Investigating the reliability of molecular estimates of evolutionary time when substitution rates and speciation rates vary

ANDREW M. RITCHIE[1 *], XIA HUA[1 2], LINDELL BROMHAM[1]

1. *Research School of Biological Sciences, Australian National University, Canberra, ACT 2600, Australia*
2. *Mathematical Sciences Institute, Australian National University, Canberra, ACT 2600, Australia*

***** Corresponding Author:

Andrew M. Ritchie

Research School of Biological Sciences, Robertson Building, 134 Linnaeus Way

Australian National University, Canberra, ACT 2600, Australia

Ph: +61 2 6125 5111

Email: Andrew.Ritchie@anu.edu.au

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Abstract

Background

An accurate timescale of evolutionary history is essential to testing hypotheses about the influence of historical events and processes, and the timescale for evolution is increasingly derived from analysis of DNA sequences. But variation in the rate of molecular evolution complicates the inference of time from DNA. Evidence is growing for numerous factors, such as life history and habitat, that are linked both to the molecular processes of mutation and fixation and to rates of macroevolutionary diversification. However, the most widely used methods rely on idealised models of rate variation, such as the uncorrelated and autocorrelated clocks, and molecular dating methods are rarely tested against complex models of rate change. One relationship that is not accounted for in molecular dating is the potential for interaction between molecular substitution rates and speciation, a relationship that has been supported by empirical studies in a growing number of taxa. If these relationships are as widespread as evidence indicates, they may have a significant influence on molecular dates.

Results

We simulate phylogenies and molecular sequences under three different realistic rate variation models – one in which speciation rates and substitution rates both vary but are unlinked, one in which they covary continuously and one punctuated model in which molecular change is concentrated in speciation events, using empirical case studies to parameterise realistic simulations. We test two commonly used “relaxed clock” molecular dating methods against these realistic simulations to explore the degree of error in molecular dates under each model. We find average divergence time inference errors ranging from 12%

of node age for the unlinked model when reconstructed under an uncorrelated rate prior, to up to 93% when punctuated simulations are reconstructed under an autocorrelated prior.

Conclusions

We demonstrate the potential for substantial errors in molecular dates when both speciation rates and substitution rates vary between lineages. This study highlights the need for tests of molecular dating methods against realistic models of rate variation generated from empirical parameters and known relationships.

1.Background

Understanding the timescale of evolution is critical to researching the processes that generate and shape the diversity of life on Earth. Analysis of DNA sequences offers the possibility of investigating the tempo and mode of evolution of all extant lineages, not just those that have a detailed fossil record. The earliest molecular dating analyses concluded that the average rate of molecular evolution was relatively constant over time in some proteins because of an apparently linear accumulation of amino acid differences when compared between species (1, 2). However, it has since become apparent that the average rate of molecular evolution can be influenced by a wide range of species traits, such as generation time, body size and longevity (3, 4), by macroevolutionary processes such as net diversification rate (5-10) and potentially also by environmental features like temperature (9-11). If the average rate of molecular evolution varies substantially and consistently between lineages, then we ideally need to incorporate rate variation in models of molecular evolution in order to accurately infer divergence times among species (12).

A wide variety of models have been developed to account for different patterns of among-lineage molecular rate variation (13-16). In the first instance, these models differ in how many different evolutionary rate parameters are estimated for different parts of the phylogeny. For example, a 'strict clock' model estimates only one rate parameter that applies to every branch in the tree, while a 'local clock' model estimates additional rate parameters for subsets of branches (17-19), and a 'relaxed clock' provides for a separate rate on every branch (20). Models also differ in how the rates on different branches are related to one another. Some models, notably in the penalized-likelihood framework, aim to minimize the overall variation of branch-specific rates or the differences between adjacent branches (21, 22). For Bayesian phylogenetic methods (23, 24), branch-specific rates are drawn from a prior probability distribution that determines which rates and patterns of relative rate variation are considered *a priori* more probable. We refer to this prior as the rate prior. Common variants include uncorrelated models, in which each branch-specific rate is an independent draw from a distribution with a common mean (20); and autocorrelated models, in which the mean of the distribution for each branch-specific rate depends on the rate drawn for its parent branch (25, 26).

Testing over the last two decades has overall supported the robustness of divergence time estimates under the Bayesian methodology to the choice of rate prior, provided that enough separate rate parameters are allowed and sufficient calibrating information is provided (27-33); but see (34). However, these studies rely heavily on simulated data or on specific and usually well-calibrated empirical examples, whereas simulations have indicated that different rate priors may give different results when calibrating information is poorer (30). Changing the rate prior in these circumstances could lead to differences in empirical dates and associated causal hypotheses. Examples of empirical studies where the choice of

rate prior appears to be a factor in differing age estimates include influenza viruses (35), the age of grasstrees (36) and the origins of Metazoa (37).

Currently used priors on rate variation are generally either uncorrelated, so that each lineage-specific rate is drawn independently from a common distribution, or autocorrelated so that lineage-specific rates depend evolve from the rates of their parent lineages according to a stochastic, random walk. However, the factors shaping variation in rate of molecular evolution are complex: in addition to random variation in rates between lineages, there is clearly also a systematic component influenced by many factors including species traits, population dynamics, environment and evolutionary history (38). Evolutionary rates among eukaryotes appear to be faster at lower elevations and latitudes (9, 39, 40) but slower in arid climates (41). Life history covaries with molecular rates across multiple axes, including body size (42-45), fecundity (46) generation time (47).and longevity (48, 49). Associations have also been observed between substitution rates and more complex species traits, such as parasitism (50), flightlessness (51) and sexual competition (52). Furthermore, substitution rates are the result of underlying microevolutionary processes, and may therefore be affected by population size (53-56).

In addition to covarying with other evolving traits, there is evidence that variation in molecular rates can be associated with macroevolutionary patterns. Relationships between the relative size of sister clades, representing their net diversification rates, and their molecular rates have been detected in several studies, include in reptiles (8, 57), birds (7, 58), flowering plants (6, 59, 60) and across Metazoa (61). Several studies have also noted relationships between the genetic divergence of species in a phylogeny and the number of intervening speciation events, which may also represent examples of this phenomenon (62-64).

The mechanism behind observed correlations between diversification and molecular evolution remains a matter of debate. One line of argument proposes that higher molecular rates could promote speciation by speeding the development of reproductive isolation (65). In this hypothesis, subpopulations become sporadically isolated over time and may accumulate alleles that may be effectively neutral on their own but deleterious in combination (66, 67). In this case, greater rates of molecular evolution could lead to faster development of reproductive isolation during periods of isolation, leading to a greater average rate of speciation (68, 69). Alternatively, higher molecular rates could reduce extinction rates by accumulating more standing genetic diversity which provides a buffer against changing environment by allowing more rapid selective response (70). Speciation rates and molecular rates could also be related through a common driving factor, such as climate or environmental productivity (5, 10). In the other causal direction, speciation itself could increase average molecular rates by producing rapid bursts of evolution (62, 63, 71). Suggested mechanisms for an increase in substitution rates during speciation involve an increase in fixation rates of mildly deleterious alleles and linked neutral mutations due to drift, or large-scale genomic divergence caused by an increase in the frequency of disruptive genomic events such as polyploidy or chromosomal rearrangements (71).

An association between substitution rates and diversification rates could affect the performance of existing divergence time estimation methods (e.g. 72). In particular, Bayesian methods divide their specification of prior beliefs about the evolutionary process between the rate prior, the prior on divergence times and tree topology (sometimes referred to as the tree prior or node time prior), and calibrating distributions on the ages of individual speciation events (23, 73). These priors are essential to estimating divergence time, because genetic

distances between sequences are the product of rate and time (the total number of character differences between related sequences). This means that even if genetic distances are correctly inferred on a phylogeny, rate and time are unidentifiable without additional constraints on joint values they can hold (74, 75). The rate prior, node time prior and calibrating distributions place either soft or hard constraints on these values, allowing researchers to infer evolutionary history on an absolute timescale. However, this means that inferred divergence times are always subject to the choice of rate, time and calibration priors even with large amounts of sequence data (76, 77). Most commonly used Bayesian molecular dating methods do not propose *a priori* relationships between the rate prior and the tree prior, so the joint distribution implied by these priors will assign relatively higher probability to long-lived, fast-evolving lineages and short-lived, slow-evolving lineages than would be expected when rates and times are related. It is therefore possible that an unmodelled relationship between rates and times could produce distributions of molecular rates and speciation times that differ substantially from those implied by common Bayesian priors, leading to widespread error in estimates of evolutionary dates.

Here, we investigate the potential influence of speciation-related rates of molecular evolution on the reliability of molecular dating studies. Rather than assuming only one specific model of the association between diversification rates and rates of molecular, we model a range of possible causal links. We include three alternative models of the association between rates of molecular evolution and speciation rates. In the first, both speciation rates and substitution rates vary over the phylogeny, but neither influences the other. In the second, we model continuous variation in a linked manner, which might represent either direct influence of one on the other (65) or an indirect association between speciation rates and substitution rates, for example if they are both influenced by environmental differences (5,

10). The third model is a punctuated model in which bursts of substitution are associated with speciation events (62, 63, 71, 78). The proportion of all substitutions associated with such bursts has recently been shown to impact Bayesian inference of clade crown ages under an uncorrelated lognormal rate prior (72).

In this study, we simulate the evolution of molecular sequences under all three models of the link between speciation and rate of molecular evolution – unlinked, continuous coevolution and a punctuated model - allowing for independent variation in molecular and speciation rates, and observe the average effects on errors in Bayesian divergence time estimates. In order to make our test have real-world significance, we also base our simulations, as far as possible, on empirically determined relationships between rates of molecular evolution and diversification rate. We parameterize our models using values taken from empirical inferences on bird data (79, 80) or which reproduce established empirical relationships between rates of molecular evolution and diversification rate in birds (81). Since the choice of rate prior may affect the results of phylogeny reconstruction (34), we also examine the effect of divergence time reconstruction under uncorrelated and autocorrelated rate priors. We reconstruct divergence times on these simulated data sets and examine the effects of different forms of correlated molecular and speciation rates on the performance of molecular divergence time reconstruction.

