Molecular Clock Dating of Deep-Time Evolution Using Complex Mixture Models

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Abstract

Molecular clocks are a fundamental technique in evolutionary biology for establishing the timing and tempo of organismal divergence. However, currently available molecular clock methods, which often rely on simple homogeneous substitution models, can produce inaccurate time estimates, particularly for deep-time or rapidly evolving lineages where substitution heterogeneity and saturation are common. Hereby, we introduce phyloHessian (https://github.com/evolbeginner/phyloHessianWrapper), a Julia-based software to enable the use of complex mixture substitution models in molecular dating. phyloHessian computes the phylogenetic Hessian matrix and integrates it into PAML-MCMCtree's approximate likelihood framework to conduct dating analyses. Simulations mimicking phylogenies at different timescales demonstrate that complex mixture substitution models significantly enhance the accuracy of divergence time and substitution rate estimates in deep-time phylogenies. This pattern remains consistent across a wide range of uncertainties associated with molecular clock analysis. Additionally, mixture models display greater robustness to model and calibration specifications compared to their homogeneous counterparts. Empirical analysis of ancient symbiont lineages Microsporidia and Rickettsiales with different substitution models shows that

mixture models, compared to homogeneous models, yield accelerated substitution rates and in some cases significantly different divergence times, leading to a revised understanding of their host association origins. Our findings underscore the importance of incorporating complex mixture substitution models for constructing reliable evolutionary timelines and elucidating the evolutionary history of deep-time or fast-evolving lineages.

Keywords

molecular clock, PAML, MCMCtree, complex mixture model, deep-time evolution, approximate likelihood, symbiont, pathogen evolution

Introduction

Historically, our understanding of evolutionary time scales relied on limited fossil records, until a significant change occurred in the 1960s when molecular clock theory was proposed (Zuckerkandl and Pauling 1965), making it possible to infer evolutionary time and rate using genetic data. While the original concept of the molecular clock assumed that the rate of gene sequence changes is constant among lineages, further evidence shows that even the same gene can exhibit variations in rate among different species, leading to the development of relaxed molecular clock models which allow for lineage-specific evolutionary rate and a series of derivative models (Thorne et al. 1998; Drummond et al. 2006; Yang and Rannala 2006). Over years, molecular clock theory has evolved, continuously increasing in complexity and accuracy, providing a powerful tool for studying biological evolution. Particularly, with the development of Bayesian phylogenetic methods and the rapid growth of genetic sequence data, Bayesian models based on Markov chain Monte Carlo (MCMC) methods have become the most widely used molecular clock dating method, which allows for a comprehensive examination of various uncertainties in analysis, including factors such as calibrations and across-lineage rate differences (Ho and Duchêne 2014; dos Reis et al. 2016).

While molecular clock approaches offer a powerful framework for inferring evolutionary timescales, the reliability of such inferences heavily depends on accurately modeling the evolution of the molecular data, such as protein sequences, alongside the incorporation of calibration information (Guindon 2020). Protein evolution is typically modelled as site-homogeneous process using empirically derived substitution matrices of amino acid exchangeabilities (e.g., LG or WAG) and fixed equilibrium frequencies, with variation in evolutionary rates modeled with a gamma distribution (Yang 1993). However, while

computationally efficient, such models oversimplify real-world biological processes by failing to capture the heterogeneity in amino acid preferences driven by diverse structural and functional constraints across sites. To address these issues, site-heterogeneous substitution models have been proposed since (Lartillot and Philippe 2004; Pagel and Meade 2004). These models account for among-site substitution heterogeneity by incorporating predefined substitution matrices for different secondary structures and solvent accessibility (Le et al. 2008; Le and Gascuel 2010), across-site rate variation (Le et al. 2012), or mixtures of amino acid frequency profiles (Quang et al. 2008; Wang et al. 2008). Of particular interest is the last category, which captures different amino acid preferences across sites, as represented by the CAT model (Lartillot and Philippe 2004) and its simplified counterparts for maximum-likelihood phylogenetics called Cxx (where xx indicates 10 to 60 profile categories) (Quang et al. 2008). By better approximating the true biological complexity of amino acid substitution, profile mixture models mitigate artifacts like long branch attraction, thereby enhancing the ability to distinguish phylogenetic signal from homoplasy and displaying higher sensitivity to substitution saturation (Wang et al. 2018; Wang et al. 2019; Schrempf et al. 2020; Baños, Susko, et al. 2024). These features make profile mixture models especially well-suited for studying microbial evolution, where deep divergences and rapidly evolving sites are prevalent. Indeed, empirical analyses of deep-time phylogenomic data show that profile mixture models often exhibit superior model fit compared to homogeneous alternatives (Williams et al. 2020; Bujaki and Rodrigue 2022).

A particular outcome of using site-homogeneous models like LG+G is that branch lengths are usually underestimated in case of extensive sequence divergence (Venditti et al. 2008; Moody et al. 2022). This happens because proteins may not use all 20 amino acids at each position and homogenous models incorrectly interpret this restricted use of amino acids as evidence of less

evolutionary change than what truly occurred over long time (Lartillot et al. 2007; Wang et al. 2008). To see this, consider a site restricted to Asp and Glu. A mixture model would identify a preference for negatively charged amino acids and infer unobserved substitutions (e.g., Asp → Glu → Asp) even if two sequences show the same amino acid, leading to longer branch lengths. Obviously, this pattern is more often observed with an increasing evolutionary scale. Studies have shown that the deepest branches of the tree of life, spanning approximately 4.0 billion years (Gyr), can display branch lengths up to twice as long under profile mixture models compared to the LG+G model (Moody et al. 2022; Wang and Luo 2025). Given that in molecular clock analysis, branch length, the expected substitutions per site, is the product of time and (average) substitution rate of that branch (dos Reis et al. 2016; Guindon 2020), an important question emerges: do profile mixture models, which often excel in deep-time phylogenetic inference, lead to more accurate divergence time and substitution rate estimates?

