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Nucleotide Substitutions during Speciation may Explain Substitution Rate Variation

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Abstract.—Although molecular mechanisms associated with the generation of mutations are highly conserved across taxa, there is widespread variation in mutation rates between evolutionary lineages. When phylogenies are reconstructed based on nucleotide sequences, such variation is typically accounted for by the assumption of a relaxed molecular clock, which is a statistical distribution of mutation rates without much underlying biological mechanism. Here, we propose that variation in accumulated mutations may be partly explained by an elevated mutation rate during speciation. Using simulations, we show how shifting mutations from branches to speciation events impacts inference of branching times in phylogenetic reconstruction. Furthermore, the resulting nucleotide alignments are better described by a relaxed than by a strict molecular clock. Thus, elevated mutation rates during speciation potentially explain part of the variation in substitution rates that is observed across the tree of life. [Molecular clock; phylogenetic reconstruction; speciation; substitution rate variation.]

Phenotypic diversification occurs at a higher rate in some clades than in others (Simpson 1945; van Valen 1985; Ricklefs 2006; Rabosky et al. 2007; Jansson and Davies 2008) and similarly, there is substantial variation across evolutionary lineages in the rate of molecular evolution (King and Wilson 1975), such as that of nucleotide sequences (Nabholz et al. 2008; Bromham 2011; Dowle et al. 2013; Sung et al. 2016). As a consequence, studies attempting to reconstruct the phylogeny of a clade often find that the sequence data do not support the assumption of a strict molecular clock, that is, constant substitution rates across lineages. For such cases, phylogenetic inference software allows one to use a relaxed molecular clock (Drummond et al. 2006; Lepage et al. 2007), which assumes that the substitution rate varies between lineages according to a statistical distribution such as a gamma or lognormal distribution. However, the relaxed molecular clock thus introduces at least one additional degree of freedom, namely the variance of the distribution of substitution rates (although some argue that an uncorrelated relaxed clock in effect adds one additional degree of freedom *per branch* (Dornburg et al. 2012; Bromham 2019; Zhang and Drummond 2020; Douglas et al. 2021). Moreover, the relaxed clock is a rather ad-hoc solution with little underlying biological reasoning (but see Lartillot and Poujol 2014; Lartillot et al. 2016; Saclier et al. 2018).

A first formal test to detect the impact of speciation on sequence evolution was formulated by Avise and Ayala (1975, 1976), who distinguished gradual evolution from "punctuated equilibria" by comparing sequence evolution in species-rich and species-poor clades. Whereas Avise and Ayala found no evidence for increased sequence evolution in species-rich clades, others did, in tetrapods (Mindell et al. 1989, 1990), sauropsids (Eo and DeWoody 2010), and angiosperms (Duchene and Bromham 2013; Bromham et al. 2015). Furthermore, substitution rates have been found to be positively associated with diversification rates (Fontanillas et al. 2007; Eo and DeWoody 2010; Lanfear et al. 2010; Lanfear et al. 2010; Ezard et al. 2013, but see Goldie et al. 2011).

Several biological processes acting at speciation could lead to accelerated sequence evolution, including, but not limited to founder effects, bottlenecks, inbreeding, hybridization, selection for an increased mutation rate, divergent selection, and local adaptation (Venditti and Pagel 2010). Here, we explore how such processes driving sequence evolution during speciation events might affect phylogenetic reconstruction; we posit that differences in apparent substitution rates between lineages are due to processes acting exclusively or predominantly during speciation. Due to (effectively) random extinction of lineages, different branches of a reconstructed phylogeny will differ in how often they experienced such short episodes of accelerated substitution rates, resulting in differences in apparent substitutions rates along these branches. Our approach is 2-fold: first, we explore whether the inclusion of substitutions during speciation affects phylogenetic inference, and, if so, which aspects of the inferred phylogenetic tree are affected. Second, we explore whether substitutions during speciation can explain variation in estimated substitution rates.

Materials and Methods

We propose a model where substitutions accumulate not only along the branches of a phylogeny but also at speciation events, including not only the internal nodes of the phylogeny but also those pruned from the phylogeny by extinction. We first make the standard assumption that gradual sequence evolution along a phylogenetic branch can be modeled as a time-homogeneous Markov process with substitution matrix:

$$\mathbf{Q}=\begin{bmatrix} -\mu_{AT}-\mu_{AC}-\mu_{AG} & \mu_{AT} & \mu_{AC} & \mu_{AG} \\ \mu_{TA} & -\mu_{TA}-\mu_{TC}-\mu_{TG} & \mu_{TC} & \mu_{TG} \\ \mu_{CA} & \mu_{CT} & -\mu_{CA}-\mu_{CT}-\mu_{CG} & \mu_{CG} \\ \mu_{GA} & \mu_{GT} & \mu_{GC} & -\mu_{GA}-\mu_{GT}-\mu_{GC} \end{bmatrix},$$

where μ_{ij} denotes the mutation rate from nucleotide i to nucleotide j. The transition probabilities of nucleotide substitutions after time t of gradual sequence evolution are then given by the matrix

$$\mathbf{P_a}(t)=\exp(\mathbf{Q}t),$$

where the subscript **a** indicates anagenetic change, that is, gradual accumulation of substitutions over time. This matrix can be multiplied with an initial probability vector at time $t=0$ to yield the probabilities for each of the four nucleotides at time t.

In addition to gradual sequence evolution over time, we assume that sequences may change rapidly during speciation. We can thus assume another matrix $\mathbf{P}_c$ (subscript c for "cladogenetic") that describes the nucleotide transition probabilities during a single speciation event.