2. Results

2.1 Simulation design

We refer throughout to simulated phylogenies, which are known without error, as the 'simulated trees', and to the phylogenies inferred from analyzing the sequences evolved along each of these simulated phylogenies as the 'reconstructed trees'. Comparison of the reconstructed trees to the simulated trees allows us to evaluate the degree to which phylogenetic inference methods correctly infer the known evolutionary history of the simulated data.

Our overall design is summarised in Figure 1. For our simulation study, we first generate phylogenetic trees in units of absolute time with evolving rates of speciation and molecular evolution. We then simulate DNA sequences along these phylogenies. Finally, we infer dated trees from the alignment of these sequences using standard methods in Bayesian phylogenetics and compare the inferred tree to the simulated tree from the known evolutionary history from each simulation.

We simulate sets of 50 phylogenetic trees and nucleotide sequence alignments under each of three different models describing the association between speciation rates and rates of molecular evolution: one "Unlinked" model in which molecular and diversification rates each vary independently, as well as two alternative mechanisms for an association between rates of molecular evolution and speciation rates ("Punctuated" and "Continuous": see Table 1). We do not consider models in which molecular rates affect rates of extinction, so in our simulations, extinction rates remain constant over the tree. For all models, we first simulate the phylogenetic tree in continuous time, together with speciation and substitution rates that vary along the tree. From this, we derive branch lengths in expected genetic divergence (substitutions/site). Finally, we simulate the evolution of nucleotide sequences along these phylogenies.

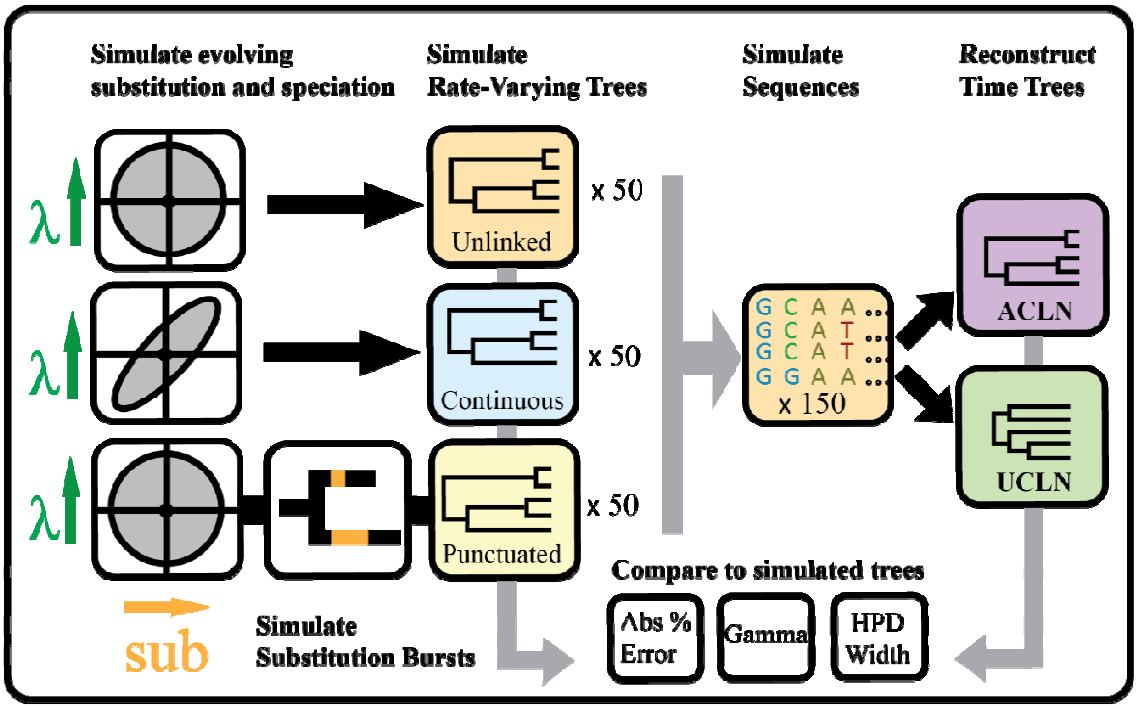

Figure 1. Flowchart of study design. Trees with branch lengths in units of time are simulated under three simulation conditions. For the Unlinked model, birth rates ( )and substitution rates (sub) vary independently, while for the Continuous model they are correlated. For the Punctuated model, trees are simulated as for Unlinked, then some of the branch length is redistributed to form bursts of substitutions occurring upon speciation. Trees and branch lengths are then used to simulate molecular sequence alignments. Time trees are then reconstructed under two rate priors, UCLN and ACLN, and compared to the original, simulated trees to determine the error (Medain abs % error), uncertainty (HPD width) and bias towards over- or under-estimation (Gamma).

Table 1. Simulation design.

Simulation type	Speciation rates covary with substitution rates?	Instantaneous covariance	Punctuated burst of substitutions at nodes?
Unlinked	*No*	0	*no*
Continuous	*Yes*	0.0044	*no*
Punctuated	*No*	0	*yes*

We lay out the conditions for each set of simulated trees. Each set consisted of 50 trees. Lineage-specific speciation rates and substitution rates evolve continuously through time via Brownian motion in all three studies. The three simulations differ in whether the covariance between speciation and substitution rates is greater than zero in the Brownian motion and whether an additional burst of substitutions occurs at nodes of the tree (speciation events).

In the Unlinked model, we simulate continuous variation in both substitution rate and speciation rate through time, but neither affects the other. Substitution and speciation rates evolve independently by Brownian motion, where each lineage continuously adds a separate random increment to its substitution rate and speciation rate in each of many small time intervals. The Unlinked model serves as a baseline to determine the level of uncertainty in molecular dates caused by the random variation in substitution and diversification rate when there is no mechanistic link between the two. In the Continuous model, speciation rates and substitution rates coevolve with each other continuously over time. The coevolution is simulated as a correlated Brownian motion, where the random increments in speciation and molecular rates added over time are dependent on one another. Consequently, substitution rate and speciation rate tend to increase or decrease together. For this model, we also ensured realism simulated many data sets with different covariance parameters, and selected a set of 50 trees that reproduced the empirical correlation of (7) between clade size and branch length (Supplementary Methods S1.1). In the Punctuated model, the genetic divergence along a branch is the sum of a gradual accumulation of substitutions at a rate that evolves as in the

Unlinked model, and a burst component at each speciation event. A speciation event occurs each time a new lineage is born, whether any of its descendants are observed in the present or not. The amount of additional branch length due to the burst is independently and randomly drawn for each speciation event. For this model, we condition our simulations to reproduce the relationship observed in (62). There it was observed that the total number of substitutions occurring along root-to-tip paths in their sample of trees correlates with the number of nodes along the path. Although this relationship could also be produced by a mechanism similar to the Continuous model (82-84), we model the mechanism proposed in (62) that the node-associated substitutions are produced in bursts associated with speciation events.

We derived plausible speciation, extinction and substitution rates for our simulations using the avian phylogeny in (85). We do not intend to make any inferences about the actual evolution of birds or the accuracy of inferred divergence times. Birds are chosen only because the relationships among traits, molecular rates and diversification rates are well studied, so that we can use these to give us an empirical ground for choosing parameter values. Branch-specific diversification rates have been estimated for many of the lineages in this tree in (80), while substitution rates have been inferred in (79) assuming covariation of molecular rates and life history. Speciation rate and molecular rate values taken from avian data have been previously used to inform realistic simulations on ecological assemblies for the purpose of estimating the effect of dating error on phylogenetic diversity estimates (86), and we make use of similar procedures here. A relationship between molecular rates and net diversification rate has also been established for birds (7, 8). We use this fact to select our Continuous data set by simulating multiple data sets and varying the covariance parameter until the relationship appears. Full details of how we used these studies to determine realistic parameterisations for our simulations are available as Supplementary Methods S1.1.

2.2 Validation of tree simulations

The simulation procedure produced trees with average characteristics similar to the avian clades used to parameterize the study (80, supplementary data, 85). Across all data sets, the mean root date of simulated trees was 28.4 million years, compared to a range of 23.8-35.6 million years for bird clades with 70-80 taxa. Simulated lineage-specific speciation rates at the tips were on average slightly lower than the speciation rate of similar clades in the bird tree (80). The range of simulated speciation rates on log scale was 0.009-0.92 compared to a range of 0.034-2.25 average log lineages/million years for bird clades with 70-80 taxa, similar taxon number to our simulated trees. The range of speciation rates within each tree was also similar to the range across the avian clades with similar taxon number, with an average magnitude of 0.44 log lineages/million years compared to 0.32-2.21 for avian clades with 70-80 taxa.

2.3 Error in reconstructed trees

We used three metrics (3.2), the gamma statistic (3.3) and the sister pair analysis (3.4) to assess the accuracy of the reconstruction of trees simulated from the Unlinked model, the Continuous model, and the Punctuated model, under uncorrelated lognormal (UCLN) and autocorrelated lognormal (ACLN) models of among-lineage rate variation (Table 2).

Table 2. Summary of results.

Rate prior	Simulation	Node age error (%)	Topo. error	HPD width (%)	Gamma Statistic	Sister pair analysis
UCLN	*Unlinked*	6.3–30.9	0–14	43–29	-1.46–1.09	Null
UCLN	*Continuous*	6.7–30.3	0–16	35–605	-1.52–0.20	Null
UCLN	*Punctuated*	8.8–80.4	0-8	42–154	-2.16–3.92	Negative
ACLN	*Unlinked*	11–417	Fixed	84.9–217.8	-7.57–4.08	Positive
ACLN	*Continuous*	9.4–170	Fixed	73.5–319.4	-4.92–3.02	Positive
ACLN	*Punctuated*	13.7–397.8	Fixed	76.7 - 144.1	-7.95–2.88	Positive

Summary of reconstructed tree characteristics. Trees simulated under three models: Unlinked , Continuous, and Punctuated. Molecular dates were then reconstructed under two methods, the uncorrelated lognormal model (UCLN) and the autocorrelated lognormal model (ACLN). We calculated three metrics for each tree and summarised these over each simulation type. The metrics were median absolute % error of node ages, topological error (Topo. Error) by number of true bipartitions not reconstructed, and uncertainty by mean highest posterior density interval width as a proportion of reconstructed node age. We compared the gamma statistics of the reconstructed trees to the simulated trees in order to detect any systematic bias in node age error. We also performed sister pair analysis to test if we can detect the positive correlation between speciation rate and substitution rate from the reconstructed trees.