To our knowledge, none of the available molecular clock programs have directly implemented complex substitution models including those profile mixture models, with PhyloBayes' CAT model being the exception—yet its high computational demands may limit its use in large-scale molecular clock analyses. We recently developed bs_inBV (Wang and Luo 2025), enabling the use of diverse substitution models in the popular MCMCtree molecular dating software by applying a bootstrap approximation of the Hessian matrix, which captures likelihood surface curvature and is required by MCMCtree's fast approximate likelihood dating procedure (Thorne et al. 1998; dos Reis and Yang 2011). However, the bootstrapping process is computationally intensive, and its approximation sometimes inadequately represents the true Hessian. Additionally, and perhaps more importantly, the relative performance of different substitution models in molecular clock dating remains unexamined through systematic

simulation studies, leaving a critical gap in our understanding of how choice in substitution model affects divergence time estimation, particularly in deep-time or fast-evolving phylogenies.

Hereby, we introduce phyloHessian, a Julia-based software that uses numerical methods to calculate accurate Hessian matrices for diverse complex substitution models and integrates them directly into MCMCtree's approximate likelihood framework for rapid molecular clock dating. We perform extensive analyses on simulation data to comprehensively compare the performance of different substitution models in divergence time and substitution rate estimation under a wide range of conditions that mimic phylogenies from relatively recent to very ancient divergences. We further analyze empirical data to assess how different substitution models affect our understanding of ancient symbiont evolution and the origins of their host associations.

Results

phyloHessian: enabling molecular clock dating in MCMCtree with complex substitution models

To address the challenges in molecular clock analysis under various substitution models, we developed a Julia-based software phyloHessian. phyloHessian first calls IQ-Tree (Wong et al. 2025) or PhyML (Guindon et al. 2010), two widely used phylogenetic reconstruction software that implement a range of mixture models, to calculate the maximum-likelihood estimates (MLE) of branch length $\hat{\theta} = \left(\hat{\theta}_1, \dots, \hat{\theta}_n\right)^{\mathrm{T}}$ under user-specified substitution model. Let $\theta = (\theta_1, \dots, \theta_n)$ be the branch lengths and $\Delta\theta = \theta - \hat{\theta}$. Next, it applies finite difference numerical methods to calculate the gradient $g = \left(\frac{\partial \ell(\hat{\theta})}{\partial \theta_1}, \dots, \frac{\partial \ell(\hat{\theta})}{\partial \theta_n}\right)^{\mathrm{T}}$ and Hessian matrix $H\left(\hat{\theta}\right) = \left(\frac{\partial^2 \ell(\hat{\theta})}{\partial \theta_i \partial \theta_j}\right)$, i.e., the first- and second-order derivatives of the log-likelihood of the phylogenetic data

evaluated at the MLEs. . Last, the resulting Hessian matrix is integrated into MCMCtree to be used with its approximate likelihood method (Thorne et al. 1998; dos Reis and Yang 2011) for divergence time and rate estimation. MCMCTree's approximate likelihood method approximates the log-likelihood surface for branch lengths using a Taylor expansion to the second order

$$\ell(\theta) \approx \ell(\hat{\theta}) + \mathrm{g}^{\mathrm{T}}\Delta\theta + \frac{1}{2}\Delta\theta^{\mathrm{T}} H(\hat{\theta})\Delta\theta.$$

To demonstrate the reliability of phyloHessian, we compared its results with those obtained from well-established phylogenetics software packages. First, we found that phyloHessian and IQ-Tree produced identical log-likelihoods under all tested complex substitution models available in IQ-Tree (Data S1). Further, we validated phyloHessian's Hessian calculation by confirming that the Hessian matrix obtained by phyloHessian and CODEML under site-homogeneous models (such as LG+G) were nearly identical (Data S2).

Profile mixture substitution models enhance time and rate estimation in deep-time and fast-evolving phylogenies: evidence from simulations

To evaluate the impact of mixture substitution models (Materials and Methods: Mixture substitution model) on divergence time and substitution rate estimates, and to compare their performance to profile homogeneous models like LG+G, we conducted extensive simulations. To simulate a range of evolutionary scenarios spanning relatively recent to very deep-time divergences, we followed our prior study (Wang and Luo 2025) to generate 30 replicates of 20-tip birth-death trees with protein sequences that were 300 amino acids (aa) in length simulated under the substitution model LG+C60+G4{1.0} for both autocorrelated-rates (AR) and independent-rates (IR) molecular clock rate model. For simplicity, we use "+G" to represent "+G4{1.0}" hereafter [IQ-Tree's default; four-category discrete Gamma distribution for the

relative rate, shape parameter α = 1.0, i.e., Gamma(1,1)]; see also Materials and Methods: Simulation procedure. We mainly focused on five calibration strategies. These five calibration strategies consisted of a single calibration at the root with both upper and lower bounds (*root_only*), fully calibrated root plus one fully-bounded internal node (*single_interval*), fully calibrated root plus one minimum-bounded internal node (*single_min*), fully calibrated root plus two fully-bounded internal nodes (*two_interval*), and fully calibrated root plus two minimum-bounded internal nodes (*two_mins*).