The processes that may accelerate sequence evolution during speciation, such as founder effects, bottlenecks, inbreeding, hybridization, and adaptation to novel environments, may well result in different kinds of substitutions than those that take place over time in established species. However, for mathematical convenience, we will here assume that we can write:

$$\mathbf{P}_{\mathrm{c}}=\exp(\mathbf{Q}\tau),$$

where τ is a parameter that measures the effect of substitutions during speciation. In other words, we assume that nucleotide sequence evolution is only accelerated during speciation events, but not qualitatively altered: the μ_{ij} used in $\mathbf{P}_c$ must be identical to those used in $\mathbf{P}_a$. The acceleration is then measured by parameter τ: a single speciation event causes as much sequence evolution as τ years of gradual evolution over time within each lineage. Thus, larger values of τ correspond to a larger experienced effect at the nodes, similar to sequence evolution along a branch of length τ. For $\tau=0, \mathbf{P}_c$ becomes the identity matrix, and our model reduces to the standard model of sequence evolution that only assumes substitutions along phylogenetic branches. Important to note here is that both daughter lineages resulting from a speciation event experience substitutions independently (see the Supplementary material available on Dryad at https://doi.org/10.5061/dryad.t1g1jwt1x for a model where substitutions in both daughter lineages are dependent on each other). Furthermore, we emphasize that we assume the speciation process to happen in a similar fashion across a tree, assuming an identical τ for all nodes in the tree. Later versions of the model could potentially relax this assumption, provided independent information about speciation dynamics.

For simplicity, we assume in our simulations that sequence evolution can be modeled as a Jukes–Cantor (JC) process (Jukes and Cantor 1969), for which **Q** is given by:

$$\mathbf{Q}=\begin{pmatrix} -\frac{3\mu}{4} & \frac{\mu}{4} & \frac{\mu}{4} & \frac{\mu}{4} \\ \frac{\mu}{4} & -\frac{3\mu}{4} & \frac{\mu}{4} & \frac{\mu}{4} \\ \frac{\mu}{4} & \frac{\mu}{4} & -\frac{3\mu}{4} & \frac{\mu}{4} \\ \frac{\mu}{4} & \frac{\mu}{4} & \frac{\mu}{4} & -\frac{3\mu}{4} \end{pmatrix}.$$

Several existing software packages (e.g., the R package phangorn, Schliep 2011; the python module pyvolve, Spielman and Wilke 2015; the R package phylosim, Sipos et al. 2011) provide algorithms to simulate sequence evolution along the branches of the phylogeny, given a rooted phylogeny and a root sequence (e.g., some arbitrary sequence assumed to represent the ancestral sequence), by applying the transition matrix sequentially along the phylogenetic tree. Here, we extend this methodology to also include substitutions accumulated at the nodes of the phylogeny. We implemented this in the R package "nodeSub," available via https://CRAN.R-project.org/package=nodeSub.

Testing the Impact of Node Substitution Models Using Simulations

To identify the amount of error in phylogenetic inference caused by assuming a (relaxed) molecular clock when substitutions actually arise (in part) during speciation, we simulated sequence evolution on known trees and then reconstructed the phylogeny from the simulated sequences, assuming strict and relaxed molecular clocks. We simulated sequence evolution with the node substitution model introduced above, with various degrees of sequence accumulation at the nodes of the tree (τ), and with various extinction rates. We then compared the resulting trees with the original true tree using a number of statistics: the gamma statistic (Pybus and Harvey 2000), the beta statistic (Aldous 2001), the mean branch length (Faith 1992; Clarke and Warwick 2001), crown age, the normalized Lineages Through Time (nLTT) statistic (Janzen et al. 2015) and the Jenson–Shannon distance metric comparing the Laplacian spectrum (Lewitus and Morlon 2016).

Phylogenetic reconstruction was performed with BEAST2 (Bouckaert et al. 2019) using the R package *babette* (Bilderbeek and Etienne 2018). BEAST2 inference was performed using default priors (see the Supplementary material available on Dryad for an example XML file), with a birth–death prior as tree prior (or a Yule prior if the extinction rate was zero), the JC nucleotide substitution model, and a strict or relaxed clock model. The BEAST chain was run for 10 million steps, whilst sampling a tree every 5000 steps. After completion, the first 10% of the chain was discarded as burn-in.

Assessing Error in Phylogenetic Reconstruction: The Twin Tree

Errors observed when comparing with the true tree include both errors incurred by the node substitution model chosen, and errors accumulated in the phylogenetic inference process even when the models used in inference are identical to those generating the data (e.g., stochasticity in substitution accumulation, stochasticity in phylogenetic tree creation). Furthermore, additional effects arising during alignment simulation might affect our findings, such as the impact of parameter values (sequence length, substitution rate, birth rate, death rate), and of multiple substitutions at the same site (the node-density effect) as well as potential biases or interactions between summary statistics. To correct for these effects, so as to isolate the error induced by using a node substitution model from other sources of error, we inferred a phylogenetic tree for a twin alignment (*sensu* Bilderbeek et al. 2021). This twin alignment has exactly the same number of accumulated substitutions as the original alignment. The total number of substitutions is tracked during the simulation of the substitution model and not just the resulting number of variable sites in the alignment. The twin alignment is based on the same true tree, but instead of using a node substitution model to generate the alignment, it results from using either a strict clock or relaxed clock substitution model. Using this twin alignment, we performed phylogenetic reconstruction with BEAST2 as for the original alignment and estimated the same summary statistics for the posterior distribution of trees. The error introduced by the node substitution model is then the difference between the error of the node substitution posterior and the error in the twin posterior. In summary, we use this twin approach as a control treatment, in order to correct for all potential sources of additional error other than that of our proposed substitution model.

Obtaining a Twin Alignment

We generated a twin alignment conditional on a phylogeny, a node substitution model, and a mutation rate. Because an alignment generated using a node substitution model (with $\tau > 0$) has accumulated substitutions at the nodes in addition to those along the branches, the overall number of substitutions accumulated is higher than for an alignment simulated using the same mutation rate and a model with substitutions only on the branches. Thus, in order to generate a twin alignment that contains the same amount of information (substitutions), we increased the mutation rate. We did this by calculating the estimated time spent at the nodes, relative to the time spent on the branches, and using this as an estimate of the expected fraction of the number of substitutions on the nodes, relative to the number of substitutions on the branches, assuming that substitutions accumulate at the same rate on both branches and nodes. That is, the mutation rate used in generating the twin alignment is calculated as:

$$\mu_{\mathrm{twin}} = \mu \left(1 + \frac{\tau(2N+H)}{\sum t_{\mathrm{branch}}} \right), \tag{1}$$

where μ is the mutation rate used in the node substitution model, τ is the time spent on the node, N is the number of internal nodes in the tree, H is the number of hidden nodes in the tree, and $\sum t_{branch}$ is the total branch length of the tree. The factor $2N$ arises from the independent accumulation of substitutions during a node substitution event for both daughter lineages.