First, we examined the median absolute percentage error (MAPE) in the ages of all nodes within each tree whose topology were correctly reconstructed (Figure 2). For trees reconstructed under UCLN, the average (min–max) MAPE was 12.2% for Unlinked simulations (6.3–30.9%). Errors for the Continuous simulation were similar at 14.2% (6.7–30.3%). Average MAPE for the Punctuated simulation was higher than for Unlinked trees at 20.3% (8.8–80.4%). For trees reconstructed under the ACLN in PAML, MAPE values were higher and range of errors were larger than for the UCLN in BEAST. Average MAPE for Unlinked trees was 96.6% (11–417%), compared to 42.7% (9.4%–170%) for Continuous trees and 84% (13.7–397.8%) for Punctuated trees.

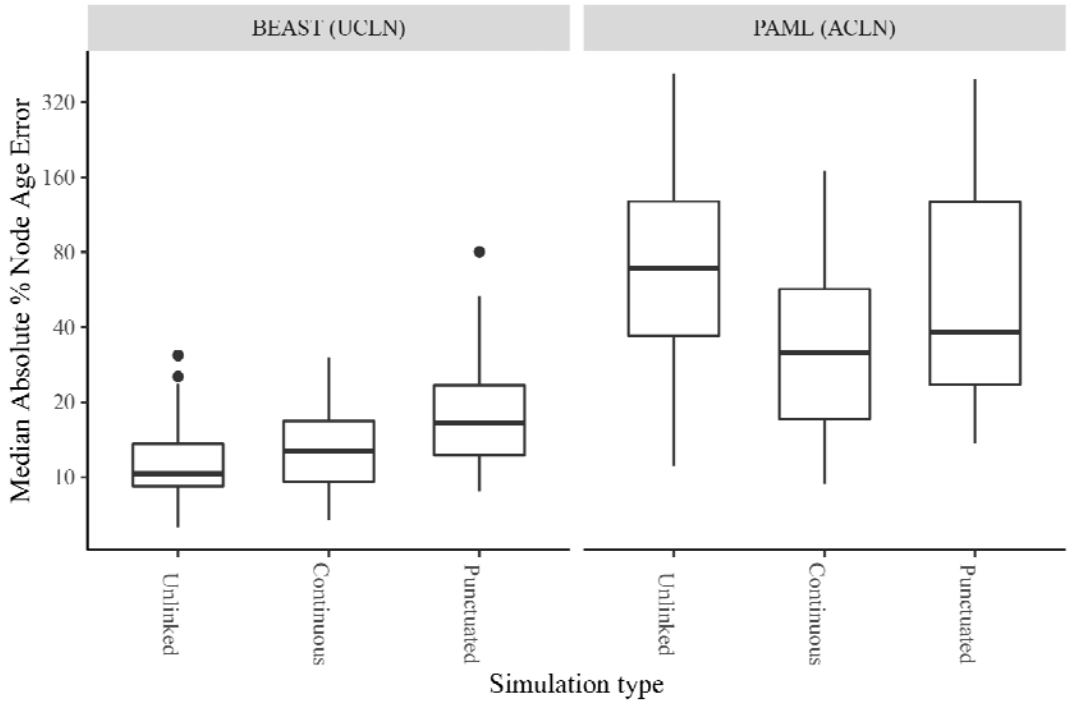

Figure 2. Median absolute percentage error of node times (on log scale) reconstructed from data sets simulated under three simulation types with different relationships between molecular evolution and speciation. The mean error is calculated only for branches whose bipartitions are shared between the true and inferred trees, and therefore ignores errors in topology which may occur in the BEAST analyses. The three simulation models are Unlinked (instantaneous covariance of molecular rates and speciation rates = 0), Continuous (instantaneous covariance = 0.0044), and Punctuated (instantaneous covariance = 0, bursts of substitutions added at speciation events). Topologies and node times were reconstructed using two different analytical methods, the uncorrelated lognormal 'relaxed clock' rate prior (UCLN) in BEAST 2, and the autocorrelated lognormal rate prior (ACLN) in PAML.

Second, for trees reconstructed under the UCLN model in BEAST, we also investigated the degree of topological error via the Robinson-Foulds distance between the true and estimated tree (87). The median background topological error for the Unlinked simulations was 6 bipartitions (0–14), compared to 5 (0–16) for the Continuous model

(Figure 3). Error was lower for the Punctuated model with a median distance of 2 bipartitions (0-8).

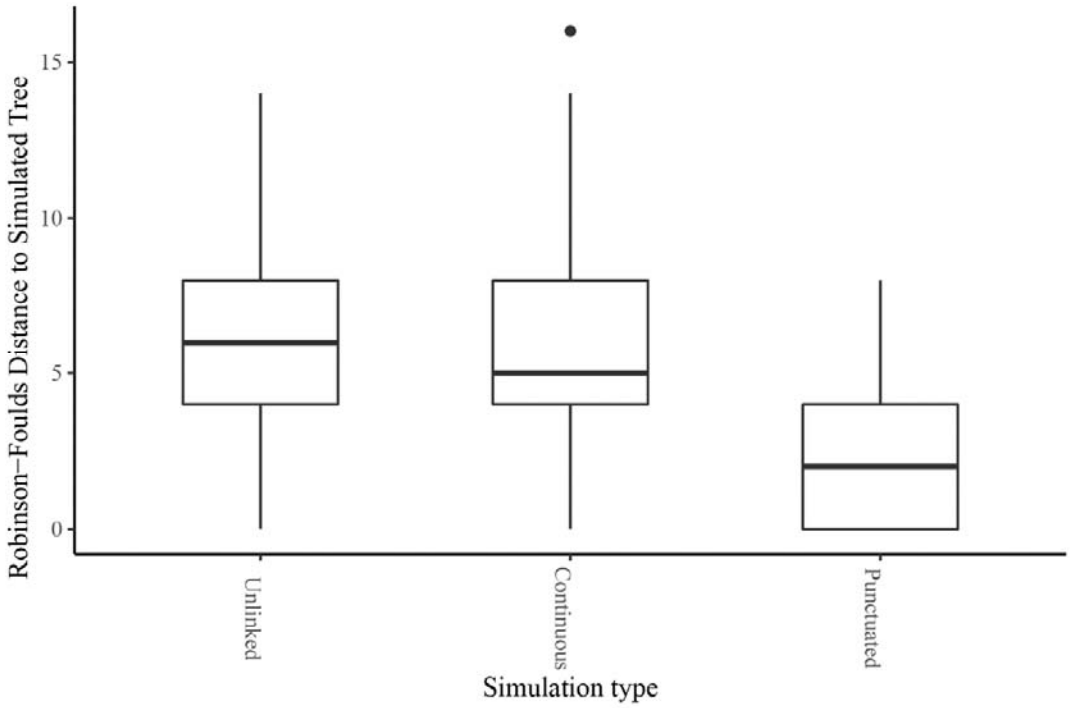

Figure 3. Topological error of trees reconstructed from data sets simulated under three simulation types with different relationships between molecular evolution and speciation. Topological error is given by the Robinson-Foulds distance (number of non-shared bipartitions). This distance incorporates both topological and branch time errors. The three simulation models are Unlinked (instantaneous covariance of molecular rates and speciation rates = 0), Continuous (instantaneous covariance = 0.0044), and Punctuated (instantaneous covariance = 0, bursts of substitutions added at speciation events). Topologies and node times were reconstructed using the uncorrelated lognormal 'relaxed clock' rate prior (UCLN) in BEAST 2. No results are available for the autocorrelated lognormal rate prior in PAML because the topology was fixed for the analysis.

Third, we calculated the uncertainty of node age reconstruction as reported by the method via highest posterior density intervals. Under UCLN (Figure 4), the average HPD interval for the Unlinked trees had a width 60% of the reconstructed age of its associated node (43 – 29%). For the Continuous trees this was 108% (35 – 605%), and for the Punctuated trees it was 70% of node age (42 – 154%). Under ACLN, the average HPD width for Unlinked trees was 120.7% of node age (84.9 – 217.8%); for Continuous trees, 112.3% (73.5% – 319.4%); and for Punctuated trees, 107.0% (76.7 – 144.1%).