Under the focal simulation scheme (see Materials and Methods: Simulation procedure), the profile mixture model LG+C60+G performed comparably to the traditional LG+G model for divergence time estimation when simulated root ages were set to either 1.0 or 2.0 billion years ago (Ga). However, for root ages ≥3.0 Ga, LG+C60+G obtained significantly more accurate time estimates than LG+G, with the performance gap becoming increasingly pronounced at 4.0 Ga (Fig. 1A). For example, under the *root_only* calibration strategy, LG+C60+G demonstrated superior time estimation accuracy (mean relative difference ± SD: 0.157 ± 0.061 for AR clock model, 0.139 ± 0.046 for IR clock model) compared to LG+G (AR: 0.193 ± 0.062; IR: 0.197 ± 0.061). Mean relative difference is defined as mean $\left(\frac{|b_i^m - b_i^{true}|}{\max(b_i^m, b_i^{true})}\right)$, where b_i^m and b_i^{true} respectively denote the inferred posterior mean age and the true value for node i. The largest discrepancies in divergence time estimation occurred under the calibration scheme *two_min*, where LG+C60+G showed lower error than LG+G (mean relative difference: AR 0.160 vs. 0.232; IR 0.147 vs. 0.259; Fig. 1A). In contrast to divergence time estimates, LG+C60+G provided much more accurate branch-specific substitution rate estimates than LG+G across all tested root ages (Fig. 1B). The error reduction obtained by LG+C60+G compared with LG+G, calculated as $\frac{\text{reldiff}_{\text{LG+G}} - \text{reldiff}_{\text{LG+C60+G}}}{\text{reldiff}_{\text{LG+G}}}$ (i.e., the error reduction was calculated using the

node-averaged mean relative differences between models), averaged across all calibration strategies, ranged from 26% to 53% under AR, and 23% to 55% under IR for root ages of 1.0 to 4.0 Ga (Table 1). The pattern held true with alternative metrics (mean branch score distance [BSD] or scatter-plot comparisons) for comparison with the simulation values (Figs. S1-S3).

Next, we explored the patterns for more complex mixture models. We combined structurally constrained substitution models EX2 (exposed/buried sites) and EX3 (highly exposed/intermediate/buried sites) (Le et al. 2008) with the predefined equilibrium frequency C20 to account for site-specific variation in amino acid profiles by 20 categories of equilibrium frequencies. This resulted in 40 and 60 different rate matrices for EX2 and EX3 respectively. When simulation was done under profile-exchangeability mixture models EX2+C20+G and EX3+C20+G, the above patterns became more pronounced (Fig. S4). In contrast to profile mixture models, when alignments were simulated under exchangeability mixture models (EX2+G, EX3+G, and LG4M), LG+G and the true simulation model performed similarly in estimating divergence times (Fig. S5).

Large sequence divergence may arise from not only long divergence time, but also rapid evolution. To test whether different time estimates are affected by varying evolutionary rates (Fig. S6), we simulated alignments on phylogenies with a fixed root age of 1.0 Ga but different mean absolute substitution rates along the phylogeny (0.25-1.0 substitutions/site/Gyr). This represents evolutionary rates from relatively low, as in most microbes, to very fast, as observed in some pathogens. We would anticipate comparable sequence divergence whether we fixed the root age at 1.0 Ga and increased the average substitution rate (from 0.25 to 1.0 substitutions/site/Gyr; Fig. S6), or fixed the substitution rate at 0.25 substitutions/site/Gyr and increased the root age to 4.0 Ga (Fig. 1). Averaged across all calibration strategies, LG+C60+G

194 displayed error reductions in substitution rate estimate of 65% (AR) and 61% (IR) at a mean
195 substitution rate of 1.0 substitutions/site/Gyr.
196
197 **Performance of profile mixture models in molecular clock dating under varied simulation**
198 **conditions**
199 Next, we evaluated the influence of multiple sources of uncertainties in molecular clock dating
200 on the performance of profile mixture and traditional homogenous models when sequence
201 alignments are simulated under profile mixture models. These involve sequence length, Gamma-
202 distributed across-site rate, birth-death process, variance in lineage-specific rates, and number of
203 sequences (see Note S1 for details).
204 We first investigated how sequence length (ranging from 100 to 2000 aa) affected the
205 accuracy of the estimates using different substitution models (Fig. S7). Although the increase in
206 divergence time estimate accuracy achieved by using LG+C60+G instead of LG+G was minimal
207 for 100-aa alignments, the improvement became increasingly apparent as the alignment length
208 extended to 2000 aa. Across all analyzed calibration strategies, the LG+C60+G model
209 consistently improved the accuracy of time estimation for the AR clock models compared to
210 LG+G. For a simulated root age of 4.0 Ga, this improvement was substantial, resulting in an
211 error reduction (see above) of 30%, 28%, 25%, and 12% for alignment lengths of 2000, 1000,
212 500, and 100 aa, respectively; the benefit was still significant but less pronounced for a younger
213 root age of 3.0 Ga, with corresponding improvements of 22%, 18%, 15%, and 7%. Both
214 LG+C60+G and LG+G models achieved higher estimation accuracy with longer sequences (i.e.,
215 lower values in *y*-axis from 100 to 2000 aa), indicating that longer sequences are more
216 informative for more accurate molecular clock analyses, consistent with the notion that mixture

models perform much better on longer alignments for phylogenetic reconstruction (Baños, Susko, et al. 2024). Regarding the substitution rate, mixture models displayed more accurate estimates across time scales for different sequence lengths (Fig. S8).

We further adjusted the gamma correction parameter (α) to modulate the variance of the across-site relative rates. Specifically, we set α to 0.5 and 1.5, which scaled the across-site variance to 2-fold ($0.5/0.5^2$) (scheme *Gamma_0.5* in Fig. 2A) and 2/3-fold ($1.5/1.5^2$) (scheme *Gamma_1.5* in Fig. S9A), respectively, compared to our focal analysis (α=1). These adjustments did not alter the patterns observed in the original analysis. Similarly, when exploring alternative birth-death process parameters, a flat birth-death process (simulation scheme: *flat_time_prior*) produced results comparable to those from the focal analysis (Fig. 2B). Under complete taxon sampling, the difference in time estimates between the LG+G and LG+C60+G models became more obvious (scheme *complete_taxon_sampling* in Fig. S9B). Moreover, increasing the standard deviation of the among-branch sequence absolute substitution rate to twice and three times its default value reduced the differences in divergence time estimation between using LG+G and LG+C60+G, despite the unchanged overall pattern (schemes *SD_lograte_0.38, SD_lograte_0.55* in Figs. 2C, S9C). In addition, although all simulations above were performed on 20-tip trees for simplicity, the analysis was extended to 40-, 60-, 80-, and 100-tip trees, with similar patterns found (Fig. S10).