During simulation of node substitution alignments, we kept track of the substitutions accumulated at each node and branch, which allowed us to directly measure the contribution of substitutions accumulated at the nodes [i.e., $\tau(2N+H)$] relative to those accumulated at the branches (i.e., $\sum t_{\mathrm{branch}}$) This provided us with an estimate of μ_{twin}, and with an estimate of the total number of substitutions arising during simulation of the alignment. We then used the obtained estimate for μ_{twin} to generate twin alignments, again tracking all substitutions, until we obtained an alignment exactly matching the number of accumulated substitutions of the alignment simulated with the node substitution model.

Node-Density Effect

The method we used to simulate substitutions along branches (and nodes) ignores repeated mutations at the same site, which may lead to a node-density effect. Because the node-density effect can mask the effect of node substitutions, we made sure in two distinct ways that our results are not affected by this effect. Firstly, by using a twin alignment, any resulting node-density effects are mirrored in the twin alignment as well, ensuring that any additional errors picked up do not reflect errors induced by the node-density effect. Secondly, we repeated our analysis using a different simulation method that explicitly tracks repeated mutations for the JC model (see Supplementary material available on Dryad for details and results). We find that this more explicit simulation method yielded virtually identical results to the more general approach described in the main text.

Simulation Settings

We generated birth–death trees with varying degrees of extinction rate d in [0, 0.1, 0.3, 0.5] and a single speciation rate of $b = 1$. Trees were simulated conditional on 100 tips, using the function sim.bd.taxa from the R package TreeSim (Stadler 2011). Across all settings, we simulated sequences of 10 kb, with $\mu = 0.001$.

Varying the Time Spent on the Nodes Relative to the Crown Age

We varied τ in [0, 0.01, 0.05, 0.1, 0.2, 0.4] times the crown age (e.g., when the crown age of the simulated tree is 3 myr, $\tau =$ [0, 0.03, 0.15, 0.3, 0.6, 1.2] myr). Again, for each combination of τ and extinction ($d = [0, 0.1, 0.3, 0.5]$) we simulated 100 trees and for each tree, we generated one node substitution alignment and one twin alignment.

The Impact of Tree Balance

In unbalanced trees, some terminal branches are connected by many more past branching events to the root of the tree than are other terminal branches. Hence, we expect that balance of a tree might have a substantial effect on the error in phylogenetic inference: less balanced trees are expected to have higher error. To test this, we compared fully balanced ($\beta = 10.0$) with extremely unbalanced "caterpillar" trees ($\beta = -2$). We did so by simulating the branching times of a birth–death tree and assigning these to a fully balanced or fully unbalanced topology. Thus, the only difference between the trees is the topology. Then, for both the balanced and unbalanced tree a node substitution alignment was generated, with the same number of total substitutions, and setting τ as a function of crown age.

As an extra check, we also generated a node substitution alignment for the original birth–death tree from which the branching times were used. For all three alignments, we inferred a phylogenetic tree as in the other scenarios and compared the error in phylogenetic inference. For caterpillar trees with extreme unbalance we were unable to calculate the Laplacian Spectrum, hence we omitted the Laplacian Spectrum summary statistic in this analysis.

Support for Strict and Relaxed Clock Models

To test whether the alignment originating from a process with node substitutions was better described by a relaxed than by a fixed clock model, we repeated the analysis, but now inferred the marginal likelihood of the relaxed clock and strict clock models using the "NS" package for BEAST2, which applies Nested Sampling to obtain the marginal posterior likelihood for both models (Russel et al. 2019). We used the function "bbt_run" from the *babette* package (Bilderbeek and Etienne 2018) in combination with the function "create_ns_mcmc" from the *beautier* package (Bilderbeek and Etienne 2018). This performs a Nested Sampling MCMC run using BEAST2 (an example XML file outlining the default settings used can be found in the Supplementary material available on Dryad), which runs until convergence is detected. Then, we converted the obtained marginal likelihoods to a relative weight for each model (by dividing both marginal likelihoods by their sum), which allows for the comparison of posterior support for each model across parameter settings and trees.

Empirical Example

As an illustration of the impact of node substitutions on a real phylogeny (rather than a simulated one), we applied our model to an empirical data set which is feasible under the assumption that there is no extinction (see below). The data set consists of sequence data (Ast 2001; Fitch et al. 2006) of 35 species of Australian monitor lizards, of the family of *Varanidae*, which covers all known species of *Varanidae* occurring in the Indo-Australian realm. For each species, mitochondrial DNA was retrieved from GenBank, consisting of ND4, 16S, and CO1 genes. Sequences were aligned using the "—auto" setting for mafft (Katoh and Standley 2013) and concatenated for ease of use. Assuming a substitution rate of 3.35×10^{-9} per site (Eo and DeWoody 2010), we inferred a maximum likelihood tree from the alignment, using the R package phangorn (Schliep 2011), assuming a generalizd time reversible (GTR) model of substitution. This yielded a reference tree, assuming no node substitutions.

Then, we made use of a new feature of phangorn (version 2.7.1.2, added upon our request), which allows for the incorporation of node substitutions under the assumption that there is no extinction, because then all nodes where node substitutions occur are observable in the tree, and the branches connected to these nodes can all be extended by a length of τ. Thus, in this new version of phangorn, one can specify a value for τ, and compute the tree likelihood (i.e., the probability of the alignment given the tree and the substitution model parameters) for this value. We explored the tree likelihood for values of τ ranging from 10^{-4} to 1 myr, for the JC and GTR substitution models.

RESULTS

Summary Statistics

We compared summary statistics of trees inferred from alignments using the node substitution model, with summary statistics of twin trees inferred from alignments with identical information content, but generated without the node substitution model (e.g., with only substitutions along the branches, and a fixed clock rate). We find that summary statistics that are influenced by branching times are affected (Fig. 1, gamma, nLTT statistic, mean branch length, and crown age). For these summary statistics, we find an increased difference with increasing τ. The impact of extinction seems to be limited, as the error in these summary statistics remains around the same level, regardless of the extinction rate used.