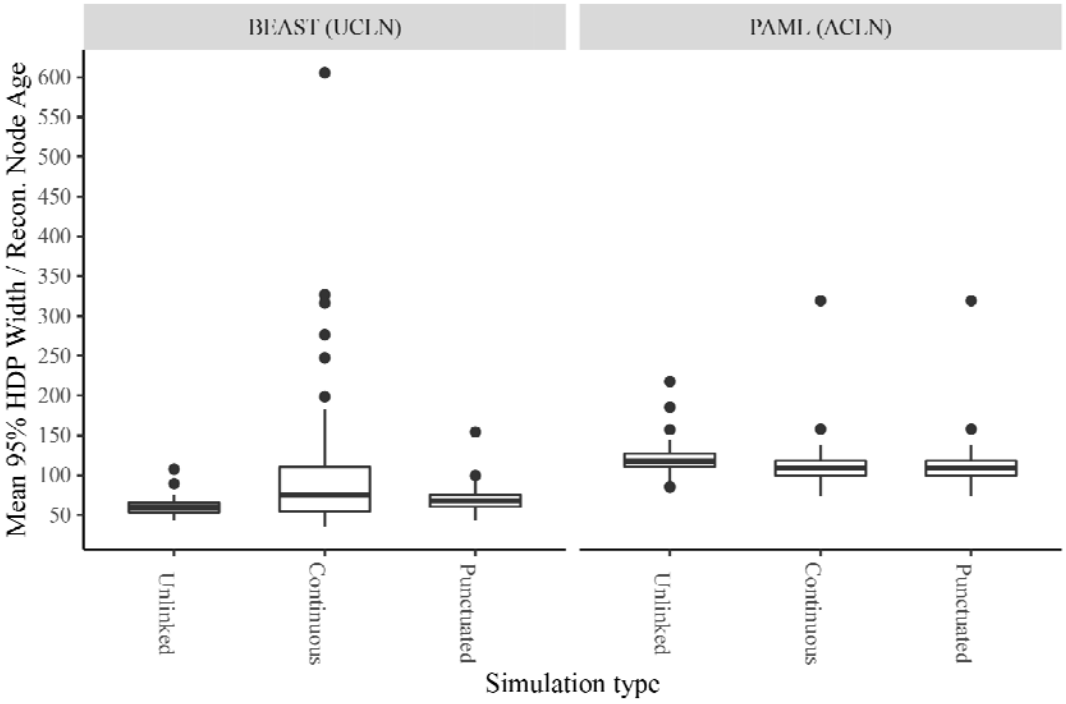

Figure 4. Uncertainty of trees reconstructed from phylogenies simulated under three models with different relationships between molecular evolution and speciation, in width as a percentage of reconstructed node age. Precision is measured by averaging the width of 95% highest posterior density (HPD) intervals as a proportion of reconstructed node height across each tree, with a larger score indicating less precise estimates. The three simulation models

are Unlinked (instantaneous covariance of molecular rates and speciation rates = 0), Continuous (instantaneous covariance = 0.0044), and Punctuated (instantaneous covariance = 0, bursts of substitutions added at speciation events). Topologies and node times were reconstructed using two different analytical methods, the uncorrelated lognormal 'relaxed clock' rate prior (UCLN) in BEAST 2, and the autocorrelated lognormal rate prior (ACLN) in PAML.

2.4 Distribution of reconstructed vs simulated node ages

If the difference in gamma values between reconstructed and true trees is negative, the reconstructed tree has shorter internal branches and longer terminal branches than the true tree, while if it is positive the reconstructed tree has longer internal branches and shorter terminal branches than the true tree. Under the UCLN, Gamma for the reconstructed Unlinked trees was distributed evenly around the gamma of the simulated tree with a mean shift of +0.11 (-1.46 – + 1.09; Figure 5). This distribution was skewed negative (shorter tips) relative to the simulated tree for Continuous trees with a mean of -0.44 (-1.52 – +0.20). This negative skew was more pronounced for the Punctuated trees at -0.94 (-2.16 – +3.92). Under ACLN, the relative change in gamma was on average negative across all three simulation models. For the Unlinked trees it was -2.78 (-7.57 – +4.08), while for the Continuous trees it was less negative at -1.39 (-4.92 – +3.02), and the Punctuated trees were again skewed negative with a mean of -2.74 (-7.95 – +2.88).

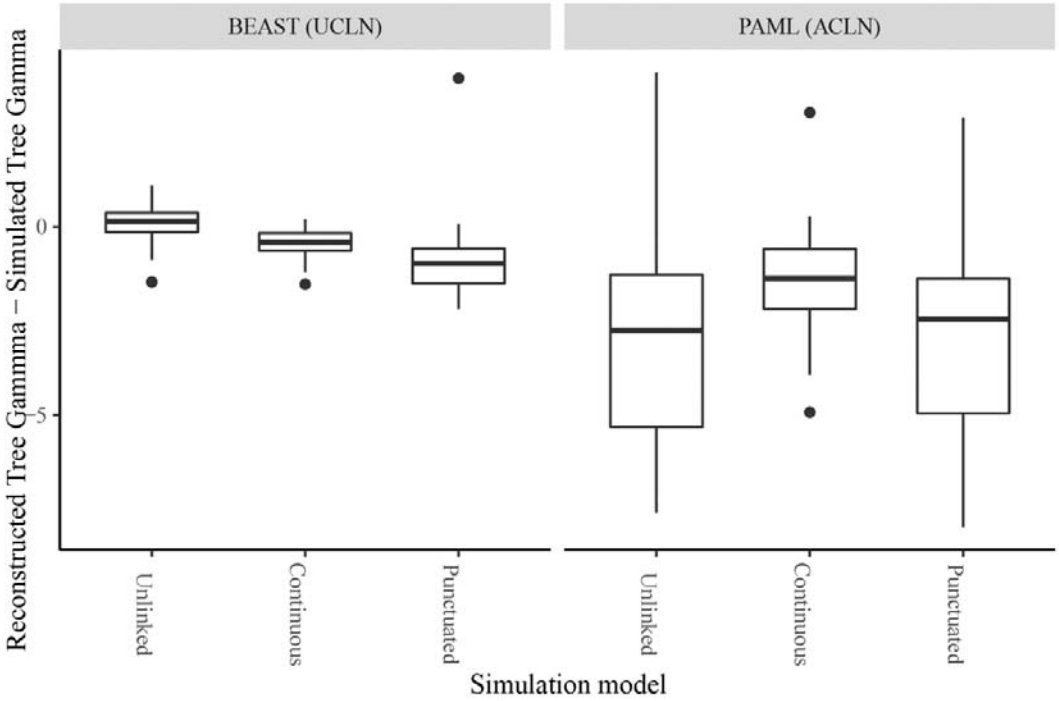

Figure 5. Difference in gamma statistic values between reconstructed and simulated trees, where simulated trees were generated under three models with different relationships between molecular evolution and speciation. Negative scores indicate that reconstructed trees have a greater proportion of their branch times in tips vs internal branches ('tippier') than the simulated tree. The three simulation models are Unlinked (instantaneous covariance of molecular rates and speciation rates = 0), Continuous (instantaneous covariance = 0.0044), and Punctuated (instantaneous covariance = 0, bursts of substitutions added at speciation events). Topologies and node times were reconstructed using two different analytical methods, the uncorrelated lognormal 'relaxed clock' rate prior (UCLN) in BEAST 2, and the autocorrelated lognormal rate prior (ACLN) in PAML.

2.5 Efficacy of sister pair correlation on reconstructed branch lengths

In order to determine whether the simulated relationship between molecular rates and diversification rates could be recovered after tree reconstruction, we regressed log clade size contrasts against contrasts in log median branch length estimates in the data set of sister pair analysis generated from reconstructed trees under UCLN or ACLN for each simulation models (see 2.8). We present the p-value and sign of slope for each regression (Figure 6). Unlinked simulated trees show no significant relationship. Significant positive relationships are found for the Continuous and Punctuated simulations; note that this was a condition for selection of the Continuous data set (Supplementary Methods). When reconstructed under UCLN, the Unlinked data set and the Continuous data set show no significant relationship, while the Punctuated data set gives a significant negative relationship, opposite to what we expect. When reconstructed under ACLN, all three of the Unlinked, Continuous and Punctuated data sets have significant positive relationships between clade sizes and median branch length estimates.

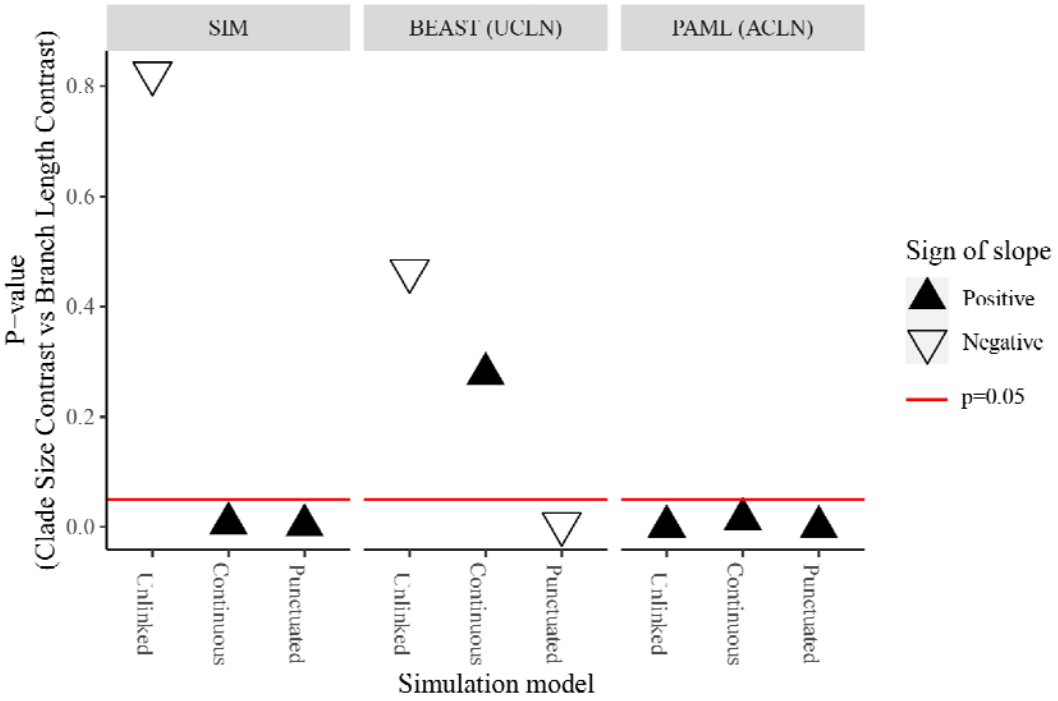

Figure 6. Slope and significance of mock sister pair analyses of clade size contrasts against contrasts in simulated branch lengths (SIM) or median branch length estimates reconstructed from simulated trees. Results are shown for branch lengths reconstructed under the uncorrelated lognormal clock (UCLN) or autocorrelated lognormal clock (ACLN). The three simulation models are Unlinked (instantaneous covariance of molecular rates and speciation rates = 0), Continuous (instantaneous covariance = 0.0044), and Punctuated (instantaneous covariance = 0, bursts of substitutions added at speciation events). Black triangles represent positive regression coefficients, while white inverted triangles represent negative coefficients. The red line indicates significance at the 0.05 level.