Profile mixture models display higher robustness to model misspecification

Further, we applied simulations to test the robustness of profile mixture models to misspecifications in calibrations or substitution models. To explore whether mis-specified calibrations would affect estimation under different substitution models when the sequences were

240 simulated under LG+C60+G, we calibrated the nodes by adjusting their ages. These adjustments
241 consisted of fixed percentage increases (shifting ages toward the past) or decreases (shifting ages
242 toward the present) (Fig. 3). In general, LG+C60+G achieved higher accuracy when calibrations
243 were older than the true divergence times (Fig. 3B). For a root age of 4.0 Ga and across all tested
244 calibration strategies, on average, the relative difference in time estimation compared to true
245 values was: 0.256 (LG+G) versus 0.195 (LG+C60+G) for the AR model, and 0.278 (LG+G)
246 versus 0.185 (LG+C60+G) for the IR model. For root age of 3.0 Ga, these values changed to
247 0.252 (LG) versus 0.202 (LG+C60+G) for AR, and 0.255 (LG) versus 0.192 (LG+C60+G) for
248 IR (Fig. 3B). Subtle differences between using LG+G and LG+C60+G were observed for root
249 age ≤ 2.0 Ga. However, when calibrations were biased towards younger ages, the LG+G and
250 LG+C60+G models generally yielded comparable accuracy for divergence time and substitution
251 rate estimates, irrespective of the root age, with significant differences arising occasionally.
252 The above analyses focused on alignments simulated under complex substitution models,
253 particularly LG+C60+G. To determine how these models perform in molecular clock analysis
254 when sequences evolve under simpler substitution models, we performed additional simulations
255 using these models and the previously described settings (varying the simulated root ages from
256 1.0 to 4.0 Ga (Fig. 3C). These analyses indicate that the profile mixture model LG+C60+G
257 displayed robust time and rate estimation, regardless of the true underlying substitution process.
258 The robustness of LG+C60+G is evident in the following comparisons. When sequences were
259 simulated under the basic LG+G model, LG+C60+G provided estimates of divergence times and
260 absolute substitution rates at least as accurate as those obtained using LG+G. Further, similar
261 results were observed when simulations were conducted with other models with factors not
262 explicitly modeled by LG+C60+G, such as invariant sites (+I) and free rate across-site

263 heterogeneity (+R) (Note S1). These findings suggest that LG+C60+G's robustness was
264 maintained even when the true evolutionary process involves additional complexities in
265 substitution models.
266
267 **Comparison between profile mixture models with PMSF (posterior mean site frequency)**
268 **and bootstrapped-Hessian approximations in molecular clock dating**
269 The computational intensity of the LG+C60+G model often necessitates the PMSF
270 approximation to accelerate phylogenetic reconstruction, especially for large datasets (Wang et
271 al. 2018). This occurs because PMSF requires the computationally intensive pruning algorithm to
272 run only once per site pattern using an approximated equilibrium frequency for each site, versus
273 *c* times for a mixture model with *c* classes. A distinct approximation, previously employed in
274 molecular clock analyses under complex substitution models, uses a bootstrap-based method to
275 approximate the Hessian matrix and is implemented in the software bs_inBV (Wang and Luo
276 2025). To assess the impact of these two approximations on molecular clock dating under profile
277 mixture models, we compared both divergence time and substitution rate estimates obtained
278 under these approximations by evaluating their relative differences from the true simulation
279 parameters.. We found that both approximations provided accurate estimates of divergence times,
280 no matter which root age was used in simulation (Fig. 4A). In terms of the time estimates, across
281 all simulated root ages, compared to LG+C60+G estimated rates, the average differences for AR
282 were 3.6 ± 1.0% (PMSF approximation; *LG+C60+G+PMSF*), 7.1 ± 0.6% (bootstrapped-
283 hessian; *LG+C60+G+bs_inBV*), and 7.3 ± 0.8% (combined use;
284 *LG+C60+G+PMSF+bs_inBV*); for IR, the differences were 1.8 ± 0.4%, 5.9 ± 0.4%, and 6.5 ±
285 0.5%, respectively. The deviations in time estimates from these approximations, particularly

PMSF, were close enough to the stochastic variation observed across independent MCMC runs of the original profile mixture model (*LG+C60+G_run2* in Fig. 4; comparisons in Fig. 4 are made relative to the initial MCMC run under LG+C60+G). The estimates of evolutionary rates exhibited slightly greater divergence from the original model with either approximation (Fig. 4B). The mean relative differences under both AR and IR models were around 5-10% for either approximation, or their combination, compared to the true parameters used in simulation.

We further compared the log-likelihood calculated by phyloHessian using Hessian computed by the finite-difference method (see Materials and Methods) and approximated via bootstrap with bs_inBV. Roughly speaking, both methods approximated the true log-likelihood of the data reasonably well. phyloHessian achieved higher Pearson correlation coefficients with the exact likelihood than the bootstrap-based method (P-value $= 0.00026$, paired Wilcoxon test; Fig. S11), on the data tested, while running ~10 times faster (assuming 1000 bootstraps for bs_inBV). More details are given in Discussion.

