rate and temperature, we used the microbial growth temperatures reported in the MA studies, which were either at or near the optimal growth temperatures reported [35]. Body temperatures of endotherms were used as their growth temperatures. For ectoderms, if the samples were raised in a temperature-controlled laboratory environment, the laboratory temperature was used as the body temperature; if the samples were collected from the wild, the average annual temperature of that area was used as the body temperature. Average body masses of plants and animals were acquired from various sources, while the cell masses of microbes were calculated from their cell volumes and the density of 1.1 g cm^{-3} (http://book.bionumbers.org/what-is-the-density-of-cells/). The total length of coding sequences in a genome was used as a proxy for the size of the functional genome of the species. Thirty-one of the 120 species analysed here do not have genome annotations so have no estimates of the functional genome size; they were excluded from the analysis in figure 1*f*.

We used the R package 'Analyses of Phylogenetics and Evolution' (APE) [50] for the PIC analysis. The species trees, encompassing both topology and branch length (in million years) information, were sourced from the TimeTree database [51]. Trait values, such as the temperature and mutation rate per site per year for each species, along with the species tree, serve as the input for APE. The resulting output consists of the corresponding PICs.

The phylogenetic relationships of 5602 bacteria were obtained from a previous study [36], which was in turn based on 120 conserved proteins and adapted from the Genome Taxonomy Database [52]. Species trios were picked from the phylogeny of the 5602 bacteria based on the following criteria: (i) the difference in growth temperature between the two ingroup species is ≥5°C and (ii) the sum of the two branch lengths connecting the two ingroup species is in the range of 0.02 to 0.2 amino acid substitutions per site in the 120 conserved proteins mentioned, ensuring that the two ingroup species are sufficiently close to each other but are not too close to cause unreliability of the substitution rate estimates. A total of 234 non-overlapping trios fit the above two criteria and were used in subsequent analysis. The protein and cDNA sequences of the 702 species (234 trios) were downloaded from the Genome Taxonomy Database [52]. Protein BLAST was performed between every pair of species in a trio, and reciprocal best hits were considered orthologues. Orthologous protein sequences were first aligned by Clustal-Omega [53], with the cDNA sequence alignment derived accordingly. The aligned cDNA sequences were concatenated and used for the calculation of substitution rates. The alignment length (after the removal of gaps) ranged from 173 727 to 2 056 436 codons for the 234 trios, with a median of 598 529 codons (electronic supplementary material, data S2). The codeml program in PAML [54] was used for the calculation of d_S, d_N and d_N/d_S of each branch of each trio tree. Specifically, the branch model, in which each branch is allowed to have its own d_N/d_S, coupled with the F3 × 4 option, was employed. All ingroups have $d_N/d_S < 0.2$, with the exception of one (*Thermodesulfatator autotrophicus*); removing the involved trio does not qualitatively alter the results.

Information on the growth temperatures of the 5602 bacteria was from a previous study [35]. Specifically, the data were collected by various stock centres and were validated by a strong correlation ($r = 0.89$) between the growth temperature of a species and the experimentally determined mean optimal temperature of the enzymes from the species [35]. This said, the growth temperature data may not be accurate for all species considered, suggesting that the significant negative association between temperature and d_N is likely stronger than observed.

When most molecules of a protein are correctly folded, the fraction (F) of molecules that are unfolded or misfolded is approximately $e^{-\Delta G/(kT)}$, where ΔG is the protein stability measured by its unfolding energy (i.e. more positive ΔG corresponds to higher stability), k is Boltzmann's constant, and T is absolute temperature [55]. Hence, to keep F constant, $\Delta G/T$ needs to be kept constant. In other words, raising the temperature imposes a higher demand for protein stability. Furthermore, a one degree increase in T has a smaller fractional change in T when T is higher, meaning that a one degree increase in T demands a smaller fractional change in ΔG when T is higher. If the impact of temperature on the nonsynonymous substitution rate is determined by the fractional change in ΔG, the same amount of temperature increase would have a smaller impact on the nonsynonymous substitution rate when the temperature is higher.

Statistical analysis and figure construction were carried out in R (https://www.r-project.org/).

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. All data used are publicly available. Intermediate results are provided in electronic supplementary material [56].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. H.L.: conceptualization, data curation, formal analysis, funding acquisition, investigation, writing—original draft, writing—review and editing; M.S.: data curation, writing—review and editing; J.Z.: conceptualization, funding acquisition, project administration, supervision, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. The authors declare no competing interests.

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