3.Discussion

3.1 Errors associated with variation in speciation and substitution rate

The addition of rate-variable 'relaxed clock' models for use as rate priors represents a significant extension to the sophistication of modern molecular dating methods (15). While there is a wide variety of rate-variable molecular dating methods, all of them to date rely on stochastic models of rate variation, such that rates of molecular evolution are expected to evolve randomly over the phylogeny. However, it is clear that real substitution rates do not always vary randomly. Instead, rates of molecular evolution are influenced by species life history, niche and environment and macroevolutionary processes. It is important to examine whether the stochastic models of rate variation can adequately model realistic biological patterns of rate variation (3, 62, 65, 88). In the case of substitution rates, this means that we must test molecular dating methods using rate simulations that are empirically based and incorporate the effects of other biological processes. Here we have focused on testing the impact of widely reported correlations between molecular rates and diversification on the accuracy of molecular date reconstruction (5-8).

We used three models, Unlinked, Continuous and Punctuated, to reflect different views on the possible links between substitution rates and diversification rates – either varying independently; covarying continuously, linked directly by common mechanisms or indirect through shared influences on rate variation; or linked by punctuated bursts of increased substitutions related to speciation events. Rather than choosing parameters arbitrarily, we parameterised our models using realistic values from the literature, or found plausible values by tuning the models to produce data sets that reproduce empirical findings

(Supplementary Methods; 7, 79, 80). We used three different metrics (Table 2) to compare the accuracy of phylogenetic inference using two population 'relaxed clock' molecular dating methods, BEAST (using an uncorrelated rates model) and PAML (using an autocorrelated rates model).

3.2 Unlinked model

The Unlinked model describes the error that can be expected from independent variation in speciation and molecular rates. Inference on trees simulated under this model produced median absolute errors in node age inference of about 12% on average under the UCLN and a very high value of 93% under the ACLN (Figure 2). The largest errors were up to 30% for UCLN and the worst instances for ACLN had errors of over 400%. The Unlinked model produced lower errors under UCLN than the other two models, with an average error of 12% (Figure 2). Gamma differences for the Unlinked model are distributed evenly around zero under UCLN, so there is no overall tendency for nodes to be over- or under-estimated (Figure 4). The sources of error in the Unlinked model are most likely to be violation of the assumptions of rate priors and node time (tree) priors. Variation in speciation rates violates the assumptions of the birth-death node time priors used in both BEAST 2 and PAML, which assume universally constant rates of speciation and extinction (89). Speciation rate variation among lineages also induces more imbalanced trees on average (90), which can lead to bias and loss of precision in node age inferences (91). While the degree and scale on which speciation and extinction rates vary remains contested (92-95), there is increasing evidence that radiations and other forms of variation are a factor in the evolution of many taxa, so that errors of this magnitude may be widespread (96-99). Substitution rates that evolve in an autocorrelated fashion also violate the assumptions of the UCLN, which assumes that the

evolutionary rate of each lineage is independent (20), and this may also induce errors in inference (30, 34).

The causes of the extremely high errors of the ACLN-based phylogenetic inference in PAML for the Unlinked trees (and throughout the study for the other models) are unclear. Examination of the output trees suggests that the most extreme errors are in trees with shallower calibrations, and that the ages of large uncalibrated clades are sometimes overestimated severely. The inferences in PAML and BEAST are not directly comparable due to numerous technical differences, such as the tree topology being fixed in PAML and BEAST placing prior distributions on speciation and extinction parameters, which are fixed in PAML. Nevertheless it is surprising that the performance of the ACLN in PAML was worse than UCLN in BEAST even though the underlying rate variation in these simulations is indeed autocorrelated. There are some indications that ACLN can be difficult to fit to data even when it is the correct model (91, 100). Another possibility is that the sensitivity of the prior to model violation in PAML was enhanced by our use of the approximate likelihood function (101). This is a second-order Taylor approximation to the likelihood evaluated at the maximum likelihood estimates of the branch lengths and other parameters. As discussed by dos Reis and Yang (101), while the approximation is very accurate under normal circumstances when the branch lengths proposed during Bayesian inference are very far from the maximum likelihood values the approximation becomes much less accurate. This can happen when the rate or node time (tree) priors are misspecified. Nevertheless, absolute error rates in other simulation studies have rarely been greater than 20% even with a deliberately misspecified rate prior and more limited calibrating information (30), so the extreme error rates we encountered could be down to analytical factors and not solely due to the rate prior itself.

3.3 Continuous model

The Continuous model has substitution rates and speciation rates that coevolve continuously rather than varying independently. Notably, we chose the degree of covariance that reproduced two well-established empirical phenomena – the correlation of clade size and branch length contrasts detected in a sister pairs analysis by (7), and the widespread relationship between branch lengths and tree nodes discovered by (62). This model produced absolute node time inference errors of 14% under UCLN, slightly higher than the Unlinked model, but the worst errors were up to 30% (Figure 2). Negatively-skewed gamma differences indicate that error generally leads to node ages being overestimated, pushing reconstructed nodes back towards the root (Figure 4). In addition to the error, which is the difference between reconstructed time estimates and the truth, we also examine how the reconstruction methods report their uncertainty about the truth through 95% highest posterior density intervals. Although not greatly different in terms of absolute error, the reported uncertainty of node time estimates was very high for this model under UCLN, with average HPD interval widths more than 107% of node height (Figure 5). This average is driven partly by some outlying trees with very high uncertainty, up to 600% of node height; the median is 75% of node height. Wide HPD intervals can be a sign of conflict between the prior and data, so it may be that covariance between diversification rates and substitution rates tends to produce patterns of branch lengths that are not well described by independent rate and time priors. The practical impact of this level of uncertainty could depend on how it is distributed within the tree and the nature of any downstream inferences; if the question at hand is the dating of major clades then high uncertainty in the age of tips may be tolerable, while the same level of uncertainty in internal branches would render the tree unusable. However, any

macroevolutionary inference that relies on the distribution of nodes throughout the tree would likely be affected (59). Under ACLN, error rates were very high (mean of 43.5%; Figure 2) but were lower for the Continuous simulations than they were for the Unlinked simulations. A reason for this could be that when substitution rate is positively correlated with speciation rate it is also negatively correlated with branch lengths in units of time. Then short terminal branches will tend to have faster rates and build up more substitutions. This in turn could enable lower variance in estimates of rate variation across the tips of the tree, which would aid in inferring the degree of autocorrelation.

3.4 Punctuated model

Levels of absolute error were highest under UCLN when speciation events were associated with large punctuational bursts of molecular evolution (mean of 20.3%, up to 80.4%; Figure 2). This implies that, if real evolutionary processes were to commonly involve such bursts of substitutions associated with speciation, or produce a similar distribution of evolutionary histories and rate variation through some other mechanism, median node age estimates reconstructed under the UCLN rate prior would be generally incorrect. Error rates were also extreme for ACLN (mean 80.4%, max 397.7%; Figure 2). The finding of high error rates in the Punctuated model corroborates a recent analysis (72), which showed that greater proportions of branch length associated with speciation events lead to greater crown age errors. Here, we show that the effect is strong enough to impact the median absolute error of all node ages, even when the crown node is calibrated. The very negative skew of Gamma away from the simulated value shows that these internal node ages are severely overestimated, leading to very distorted trees with long tips (Figure 4).

Despite having the highest degree of branch length error under UCLN, the Punctuated model had the lowest degree of topological error (a median of 2 bipartitions not correctly reconstructed; Figure 3), comparing to the Unlinked and Continuous models (median of 5 and 6 respectively, Figure 3). Intuitively, this could be because rapidly diversifying clades will normally have many short branches, making their internal relationships more uncertain. The Punctuated model adds more substitutions to every branch regardless of its duration in time, and so helps resolve these rapidly diversifying clades. The precise impact of topological error is difficult to interpret, since our investigation does not distinguish among topological errors at different levels and we do not designate any particular nodes as important. But the practical upshot of systematically lower topological error in the simulation with the most severe node age errors under UCLN is that these errors will be difficult to spot. Major topological errors are easier to diagnose by checking against classical taxonomies or other information, while little is usually known about the dates of uncalibrated nodes ahead of time.

As an alternative method of diagnosis, we tried conducting a sister pairs analysis on each set of simulated and reconstructed trees to see if an empirical relationship could be recovered (Figure 6). However, tree reconstruction under either the UCLN or ACLN rate prior disrupts the relationships between branch lengths and clade sizes displayed by the underlying simulated trees (Figure 4). Under the UCLN they are even inverted, giving a negative correlation between clade sizes and branch lengths. This could be related to the fact that lineages with many speciation events have higher variance in branch lengths under UCLN (102) which means their total length could be more strongly constrained by the node time (tree) prior. Conversely, under ACLN we found significant positive relationships between inferred branch lengths and clade size for all three models, even the Unlinked model where speciation rates and substitution rates are not positively associated. A possible cause

of this inference of a positive assertion between diversification rates and substitution rate, even in the absence of an underlying association between the two, is the node-density effect, which results from systematic underestimation of branch lengths with high uncertainty (103). The influence of the node density effect should be reduced by our procedure of selecting an even number of samples in each sister clade, but may not be eliminated by it due to the extremely high error rates under ACLN. The failure to recover the empirical correlation from branch lengths reconstructed as part of a Bayesian dating analysis suggests that this is not a useful way to test for the presence of interactions between substitution rates and speciation rates. For some data sets it may be practical to check for such correlations by reconstructing branch lengths in units of genetic divergence using a fast branch length reconstruction method that does not impose a model of timing or rate variation (e.g. 104, 105-107). However, even if such correlations can be diagnosed, there is currently no available method for correcting any associated bias. This should be an area of active research.