Divergence times and substitution rates under mixture models for the ancient pathogens Microsporidia and Rickettsiales: an empirical study

To evaluate mixture models on empirical data, we selected two ancient symbiont lineages with distinct evolutionary significance and accelerated evolutionary rates: Microsporidia, highly reduced, fast-evolving obligate intracellular parasites of animals related to fungi (Capella-Gutiérrez et al. 2012), and Rickettsiales, an alphaproteobacterial lineage that shares a close evolutionary relationship with the ancestors of mitochondria (Martijn et al. 2018; Wang and Luo 2021). Their rapid evolution combined with ancient divergence makes them ideal test cases for our analysis. Note that our primary goal was to assess how complex substitution models

308 influence the molecular clock analysis itself, leading us to test different settings rather than
309 selecting a single optimal configuration (e.g., partitions, clock model).
310 For both datasets, we partitioned the alignments into three subsets (see Materials and
311 Methods) according to a Gaussian mixture model-based clustering according to the logarithm of
312 the estimated substitution rates of each gene, and selected the best-fitting substitution models for
313 each partition under both site-homogeneous and mixture substitution models respectively. In all
314 cases, profile mixture models were associated with the lowest AIC values, with
315 LG+Cxx+F+I+R4 (where xx = 40, 50, 60) being the most frequently selected (Data S3-S4).
316 Microsporidia do not have fossil records. Thus, we applied different calibrations to only the root
317 node based on previous estimate of the occurrence time of its LCA (last common ancestor) (Note
318 S2). We found that the time estimates for Microsporidia lineages were largely consistent between
319 the best-fitting profile mixture and homogenous models (Fig. 5). The LCA of Microsporidia was
320 estimated to occur at approximately 0.41 Ga (95% HPD: 0.74-0.45 Ga) and 0.63 Ga (95% HPD:
321 0.73-0.52 Ga) under the AR and IR clock models, respectively (Fig. 5A). In contrast to the
322 similar divergence time estimates between homogeneous and mixture models, the absolute
323 substitution rate was at least 1/3 higher under the mixture model (Fig. 5B). All microsporidian
324 lineages experienced accelerated sequence changes compared to the outgroup lineages. While the
325 highest substitution rate was predicted for the branch leading to the LCA of Microsporidia under
326 the IR clock model, the AR clock model instead predicted the highest rate on the branch leading
327 to *Enterocytozoon*, a major microsporidian pathogen in human and animals. The above pattern
328 was consistent with alternative calibrations and different numbers of partitions (Fig. S12).
329 Rickettsiales also lack direct fossil evidence; however, due to their close phylogenetic
330 relationship with mitochondria, we employed the mitochondrial endosymbiosis dating strategy as

used in our prior study (Wang and Luo 2021). This approach allows estimating Rickettsiales' evolutionary timeline by incorporating eukaryotic fossils, based on the evolutionary scenario that mitochondria evolved from a lineage closely related to Alphaproteobacteria (and thus Rickettsiales). Particularly, under the AR clock model, the time estimates of Rickettsiales lineages under the mixture substitution model differed by an average of >20% compared to those from the homogeneous alternatives (Fig. 6A). In general, the ages of most lineages were estimated to be older under the profile mixture model (Fig. 6A). For example, under the AR clock model, the LCA of Rickettsiales was estimated to occur at 2.67 Ga (95% HPD: 2.97-2.35 Ga) by the mixture model, whereas the homogeneous substitution model estimated it at 2.26 Ga (95% HPD: 2.57-1.94 Ga). Similarly, eukaryotes were estimated to originate at 2.29 Ga (95% HPD: 2.57-1.98 Ga) under the mixture model, compared to 1.74 Ga (95% HPD: 1.98-1.50 Ga) under the homogeneous model (Fig. 6A). The above pattern holds under alternative calibration settings (Fig. S13). With a single partition (Fig. S13A, S13B), the difference in time estimates between different substitution models was less obvious. In all calibration schemes, the substitution rates under the mixture models were roughly 1.5 to 2 times as fast as those estimated under homogeneous models (Fig. 6B).

A recent study (Baños, Susko, et al. 2024) suggests that the empirical frequency estimated from the alignment (+F) might mislead phylogenetic reconstruction for profile mixture models. As an additional analysis, we re-performed molecular clock dating with the best-fitting mixture model without the empirical frequency option. Similar patterns were found (Figs. S14-S15).

Discussion

Approximate likelihood dating with complex models

The present study introduces phyloHessian, enabling Hessian calculation under diverse mixture and non-mixture amino acid substitution models for molecular clock dating using MCMCtree's approximate likelihood method (Thorne et al. 1998; dos Reis and Yang 2011). A key question is whether MCMCtree's approximate likelihood method performs equally well as exact likelihood under mixture substitution models in terms of divergence time estimation. While previous research suggests that this holds true for homogeneous models (dos Reis and Yang 2011), a full comparison is impossible due to the lack of software directly implementing complex substitution models. Therefore, we compared the phylogenetic likelihoods of bootstrap trees calculated by the approximate and exact likelihood methods under LG+C60+G using phyloHessian. The approximate likelihood values obtained from phyloHessian (using finite differences) generally showed good agreement with those derived from both the exact method and bs_inBV, despite minor discrepancies (Fig. S11). Further, phyloHessian sometimes outperformed bs_inBV, with bs_inBV showing markedly lower log-likelihood in occasional cases (Fig. S11). Discrepancies in time and rate estimates between the Hessian-based method (phyloHessian) and the bootstrap approximation (bs_inBV) stem from three factors (Fig. 4): the inherent limitation of bs_inBV due to the finite number of bootstrap replicates; the statistical assumption in bs_inBV that the gradient is zero when approximating the Hessian via the covariance matrix, an assumption that is invalid when the MLEs of branch lengths are near zero (dos Reis and Yang 2011); and the difference in parameter handling, as MCMCtree fixes substitution model parameters (such as the parameter α for the gamma distribution for relative rates) at their MLEs while bs_inBV re-estimates all parameters in each bootstrap, resulting in phyloHessian using only the branch

length Hessian versus bs_inBV approximating the full parameter Hessian. A more thorough examination in time estimates, instead of the phylogenetic likelihood, is necessary to fully address the question in future.

It was when we were finishing writing this manuscript that we became aware, through personal communication, of another computational software, IQ2MC (Demotte et al. 2025), implemented in the newest version of IQ-Tree (v3.0.1). IQ2MC leverages the highly optimized computation of IQ-Tree's phylogenetic library enabling efficient Hessian matrix estimation under not only amino acid, but nucleic acid and partitioned models, unlike phyloHessian which is restricted to amino acid models. Due to these computational advantages, IQ2MC was markedly faster than CODEML and phyloHessian (Data S5). However, our software, phyloHessian, offers more flexible settings in molecular dating by alternative Hessian calculation algorithms and interactions with other software like PhyML and bs_inBV. Its code for phylogenetic likelihood calculation under complex substitution models would be a valuable resource for phylogenetic software development within the Julia ecosystem.