The Impact of Tree Balance

Tree balance clearly influences the sensitivity of inference to node substitutions (Fig. 2). The inference error is larger for unbalanced trees, again only for the gamma and the nLTT statistic. Fully balanced trees show slightly less error than birth–death trees. Overall, all three types of trees show an increased error when

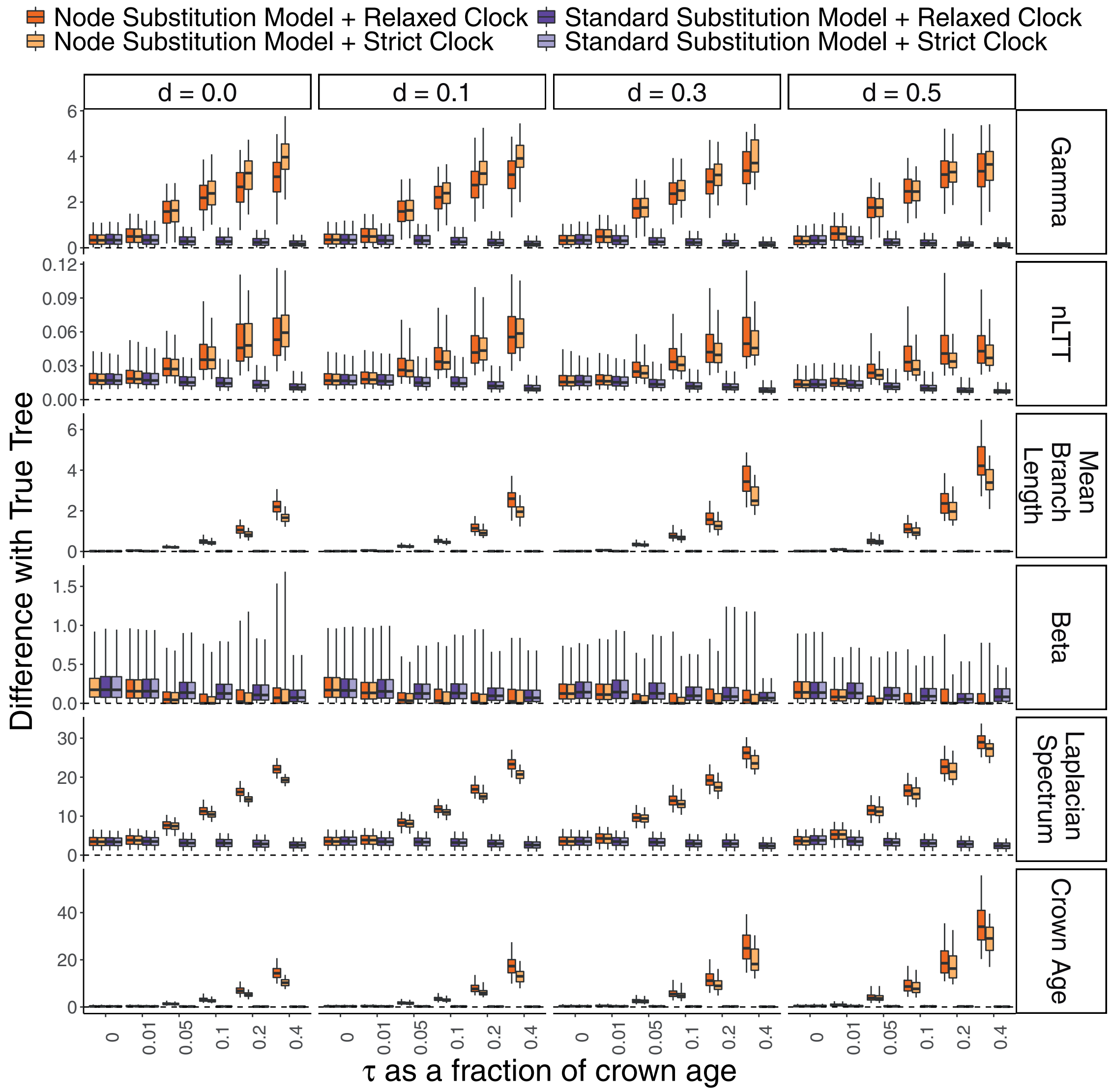

FIGURE 1. Difference in summary statistic values for trees inferred from an alignment generated with node substitutions, and twin trees that were inferred from an alignment generated without node substitutions, both compared with the summary statistics of the true tree. We explored τ (the amount of time spent on each node) as a fraction of crown age (horizontal axis), and the impact of extinction (d, columns). The summary statistics are the beta and gamma statistic, Laplacian spectrum, mean branch length, nLTT statistic, and crown age. The figure shows that with increasing τ, trees inferred from an alignment generated with node substitutions show larger differences with the true tree than trees inferred from an alignment generated without node substitutions. Differences with the true tree are larger for trees inferred using the strict clock model than for those using the relaxed clock model, but only for the alignment generated with node substitutions.

alignments are generated using the node substitution model. Errors are particularly large for the beta statistic, but that is expected because it measures the topological features of the tree that we modified artificially.

Support for Strict and Relaxed Clock Models

We compared the relative support for each model, reflected by the relative weight of the marginal likelihood. With an increasing amount of time spent at the nodes τ, the median weight of the relaxed clock model increases for the node substitution alignment, with generally (across extinction rates) a higher weight than the strict clock model for values of τ that are equal or larger than 0.1 times the crown age (Fig. 3). For the twin alignment, the strict clock model is preferred, as expected, because this is the generating model.

For low values of τ (smaller than 0.1 times the crown age), we do not find any effect of the balance of the tree on the marginal likelihood of the relaxed clock model (Fig. 4), in line with our finding above. However, for intermediate values of τ (0.1 and 0.2), we find that unbalanced trees tend to have a higher marginal weight for the relaxed clock model. For high values of τ (0.4), we find that the marginal weight for the relaxed clock model is always higher, regardless of the balance of the tree.