The true role of punctuational bursts or coevolution of speciation and substitution rates in macroevolution is currently a matter of debate. The primary empirical evidence is the analysis of a large selection of empirical phylogenies in (63) and (62). These studies have found that the length of root-to-tip paths in the phylogenies in substitutions per site is frequently higher when there are more speciation events on the path. The relationship remains even after removing the node-density effect that can generate spurious correlations between branch lengths and node densities across clades (103, 108). Regardless of the mechanisms, we have shown that if there were punctuated busts of substitutions associated with speciation events, we could expect it to impact the accuracy of molecular date estimates, since any mechanism that leads to similar patterns of rate variation and apparent correlation between branch lengths and speciation events would produce similar effects.

3.5 Molecular dating methodology

Our study contrasts two common reconstruction methods, rather than varying aspects of data choice. Many aspects of data choice and analytical method could exacerbate or reduce the errors found in this analysis. For example, our alignments are shorter than many used in actual Bayesian phylogenetic analyses but also likely more informative due to being simulated by a simple method without invariant sites or many real sources of substitution model misspecification. Shorter or less informative alignments could lead to greater or more widespread error. Perhaps the most important element that we did not explore was the influence of calibrations. Calibration choice and positioning, as well as the shape of the calibrating distribution used, is often the most influential factor influencing divergence time reconstruction (e.g. 32, 109) and is the source of many disagreements in published molecular dating studies, including high-profile dating studies in birds (110-112) mammals (113, 114), and insects (115-117). Simulation studies have suggested that calibrations can rescue analyses strongly affected by rate prior misspecification (30), although others have found that sufficiently misspecified rate priors cannot be rescued by more calibrations (34). The largest and most data-rich molecular dating studies may have many more calibrations than we implement (e.g. 139, 118). However our trees are smaller than in these studies, and our calibrations are guaranteed to be correctly placed, have hard constraints, and are centered on the true value. We also ensure the root is calibrated, which has been shown to have the largest effect on accuracy in several studies (30, 34, 119). We therefore believe that our method overall represents a balance between realism and conservatism in determining the amount of calibrating information available to inform divergence time estimates. However, any

extension of the analyses undertaken in this study would certainly indicate investigation of the effects of both alignment length and calibration numbers and positioning.

4. Conclusions

Our results here show the value of testing molecular phylogenetic and dating methods against complex simulation models informed by empirically determined patterns of rate variation. We have demonstrated that commonly applied Bayesian divergence time estimation methods can experience average errors of 12% in node dates under a model in which speciation and molecular rates vary independently, 14% when substitution rates are linked to speciation rates and 20% under a punctuational model when analysed using an uncorrelated rates prior (UCLN) in BEAST 2. Errors were much higher under all models when trees were analysed with an autocorrelated rate prior (ACLN) in PAML. As demonstrated by the negative skewness of gamma statistics, for trees with links between speciation and molecular evolution these errors could lead to systematic overestimation of node ages. Regardless of the mechanism generating the association between speciation rates and substitution rates, we show that the potential for divergence time estimation error associated with known empirical relationships between molecular evolution (manifest in substitution rate) and macroevolution (manifest in the topology and branch lengths of phylogenetic trees) can be significant and should be taken into account. Future work should see a broader range of sophisticated divergence time estimation methods be tested against a wider variety of more empirically realistic simulated data, including geographic and environmental biases, concerted changes in population size, and directional changes in life history characteristics. Ultimately the goal should be to produce new models that relax the assumption of independence between priors on divergence times and priors on substitution

rates. These will lead to greater confidence in applications that depend on robust estimates from molecular dating procedures.

5.Methods

5.2 Simulation trees and alignments

To simulate the phylogeny, we adopt a forward approach, starting at the root of the phylogeny and moving forward in time, as lineages divide into two daughter lineages, go extinct, and evolve in speciation and substitution rate as a continuous time Markov process. In each step, we draw the time until the next event from an exponential distribution with rate equal to the sum of the rates of all events in all lineages. Then, for every lineage *l* that is present at time *t*, one of the following three events may take place:

(i) The lineage gives rise to two daughter lineages with rate $\lambda(l, t)$, the current speciation rate of lineage *l* at time *t*. Both daughter lineages initially retain the same speciation rate and trait values as their parent lineages.

(ii) The lineage becomes extinct with rate μ, which is constant over the tree.

(iii) The lineage updates its speciation rate and substitution rate with rate q, which is constant throughout the tree. The update process adds an increment to the current speciation rate, which is a random draw from a bivariate normal distribution with mean 0 and an instantaneous variance-covariance matrix Σ multiplied by the time since last jump. Until one of these events happens, the lineage continues with the same speciation and molecular rates. High values of q make for longer simulations but produce a better

approximation to Brownian motion. We found that q had no influence on observed speciation or substitution rate variance or average tree age for $q > 10$. For our final simulations, we selected $q = 50$ to allow a significant margin of safety.

Specifically, the instantaneous variance-covariance matrix takes the form:

$$\Sigma = \begin{bmatrix} \sigma_\lambda^2 & \mathrm{Cov}(\lambda, r) \\ \mathrm{Cov}(\lambda, r) & \sigma_r^2 \end{bmatrix}$$

where σ_λ^2 describes how fast the speciation rate evolves and σ_r^2 describes how fast the substitution rate evolves. $\mathrm{Cov}(\lambda, r) = \rho\sigma_\lambda\sigma_r$, where ρ is the correlation coefficient between speciation rate and substitution rate, so $\rho = 0$ in the Unlinked Model and the Punctuated model. We employ the Generalised Sampling Approach (120) to ensure a correct distribution of trees with a given number of tips, which is set to 75, the approximate mean size of the avian clades taken from (80). Details of the approach is in Supplementary Methods S1.2.

In the Punctuated model, we simulate an initial tree as per the Unlinked sample, but without removing extinct taxa. We generate a burst of substitutions following each speciation event. We generate Punctuated trees to match the formula found by (62), in which $16 \pm 5.4\%$ of the tree length (sum of all branch lengths) results from node-associated bursts (Supplementary Methods S1.3).

Once we have simulated a tree, we simulate the evolution of nucleotide sequences along the tree, given that branch lengths in the tree represent the expected number of substitutions per site along the branch. The branch lengths in this tree represent the expected number of substitutions per site along the branch. We simulate alignments of 2000 bases, which is equivalent to a 6000-base coding alignment, since most substitutions in a coding alignment occur in the third codon sites. A 6000-base coding alignment is about the size of

the concatenated coding sequences of a mitochondrial genome. Previous literature reviews have shown this to be a common length for phylogenetic data sets in published ecological studies, and a tractable length for large Bayesian simulation studies (86). We parameterize our sequence simulations using realistic among-site rate variation values from birds (Supplementary Methods S1.4).

5.3 Calibrations

In addition to the trees and sequence alignments, we need calibrations to provide information on absolute time to allow substitution rates to be identifiable from absolute ages of nodes. For simulated trees of similar size to ours, diminishing accuracy returns for adding new calibrations are achieved between 1 and 5 calibrations, especially if one is at the root (30). Since our calibration dates are known with more accuracy than those in a real analysis, and we remove the possibility of incorrectly assigned calibrations, we choose to assign two calibrations per tree. For each tree, we choose calibrations at the root node and at one randomly chosen internal node. To ensure that the calibration is randomly chosen across the nodes of the simulated trees, we first decide the level of the calibrated node as a random integer between 1 and the largest number of nodes along any root-to-tip path in the tree. Then we randomly pick the calibrated node from all the nodes at that level.

For analyses in both BEAST 2 and PAML, we apply a hard-bounded uniform prior for the age of each calibrated node. Both calibrations have bounds at the true age ± 15%. Note that this is a conservative test of molecular dating because in the simulated datasets we have accurate knowledge of the true date of the speciation event. In reality, calibrations may be misplaced or incorrectly dated, and may only provide maximum or minimum bounds.

5.4 Phylogenetic inference

We generate a set of 50 trees under each of the Unlinked, Continuous and Punctuated simulation types. After sequence simulation, this gives us 50 sequence alignments for each of the three models.We infer divergence times from the simulated sequences using common Bayesian molecular dating procedures. First, we generate posterior distributions of tree topologies and divergence times using two common molecular dating methods: the uncorrelated lognormal rate prior (UCLN), implemented in the software package 'BEAST 2' (BEAST 2; 121), in which rates for each branch are independent draws from a common lognormal distribution, and the autocorrelated lognormal rate prior (ACLN), implemented in the 'mcmctree' program in the PAML software suite (105), in which the rate of each branch is lognormally distributed with a mean equal to the rate of its parent branch. To speed analysis, we used the approximate likelihood algorithm implemented in mcmctree. The PAML analyses differ from the BEAST analyses in that PAML requires a fixed tree topology, which in this case was the topology of the true simulated tree. Therefore only BEAST incorporates the possibility of topological inference errors. However, the three simulation models can be compared within each reconstruction method. Details of the inference method are available as Supplementary Methods (S1.5).

5.5 Error in reconstructed trees

For each reconstructed tree, we calculated accuracy in the estimation of node times using the median absolute percentage error (MAPE) of inferred node times for clades that are present in both true and reconstructed trees. The median was chosen rather than the mean to reduce the impact of extremely high values for the estimates of shallow nodes. Since these

are only calculated for correctly reconstructed branches and do not capture errors in reconstructing topology, we also calculate the Robinson-Foulds distance or number of non-shared bipartitions, which only reflects typographical error (87).

Model misspecification in Bayesian molecular dating methods can sometimes be associated with higher uncertainty in the reconstructed node ages, as measured by the width of Bayesian credible intervals. This is undesirable because the reconstructed history is less useful as an explanatory tool and less informative in downstream analyses, but it can also be a simple way to diagnose problems with the specified model. We examined the uncertainty with which node ages were reconstructed by the two Bayesian molecular dating methods. We scored uncertainty by averaging the width of the reconstructed 95% highest posterior density (HPD) intervals as a proportion of reconstructed node height across each tree, with a larger score indicating less precise estimates.