Other methods, such as McmcDate (Mahendrarajah et al. 2023), also accelerate molecular dating analyses by approximating the phylogenetic likelihood. It is in concept similar to bs_inBV (Wang and Luo 2025), but operates in a Bayesian framework. A key advantage of bs_inBV and McmcDate over phyloHessian and IQ2MC is their ability to accommodate substitution models not yet available in specific phylogenetics software, such as FunDi (Gaston et al. 2011) or CAT-PMSF (Szánthó et al. 2023). However, these approximations via bootstrap or MCMC sampling may sacrifice computational speed and accuracy to some extent (Fig. 4), as discussed above.

Deep-time molecular clock dating: the influence of complex mixture models

Our extensive simulations demonstrate that the advantage of using the complex mixture substitution model for divergence time estimation becomes increasingly significant as evolutionary timescales deepen (Figs. 1-4). Particularly, when the root age was earlier than 2.0 Ga, the difference became obvious. This highlights the importance of substitution models that better fit the data for dating deep-time phylogenies. On the other side, the substitution rate was almost always estimated higher and more accurate under mixture models than under homogeneous models. The underlying logic is straightforward. Mixture models typically estimate longer branch lengths than homogeneous models (Venditti et al. 2008; Moody et al. 2022; Wang and Luo 2025). Because branch length equals substitution rate multiplied by time (dos Reis et al. 2016; Guindon 2020), the underestimated branch lengths under simpler substitution models must be attributable to changes in either the rate, the time, or both. Given that molecular clock analyses typically constrain the time estimates through fossil-based calibrations, the increased branch lengths under mixture models primarily result in higher rate estimates, with a smaller impact on the time estimates themselves.

The case in empirical data analysis is more complex. Unlike simulations with known parameters, empirical data could arise from processes so complex that even the best-fitting models are inadequate. This notion is supported by the frequent selection of the most highly parameterized models as the best fit, implying that the complexity of the real process the sequence is generated far exceeds the capabilities of our current models. Consequently, they could make little difference in the time estimates compared to homogeneous models, because both might be an oversimplification of the true model. In such cases, it would be worth applying models like UDM (Schrempf et al. 2020) with more profile mixtures than C60, or the GTRpmix exchangeability matrix and its derived models (Baños, Wong, et al. 2024). While some of these

422 models have been integrated into phyloHessian, a comprehensive evaluation of their applications
423 in molecular dating analysis on empirical data remains a task for future research.
424 While the "mean relative difference" metric used to compare between models takes the
425 absolute value, it is possible to tell a presumable bias in the time estimates from Figs. S2-S3: the
426 posterior ages estimated by the homogeneous model seemed to overestimate the divergence
427 times in scenarios mimicking deep-dating evolution in simulation. As such, it would be very
428 important to have appropriate maximum time bounds on internal nodes (Wang and Luo 2021;
429 Moody et al. 2022). If indeed in current molecular clock models there is a systematic bias in
430 overestimating the divergence times, it might also explain the observation that mixture models
431 displayed greater improvements in time estimation for alignment simulated under mixture
432 models in the absence of maximum time bounds (*single_min*, *two_min*) compared to the case
433 where internal nodes are fully calibrated (*single_interval*, *two_intervals*; Fig. 1A). In contrast to
434 simulations, the Rickettsiales data reveals a contrasting pattern where mixture models led to
435 older age estimates (Fig. 6). We suspect that one important factor lies in the calibration settings.
436 In simulations, we have complete control over the calibration settings. Real-world data
437 introduces complexities absent in simulation because the available calibrations are not uniform in
438 their quality. While some might accurately reflect the true age of the node, others could be overly
439 young, overly old, or simply uninformative. This complex interplay between substitution models
440 and the quality and number of time calibrations, along with other variables such as the clock
441 model and sequence length, likely makes the impact of mixture substitution models on posterior
442 age estimates case dependent.
443 In summary, while the present study indicates significant impacts of complex profile
444 mixture substitution models on divergence time inferences for deep-time or fast-evolving

445 lineages, the importance of these models on time estimates cannot be overstated; their effect
446 likely requires consideration on a dataset-specific basis. Therefore, we recommend rigorous
447 molecular clock analyses employing different substitution models to assess their impact on
448 divergence time and substitution rate estimates in future research. Note also that while our
449 simulations used an LG+C60+G model, the LG exchangeabilities themselves were not estimated
450 in the presence of profile mixtures (Le and Gascuel 2008). Ideally, for practical applications, it
451 would be more appropriate to use models that directly estimate exchangeabilities under a
452 corresponding profile mixture model for such cases (Baños, Wong, et al. 2024).
453

454 **Implications for the evolution of ancient symbionts**

455 For empirical datasets of Microsporidia and Rickettsiales, profile mixture models, which display
456 much higher model fit to data than their homogeneous counterparts, produced significantly
457 higher substitution rates than estimated by homogeneous models (Figs. 5B, 6B). The difference
458 suggests that bacterial pathogens/symbionts could experience relatively high evolutionary rates
459 up to 1.0 or 2.0 Gyr. This hints that factors like elevated mutation rates, selection, or persistently
460 reduced effective population sizes, which are often responsible for rapid sequence divergence,
461 can have more profound and long-lasting effects on the evolutionary trajectory of symbiotic
462 microorganisms than previously thought. Studying the substitution rates will also improve our
463 understanding of microbial lifestyle evolution.