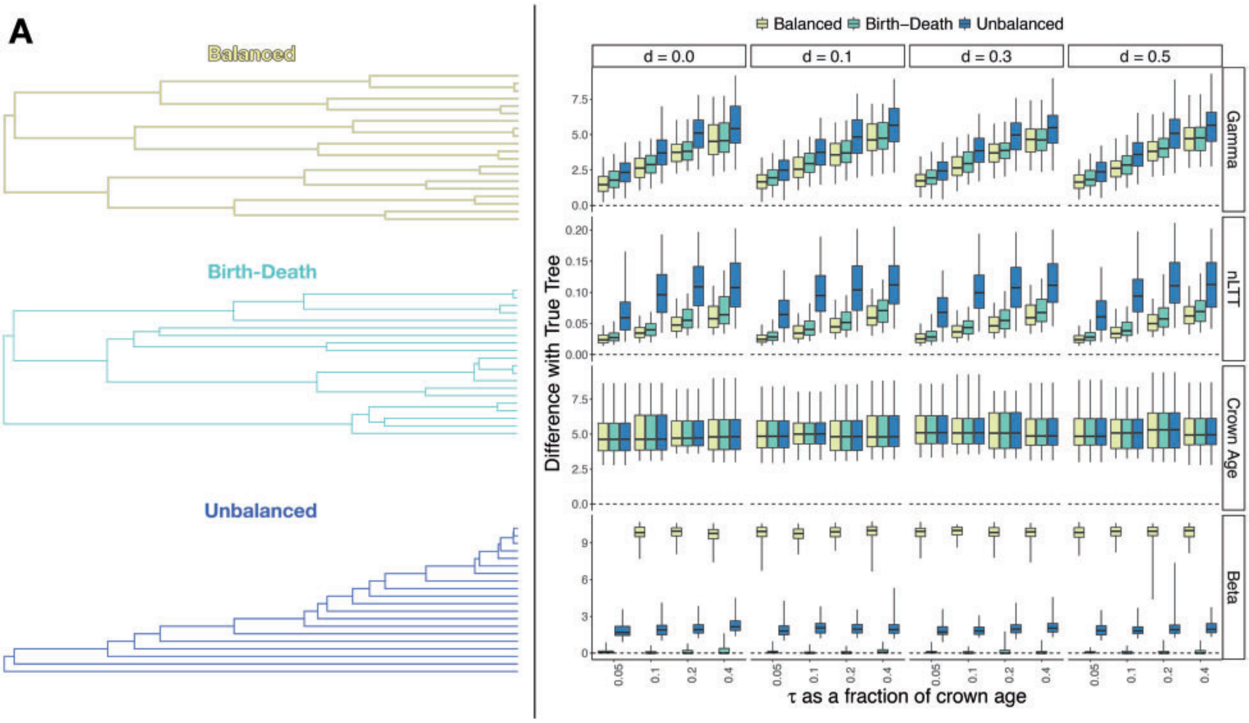

FIGURE 2. Effect of the node substitution model for phylogenies differing in balance (a) example plots of a randomly generated birth-death tree (top), a fully balanced tree generated using the same branching times as the birth–death tree (middle), and a very unbalanced tree generated using the same branching times as the birth–death tree (bottom). Shown are trees with 20 tips for illustrative purposes, but results in B are from trees with 100 tips. b) Difference in summary statistic with the true birth–death tree for phylogenetic trees inferred from alignments generated using the node substitution model on either balanced, unbalanced, or random trees. We explore τ as a fraction of crown age (horizontal axis), and the impact of extinction (d, columns). The dotted line indicates zero difference with the true tree. The summary statistics are the beta and gamma statistic, nLTT statistic, and tree height. Balanced and birth–death trees tend to have similar inferred error, whereas unbalanced trees differ strongly, with a much larger error for the gamma and nLTT statistic.

Empirical Example

We first verified that extinction was low by fitting a birth–death model to the maximum likelihood tree inferred without node substitutions. Here, we found an estimate for d/b of 0 (95% confidence interval [CI]: −1.65, 0.24), and for $b-d$ of 0.013 (95% CI: 0.0095, 0.01889), which together indicate that extinction is low indeed. This provides justification for using the likelihood computations in the new version of phangorn which assumes that the extinction rate is zero.

Next, we inferred τ and found a nonzero estimate for τ of 0.74 myr when using the JC model, and 2.53 myr when using the GTR model (Fig. 5a,d). Comparing the resulting trees for these ML estimates, we find that the crown age of the tree is inferred to be much lower. Without node substitutions, the crown age is estimated to be 48.22 myr for the JC model and 46.98 myr for the GTR model. When including node substitutions, the crown age shifts to 33.9 myr for the JC model and 34.1 myr for the GTR model. Rescaling of the trees relative to the crown age (Fig. 5c,e) shows that including node substitutions does not merely rescale all branching points proportional to the newly inferred crown age, but that the relative positions of the different branching points shift as well.

DISCUSSION

We have shown that an increased substitution rate during speciation events potentially provides a mechanistic explanation of variation in substitution rates across the branches of phylogenetic trees. Trees inferred from alignments generated with this substitution model differ substantially from trees inferred from alignments generated with a standard substitution model, especially concerning branching times. Furthermore, we find that this new substitution model can potentially explain widespread support for relaxed molecular clocks.

If sequence evolution mainly occurs during speciation, this would lead to a correlation between species richness and substitution rate. However, this correlation could also be an artifact of phylogenetic reconstruction known as the node-density effect (Fitch and Bruschi 1987; Fitch and Beintema 1990). The node-density effect reflects the inability to detect multiple mutations occurring at the same site, thus causing an underestimate of the true branch length, especially for longer branches where the probability of multiple mutations occurring at the same site is higher. Because species-rich parts of phylogenies tend to have shorter branches, sequence evolution in these species-rich parts is less underestimated than in species-poor parts, causing a correlation between