5.6 Distribution of reconstructed vs simulated node ages

We examine the direction of error by calculating the gamma statistic for each tree and comparing to the true tree (122). The gamma statistic is a description of how the times between branching events in a tree whose tips are all extant taxa change as they become closer to the present. It summarises information relating to the pattern of diversification, following a standard normal distribution for a tree with a constant rate of speciation and no extinction. Trees with higher values have a relatively greater proportion of their length in older branches, while those with lower values have relatively more of their length in younger branches. The statistic therefore tells us whether the ages of internal nodes within each tree

tend to be older or younger on average than the true times while accounting for the different scale of this bias for older and younger nodes.

5.7 Efficacy of sister pair correlation on reconstructed branch lengths

We checked to see if we can use the reconstructed branch lengths, which are the median of posterior branch lengths calculated by BEAST or mcmctee, to detect the correlation between branch lengths and clade sizes found by sister pair analysis on the simulated trees (see *Simulating realistic rate variation* above). This could be used as a rapid post-hoc check to see whether published dated trees could be affected by reconstruction error that is associated with substitution and speciation rate covariation. Data sets for the sister pair analysis are generated from the 50 simulated trees under each of the three simulation models, where each tree gives a pair of sister clades and the sister clades are the two clades that split at the root. So, under each simulation model, we have a data set of 50 sister pairs. We then regress contrasts in log clade sizes against contrasts in the log of the median branch length estimates from BEAST or mcmctree.

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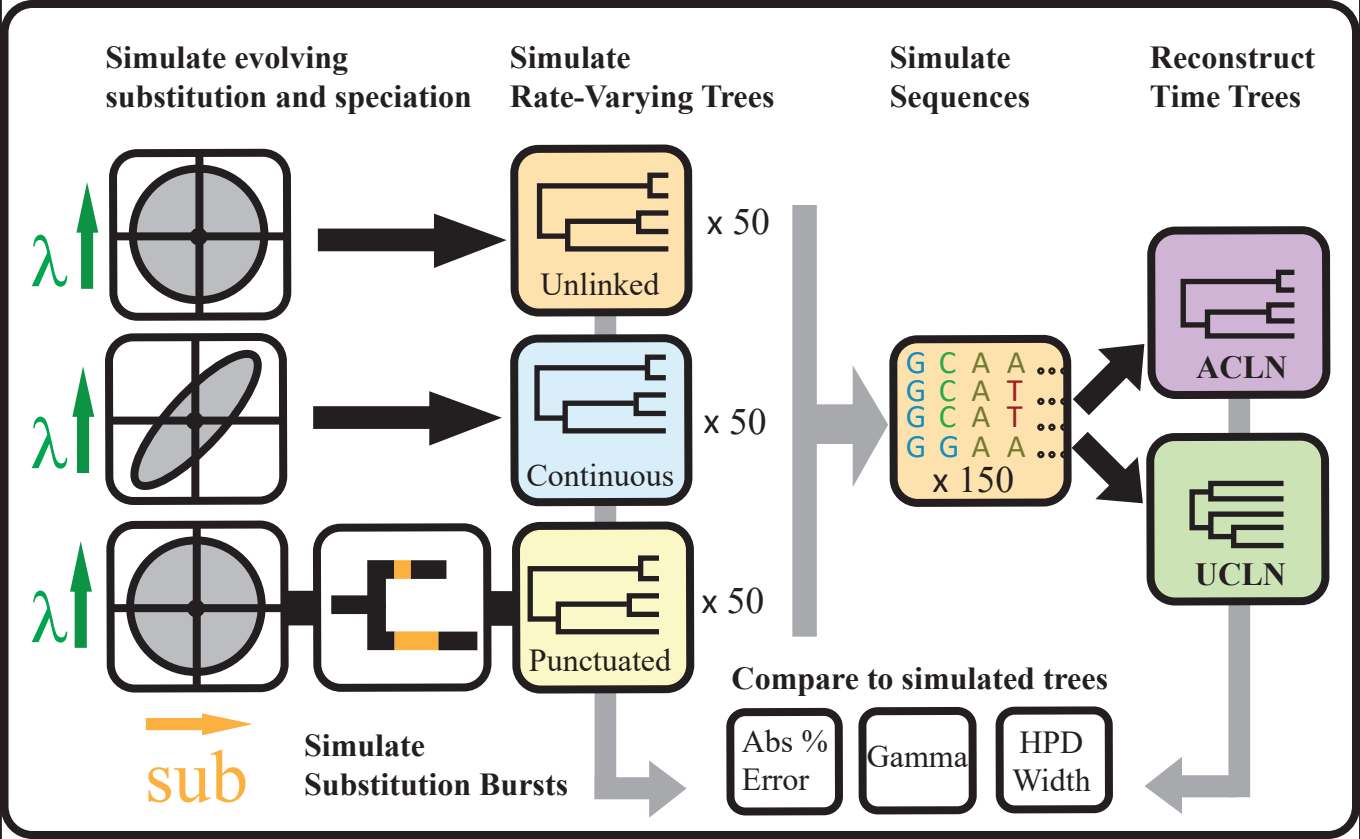
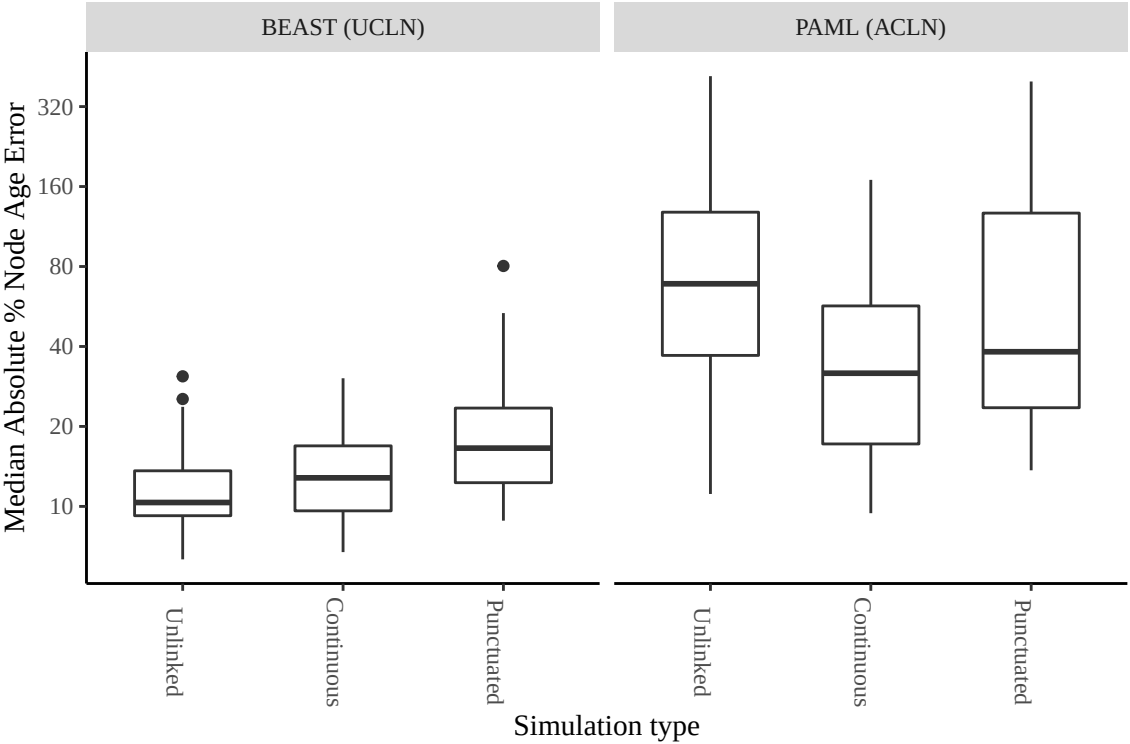
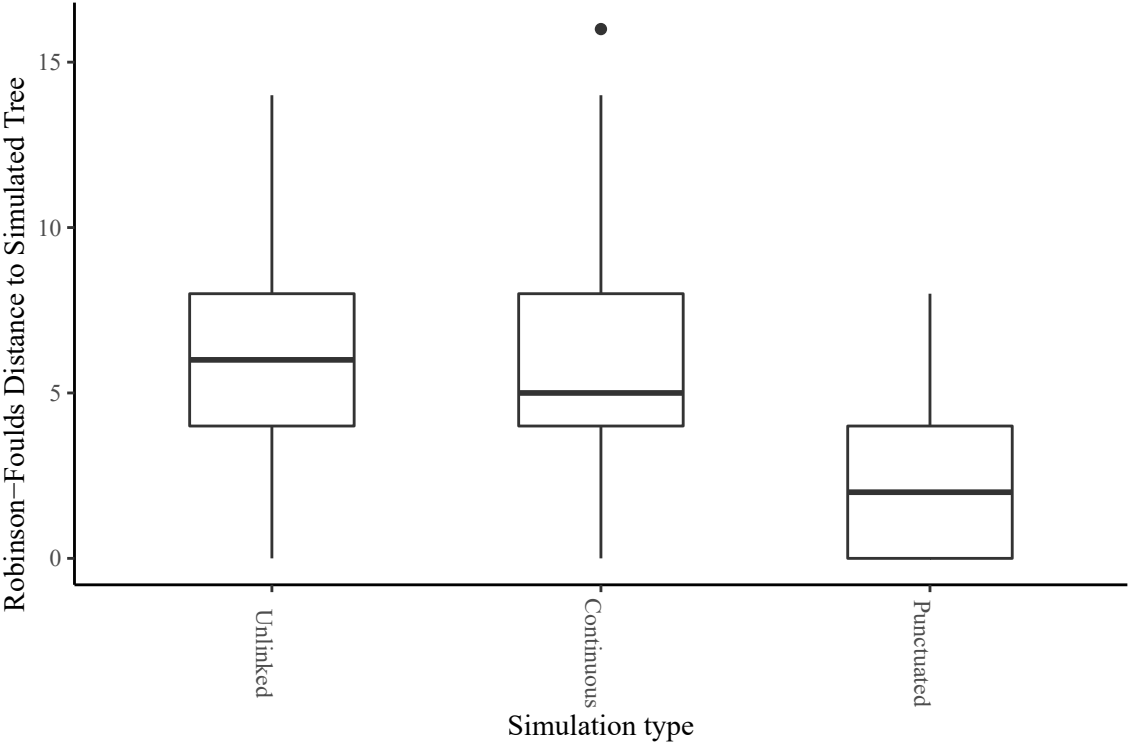
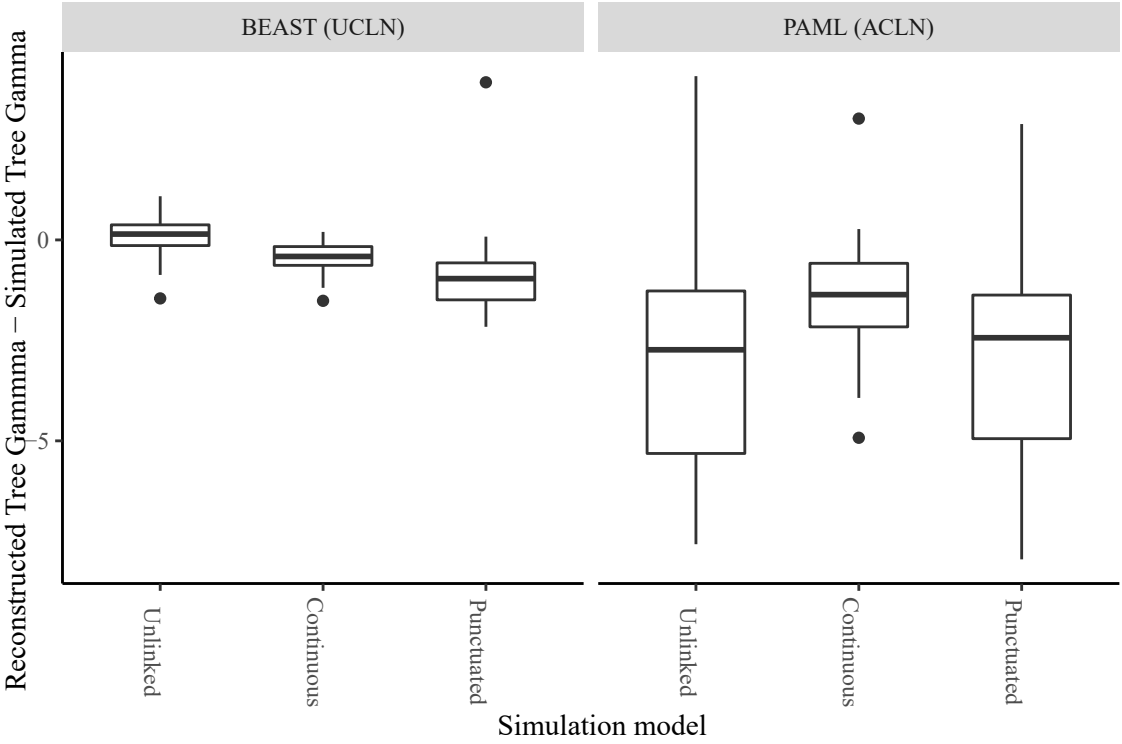
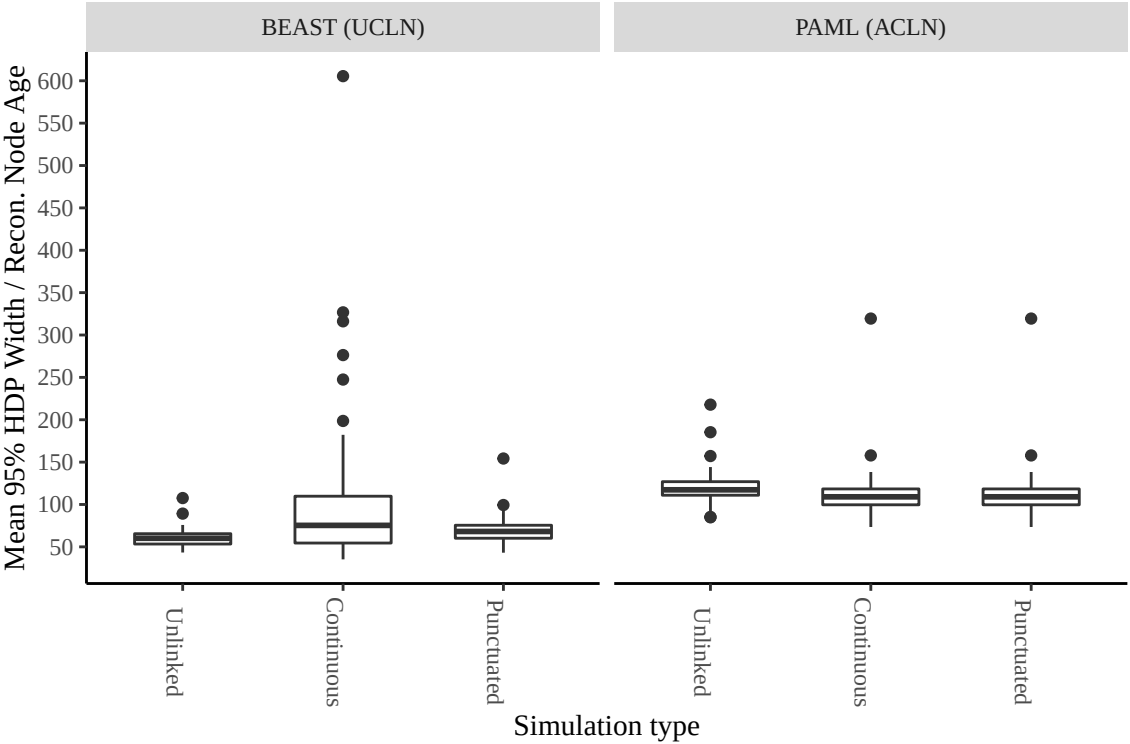
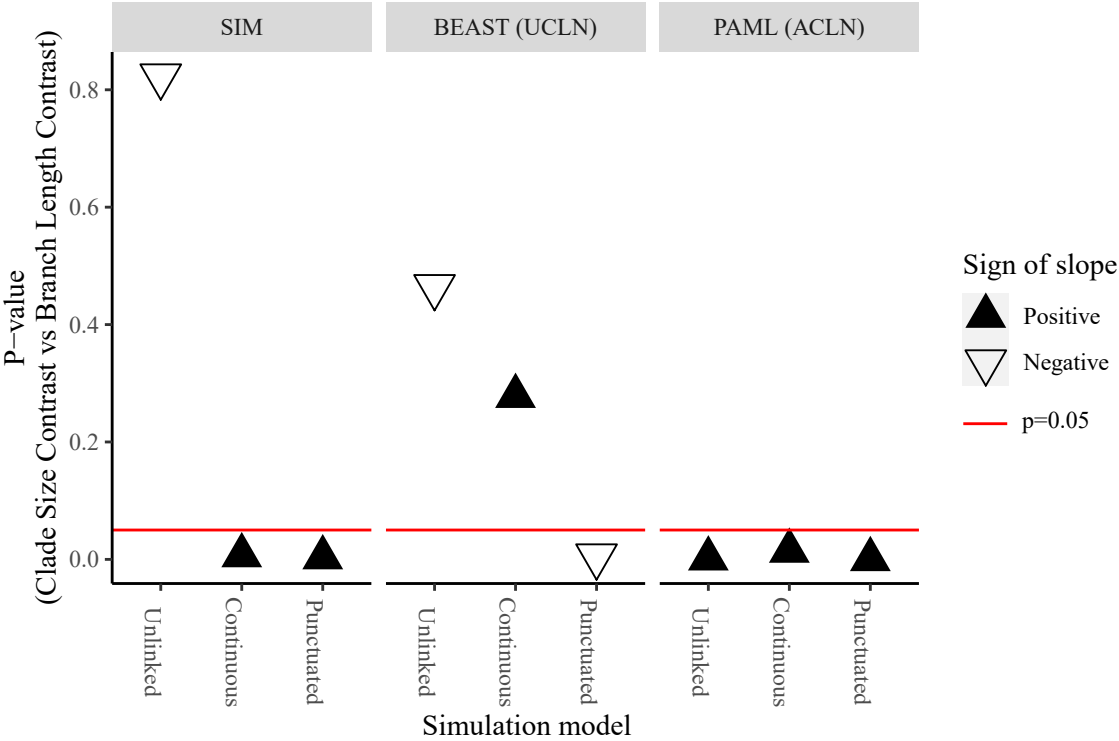