464 One of the merits of the mitochondria symbiosis-based dating method for Rickettsiales
465 evolution is that it co-estimates the divergence times for both Rickettsiales and their eukaryotic
466 hosts with a unified dataset (Wang and Luo 2021). Notably, under profile mixture models, the
467 origin time of Rickettsiales was estimated at 2.5-2.0 Ga, roughly 0.5 Gyr earlier than that of the

last eukaryotic common ancestor (LECA) using homogeneous models estimated in both the current (Fig. 6) and previous studies (Parfrey et al. 2011; Betts et al. 2018; Wang and Luo 2021; Moody et al. 2024). Hence, if the revised time estimate is accurate, it implies that the Rickettsiales' LCA existed before LECA emerged. This interpretation is consistent with recent comparative genomics findings suggesting that early Rickettsiales initially adopted a free-living or non-obligate symbiotic lifestyle (Castelli et al. 2024), with subsequent evolutionary events leading to independent colonizations of eukaryotic hosts—first protists, then animals (Castelli et al. 2016; Wang and Luo 2021). Alternatively, it is possible that early Rickettsiales were associated with unknown host organisms belonging to eukaryotic stem groups (i.e., extinct or unknown lineages that diverged before LECA but after the split with the closest living relatives of eukaryotes). This hypothesis points to previously unrecognized interactions between stem-group eukaryotes and prokaryotes, and hints at complex prokaryotic engagement as a foundational trait of eukaryotic ancestors, offering insights into the origin of eukaryotes.

Materials and Methods

Mixture substitution model

In a mixture model, each category consists of a rate matrix M, an equilibrium frequency vector F, and a relative substitution rate R. Additionally, the computation of the likelihood necessitates the inclusion of branch lengths θ, and the tree topology τ. Let the full parameter vector be denoted by $\mathbf{\Theta} = (R, F, V, \theta, \tau)$. Denote by $\mathcal{R}$, $\mathcal{F}$, and $\mathcal{V}$ the number of categories of exchangeability matrices, equilibrium frequencies, and relative substitution rates, respectively. Denote by $\mathcal{S}$ the number of alignment sites. Hence, the full likelihood of the phylogeny is given as

$$P(D|\boldsymbol{\Theta}) = \prod_{s=1}^{\mathcal{S}} \sum_{r=1}^{\mathcal{R}} \sum_{f=1}^{\mathcal{F}} \sum_{v=1}^{\mathcal{V}} P\left(D_s \middle| R_r, F_f, V_v, \theta, \tau\right) P\left(R_r, F_f, V_v\right), \tag{1}$$

where $P\left(R_r, F_f, V_v\right)$ indicate the mixture weights (Guindon 2019). Model constraints are enforced by restricting the set of allowed combinations (r, f, v), with the mixture weights normalized over this restricted set. Under independence, this gives $P(R_r, F_f, V_v) = P(R_r)P(F_f)P(V_v)$, whereas for linked models such as LG4M or LG4X, combinations with $f \neq v$ are excluded.

Numerical calculation of the Hessian matrix

Two primary numerical methods are commonly employed for computing the Hessian matrix. Let $\hat{\theta} = \left(\hat{\theta}_1, \dots, \hat{\theta}_n\right)$ indicate the vector of the MLES of branch lengths, and $\ell\left(\hat{\theta}\right)$ be the log-likelihood of the phylogeny. The outer product of scores (OPS) method (Seo et al. 2004; dos Reis and Yang 2011) estimates the Hessian matrix indirectly using the first derivatives (scores) of the log-likelihood to sum the contributions of the log-likelihood at each site:

$$H\left(\hat{\theta}\right) \approx -\sum_{s=1}^{\mathcal{S}} \left(\frac{\partial}{\partial \theta} \ell_s\left(\hat{\theta}\right) \frac{\partial}{\partial \theta^T} \ell_s\left(\hat{\theta}\right) \right). \tag{2}$$

Each component of the score vector $\frac{\partial}{\partial \theta} \ell_s\left(\hat{\theta}\right)$ is numerically calculated by the central finite difference method as

$$\frac{\partial \ell_s}{\partial \theta_i} \approx \frac{\ell_s\left(\hat{\theta} + h e_i\right) - \ell_s\left(\hat{\theta} - h e_i\right)}{2h} + \mathcal{O}(h^2),$$

where e_i is the i-th standard basis vector, and h is a small step size, typically the cube or square root of machine precision.

The second way is by a direct second-order derivative method. It calculates $H(\hat{\theta})$ via finite differences of the log-likelihood function by

$$H(\hat{\theta}) = \sum_{s=1}^{\mathcal{S}} \frac{\partial^2}{\partial\theta\partial\theta^T} \ell_s(\hat{\theta}), \tag{3}$$

where diagonal elements and off-diagonal elements are respectively calculated by the central finite difference method as

$$H_{ii}(\hat{\theta}) \approx \frac{\ell(\hat{\theta} + he_i) - 2\ell(\hat{\theta}) + \ell(\hat{\theta} - he_i)}{h^2},$$

$$H_{ij}(\hat{\theta}) \approx \frac{\ell(\hat{\theta} + he_i + he_j) - \ell(\hat{\theta} + he_i - he_j) - \ell(\hat{\theta} - he_i + he_j) + \ell(\hat{\theta} - he_i - he_j)}{4h^2}.$$

Under regularity conditions, $H(\hat{\theta})/\mathcal{S}$ converges to the expected Fisher information for a single site $I(\theta) = -\mathrm{E}\left[\frac{\partial^2}{\partial\theta\,\partial\theta^T}\ell_s(\theta)\right]$ regardless of whether it is calculated using Eq. (2) or (3) (Pawitan 2001):

$$\frac{1}{\mathcal{S}}\sum_{s=1}^{\mathcal{S}} \frac{\partial^2}{\partial\theta\partial\theta^T} \ell_s(\hat{\theta}) \simeq -\frac{1}{\mathcal{S}}\sum_{s=1}^{\mathcal{S}} \left(\frac{\partial}{\partial\theta}\ell_s(\hat{\theta})\frac{\partial}{\partial\theta^T}\ell_s(\hat{\theta})\right). \tag{4}$$

In both methods described as above, the error term is of order $\mathcal{O}(h^2)$. To further improve the numerical accuracy of the second-order derivative method, particularly when dealing with very short branches, we derive the central finite difference calculation of the Hessian at $\mathcal{O}(h^4)$. For the OPS method, this is simply

$$\frac{\partial\ell}{\partial\theta_i} \approx \frac{-\ell(\hat{\theta} + 2he_i) + 8\ell(\hat{\theta} + he_i) - 8\ell(\hat{\theta} - he_i) + \ell(\hat{\theta} - 2he_i)}{12h} + \mathcal{O}(h^4) \tag{5}$$

followed by summing the contributions of the log-likelihood at each site according to Eq. (2). Regarding the direct second-order derivative method, the result is more complex and is detailed in Note S3.3.