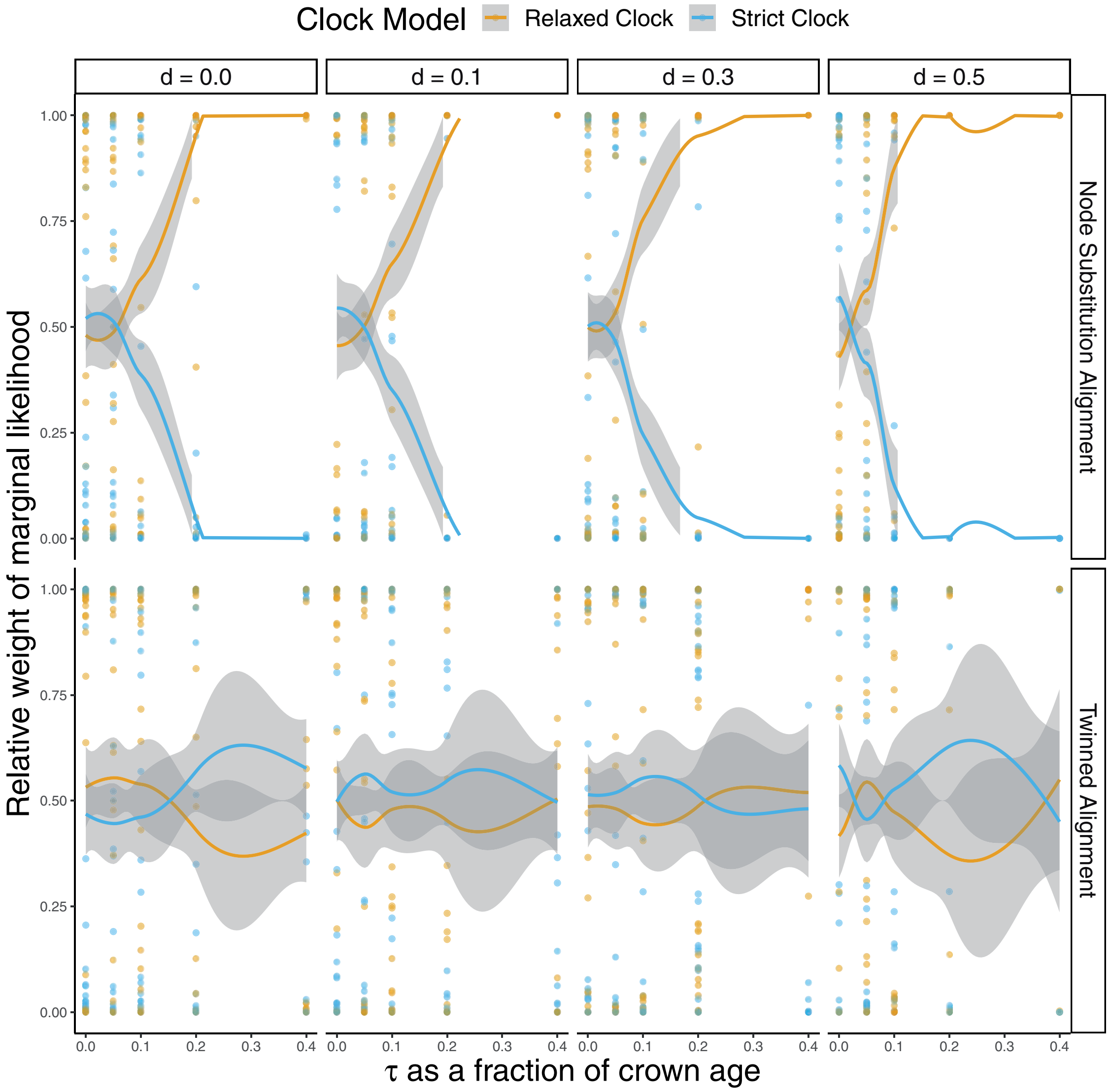

FIGURE 3. Marginal likelihood weight of the relaxed and strict clock model for varying time spent on the nodes (τ), where τ gis chosen as fraction of the crown age. Alignments generated with a node substitution model (top row) are compared with alignments generated without node substitutions (bottom row). Per parameter combination, 100 replicate trees were analyzed. Because many dots are plotted on top of each other, we use solid lines to indicate the best fitting locally estimated scatterplot smoothing (LOESS), and the 95% confidence interval (grey shaded area) of the LOESS curve. As the time spent on the nodes increases, posterior support for the relaxed clock model increases, but only if the alignment was generated with a node substitution model.

the number of observed substitutions and species diversity. Pagel et al. (2006) tested for the impact of speciation events, and of the node-density effect in 122 phylogenies, spanning 4 taxa. Using previously demonstrated methodology to detect the node-density effect (Webster et al. 2003; Venditti et al. 2006), they showed that in 57 of the 122 examined phylogenies, they could detect a signature of increased sequence evolution during speciation events. However, this was the result of the node-density effect in 22 out of these 57 phylogenies. Here, disentangling sequence evolution during speciation from confounding factors such as the node-density effect, but also stochasticity in tree simulation, stochasticity during alignment simulation, and error or bias in tree inference, has proven to be a nontrivial endeavor. In order to assess the impact of node substitutions, we therefore separated error due to assuming an alternative substitution model from the errors introduced by the factors mentioned above. To do so we extended the twinning approach (introduced by Bilderbeek et al. 2021) to assess the impact of choosing a different tree prior to explore the impact of a different substitution model. The twinning approach succeeds by replicating the chosen analysis pipeline, but using *control* data that have been generated using known models and priors. The impact of the node substitution model then follows from the difference between results obtained with the node substitution model and results obtained