Simulate evolving substitution and speciation
Simulate Rate-Varying Trees
Simulate Sequences
Reconstruct Time Trees
λ
λ
λ
Unlinked
Continuous
Punctuated
x 50
x 50
x 50
G C A A ...
G C A T ...
G C A T ...
G G A A ...
x 150
ACLN
UCLN
sub
Simulate Substitution Bursts
Compare to simulated trees
Abs % Error
Gamma
HPD Width


BEAST (UCLN)
PAML (ACLN)
Median Absolute % Node Age Error
320
160
80
40
20
10
Unlinked
Continuous
Punctuated
Unlinked
Continuous
Punctuated
Simulation type


Robinson−Foulds Distance to Simulated Tree
15
10
5
0
Unlinked
Continuous
Punctuated
Simulation type


BEAST (UCLN)
PAML (ACLN)
Reconstructed Tree Gammma − Simulated Tree Gamma
0
−5
Unlinked
Continuous
Punctuated
Unlinked
Continuous
Punctuated
Simulation model


BEAST (UCLN)
PAML (ACLN)
Mean 95% HDP Width / Recon. Node Age
600
550
500
450
400
350
300
250
200
150
100
50
Unlinked
Continuous
Punctuated
Unlinked
Continuous
Punctuated
Simulation type


SIM
BEAST (UCLN)
PAML (ACLN)
P−value
(Clade Size Contrast vs Branch Length Contrast)
0.0
0.2
0.4
0.6
0.8
Unlinked
Continuous
Punctuated
Simulation model
Sign of slope
Positive
Negative
p=0.05