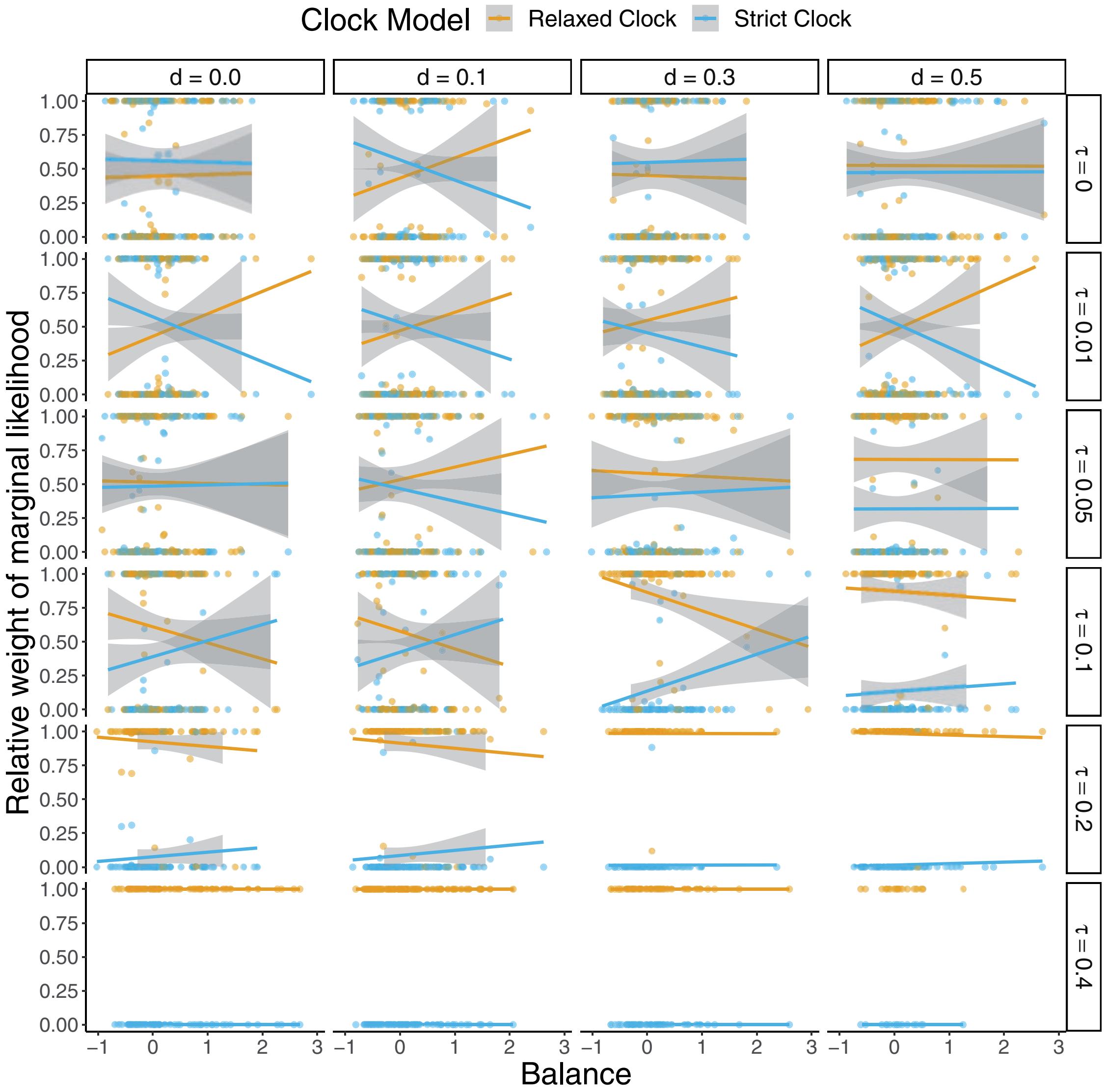

FIGURE 4. Marginal likelihood weight of the relaxed clock model for trees of varying balance, split out across different extinction rates (d = [0, 0.1, 0.3, 0.5]), and time spent on the nodes (τ), where τ gis chosen as fraction of the crown age (e.g., τ=0.1 reflects a node time of 10% of the crown age). Per parameter combination, Solid lines indicate the best-fitting linear regression and the 95% CI (grey-shaded area) of regression. With increasing values of τ, the relative weight of the relaxed clock model becomes larger. For smaller values of τ, the relative weight of the relaxed clock model is negatively correlated with the balance of the tree, with unbalanced trees having a higher relative weight.

with the twin (control) pipeline: errors are then due to model misspecification, and not stochastic uncertainty produced by the analysis pipeline. Our results show thus that when we correct for the background effects of (amongst other factors) the node-density effect, we observe strong effects of node substitutions. However, we expect that for small values of τ, the impact of node substitutions might become comparable to the node-density effect, and disentangling these sources of substitution rate variation might become difficult.

One might expect that a high extinction rate, by elevating numbers of hidden nodes, would lead to a greater impact of node substitutions. It may therefore be counterintuitive that in our simulation study, we did not find such an effect of higher rates of extinction. However, we conditioned our alignments on the same total number of substitutions, to ensure that alignments with and without node substitutions contained the same information content. Thus, with higher extinction and hence more hidden nodes, relatively fewer substitutions occur on the observed nodes. Because the number of hidden nodes is proportional to branch length (Equation 2), the number of hidden nodes is interpreted as substitutions on the branches. Potentially, this provides a way to distinguish between phylogenetic models: although every constant-rate birth–death model has a corresponding zero-extinction model with a time-varying speciation rate that yields the same probability of the reconstructed tree (Nee et al. 1994; Louca and Pennell 2020), the resulting alignments under the node

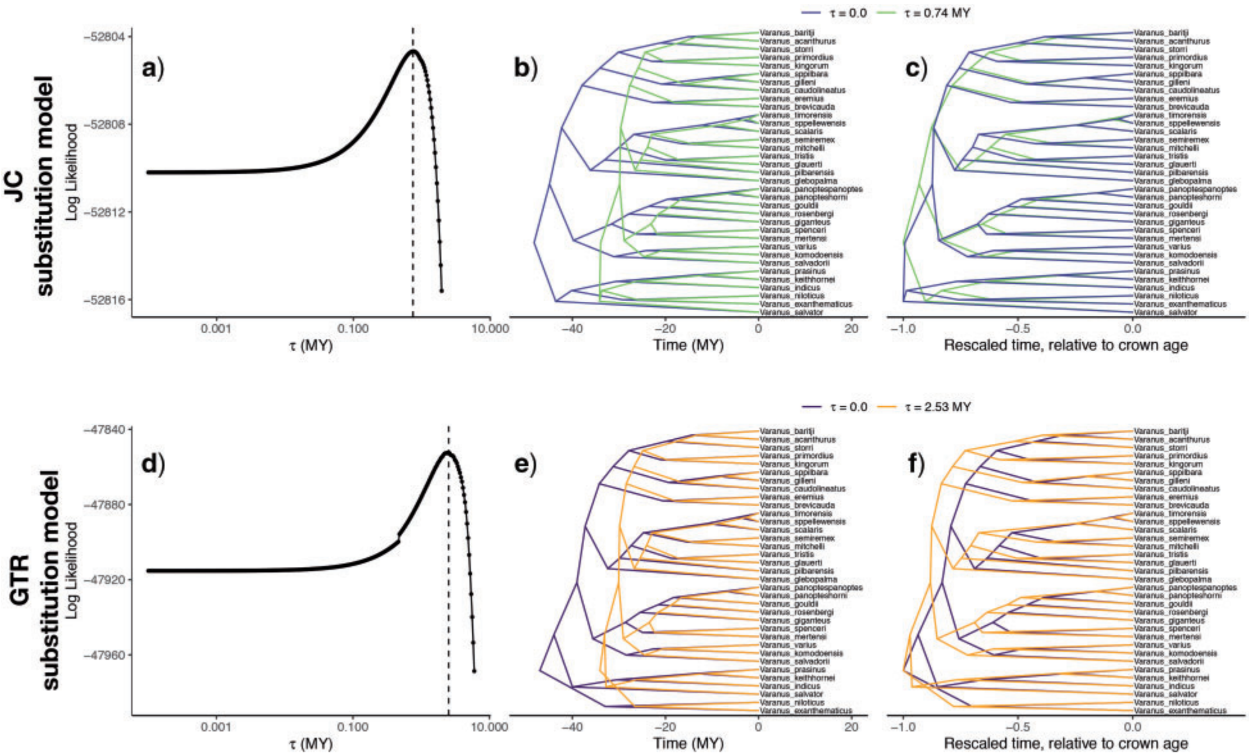

FIGURE 5. Results applying our node substitution model to an alignment consisting of all 35 species of Varanidae occurring in the Indo-Australian realm, assuming no extinction. Likelihood profiles with respect to τ of the JC (a) and the GTR model (d) are shown. b and e), The inferred trees for both $\tau = 0$ and for the maximum likelihood value of τ, for the JC and GTR substitution models, respectively. c and f), These same inferred trees, but here the branching times have been rescaled with respect to the crown age.

substitution model will not be similar. Because the birth–death tree includes extinction events, substitution patterns will be different from those of the tree generated with the time-varying speciation rate model.

Distinguishing phylogenetic models will become more feasible if some of the simplifying assumptions made here are relaxed. The model we propose here takes the simplest form, assuming a JC (Jukes and Cantor 1969) substitution matrix, identical substitution rates, identical substitution matrices between nodes and branches, and constant birth–death rates over time. These assumptions were made as a most basic starting point, but can be relaxed in future analyses, for instance by introducing a different substitution matrix at the nodes, or by studying the effect of node substitutions on trees that are generated by diversity-dependent speciation rates (Etienne et al. 2012). By starting with the most tractable version of the node substitution model, we have provided a first proof of concept of the potential impact of node substitutions without overcomplicating matters.

Previous methods have applied rather ad-hoc corrections to account for differences in substitution rates across different branches in the same phylogeny, typically referred to as the "relaxed clock" approach. These methods provide satisfying statistical solutions to account for variation in substitution rates, but refrain from providing biological explanations for this observed phenomenon. The node substitution model we introduce here provides this explanation: branches that have accumulated a number of "hidden" branching events, for example, speciation events of species that have subsequently gone extinct, have a higher number of accumulated substitution events during these "hidden" speciation events. When we compared the marginal likelihood of the relaxed clock model versus the strict clock model for alignments generated with the node substitution model, we found that marginal likelihoods for the relaxed clock model are much higher. This indicates that our proposed process of accumulating substitutions during speciation events can generate patterns in the alignment that are picked up by phylogenetic methods as evidence for a relaxed clock model, without actually using a relaxed clock model.

The notion of accelerated evolution during speciation events ties in closely with the theory of punctuated equilibrium; where Eldredge and Gould (1972) proposed that evolution perhaps is not a gradual process, but rather a process with distinct bursts of phenotypic and morphological change. Their theory was influenced by ideas like Lerner's "genetic homeostasis" (Lerner 1954), which had earlier inspired Mayr (1954) to suggest that the formation of new species involves "genetic revolutions." Our framework provides a step toward being able to test this notion, where information on the estimated fraction of substitutions accumulated at the nodes can directly inform us about whether the majority of substitutions is accumulated over long periods of time in established lineages (e.g., along branches), or during speciation (e.g., at the nodes).

To infer whether node substitutions really occur, we should fit the node substitution model to empirical sequence alignments, and find a nonzero estimate for τ. However, the computation of the likelihood of our model (and estimation of associated τ values), is nontrivial because it requires integration across the enormous state space of complete trees (trees including extinct species). Manceau et al. (2020) have taken a first step toward formulating such a likelihood. They introduced an alternative solution for punctuated equilibrium-like patterns in molecular evolution through the implementation of spikes of substitution, for example, moments in time at which there is an increased substitution rate. They let these moments occur at speciation events and also model the probability of such an event happening at a speciation event (rather than assuming that they always occur, as we did here). However, they have to assume both the topology and branching times to be fixed. We have provided an alternative inference approach that does not require topology or branching times to be fixed, but assumes extinction to be zero. The absence of extinction greatly reduces computational complexity and allows us to use maximum likelihood to infer the most likely tree, using the R package *phangorn* (Schliep 2011). We inferred a phylogenetic tree via maximum likelihood for 35 species of *Varanidae* and recovered a nonzero estimate for τ. Furthermore, we found that the resulting tree was substantially different from a tree with $\tau = 0$; not only were the crown age and branching times drastically different, the relative position of the branching times was also affected. As expected from our simulation results, topology of the tree was not affected.

In order to be able to infer the phylogenetic tree, we had to make several restricting assumptions. Firstly, as stated above, we had to assume extinction to be zero. This ignores any effects that hidden nodes might have. Yet, it seems unlikely that in the 40 myr since the origination of the clade of *Varanidae*, no extinctions took place. Secondly, we were limited to only using a strict clock (other clocks are not yet incorporated in phangorn). Future work could explore how the incorporation of a relaxed clock in the maximum likelihood framework we used impacts our findings, particularly whether using a relaxed clock could mitigate some of the differences we recovered.

The present study aims to demonstrate that substitutions accumulated during speciation might explain the prevalence of the relaxed molecular clock in phylogenetic analysis. We found that substitutions during speciation may profoundly affect phylogenetic inference: if node substitutions are not taken into account, branching times tend to be overestimated, even when a relaxed clock is used to counteract the effect of "hidden nodes." This suggests that the incorporation of a node substitution model may improve phylogenetic inference.

With our introduction of the node substitution model, we hope to stimulate discussion on the biological explanation of variation in substitution rates within and across phylogenies. Furthermore, we hope to have set a first step in improving our understanding of this variation and improving phylogenetic inference as a whole.

Data Availability

R code to simulate the node substitution model has been made available as an R package called "nodeSub," and can be found here: https://CRAN.R-project.org/package=nodeSub. All code used in simulations, and scripts used to visualize obtained results, are available on dryad via: https://doi.org/10.5061/dryad.t1g1jwt1x.

Supplementary Material

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.t1g1jwt1x.

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