In phyloHessian, the default algorithm is the OPS method, which is employed by CODEML in PAML v4.3+. Alternatively, users can specify the second-order derivative method, as used in PAML 4.1 and earlier versions. Users can also specify the order of the error term, allowing balancing computational accuracy with speed based on their needs. If not otherwise indicated, the computation of all Hessian matrices in the present study was performed using the OPS algorithm.

Simulation procedure

We evaluated the performance of different substitution models using simulated data that mimics deep-time evolution (Fig. S16). In our focal simulation scheme, for each substitution model used in sequence evolution simulation (details provided below), we generated 30 timetrees each with 20 tips, using the R package TreeSim (Stadler 2011). Following our previous work (Wang and Luo 2025), timetrees were created under a birth-death model with birth and death rates of 0.4 and 0.2 lineages per 0.1 Gyr as estimated in a prior study of prokaryotic lineage diversification (Scholl and Wiens 2016), and with a taxon sampling proportion of 10% (see Note S1 for alternative settings). Simulated trees were generated with true root ages of 1.0, 2.0, 3.0, and 4.0 Ga to represent a range of evolutionary timescales approaching the age of Earth (~4.5 Ga).

For each timetree, branch-specific substitution rates were sampled under both the independent rates (IR) and autocorrelated rates (AR) model. For the IR mode, following our previous study (Wang and Luo 2021), the rate follows lognormal distribution with parameters $\mu_{\mathrm{IR}} = -3.7$ and $\sigma_{\mathrm{IR}} = 0.2$, such that the mean of rate is $\mathrm{e}^{\mu_{\mathrm{IR}}+\frac{\sigma_{\mathrm{IR}}^2}{2}} \approx 0.025$ substitutions/site/0.1 Gyr, with a standard deviation of $\sqrt{\left(e^{\sigma_{\mathrm{IR}}^2}-1\right)e^{\left(2\mu_{\mathrm{IR}}+\sigma_{\mathrm{IR}}^2\right)}} \approx 0.005$ substitutions/site/0.1 Gyr. This roughly corresponds to empirical observations that tip-to-root distances in the bacterial tree of

552 life, constructed with universally conserved orthologs, are roughly 1.0 amino acid
553 substitutions/site (Moody et al. 2022; Wang and Luo 2025), assuming an origin of early life at
554 around 4.0 Ga (Battistuzzi and Hedges 2009; Marin et al. 2017; Betts et al. 2018; Moody et al.
555 2024). The methodology used to determine the simulation parameters for the AR model is
556 described in detail in the next section.

557 Alignments of 300 amino acids (aa) were simulated using AliSim (Ly-Trong et al. 2022)
558 according to the timetree generated by simulation, under various substitution models. In the focal
559 analysis, we employed the LG+C60+G4{1.0} model. This indicates LG substitution model plus
560 C60 profile mixture models (60 empirically derived amino acid site frequency profiles) (Quang
561 et al. 2008) and across-site rate variation under a discrete gamma distribution with four relative
562 rate categories parameterized by $\alpha = 1.0$, hence Gamma(1,1), meaning that the mean and
563 variance of the across-site relative rates are $\frac{1}{1} = 1$ and $\frac{1}{1^2} = 1$ respectively (Yang 1994). Further,
564 we varied several simulation parameters, including sequence length, number of taxa, calibration
565 strategies, birth-death process parameters, across-site rate heterogeneity, molecular clock
566 settings, and model misspecifications. These are detailed in Note S1.

567

568 **Determining the parameters for simulation under the AR clock rate model**

569 Denote the absolute rate of sequence evolution (measured in substitutions per site per time unit)
570 under IR and AR respectively by the vectors $X_{\mathrm{IR}} = \left(X_{1,\mathrm{IR}}, \dots, X_{n,\mathrm{IR}}\right)$ and $X_{\mathrm{AR}} = \left(X_{1,\mathrm{AR}}, \dots, X_{n,\mathrm{AR}}\right)$
571 where n represents the number of branches. Their log-transformed values are given by
572 $Y_{\mathrm{IR}} = (Y_{1,\mathrm{IR}}, \dots, Y_{n,\mathrm{IR}})$ and $Y_{\mathrm{AR}} = (Y_{1,\mathrm{AR}}, \dots, Y_{n,\mathrm{AR}})$. To make simulations under IR and AR models
573 more comparable, we require that the expectations of the sample mean and sample variance of

the log-transformed rate under the two models to be equal, respectively, given the same phylogeny. Mathematically, this requires that

$$\mathrm{E}(\bar{Y}_{\mathrm{AR}}) = \mathrm{E}(\bar{Y}_{\mathrm{IR}}), \quad \mathrm{E}(s_{\mathrm{AR}}^2) = \mathrm{E}(s_{\mathrm{IR}}^2) \tag{6}$$

where the sample variances of the log-transformed rates are denoted by s_{AR}^2 and s_{IR}^2.

For the IR model, it is assumed that the rate for each branch, denoted $X_{\mathrm{IR}} = [X_{1,\mathrm{IR}}, \dots, X_{n,\mathrm{IR}}]$, is independently and identically (*i.i.d.*) distributed according to a lognormal distribution with parameters μ_{IR} and σ_{IR}^2, such that the rate for each branch can be expressed as $X_{i,\mathrm{IR}} \sim \mathrm{Lognormal}(\mu_{\mathrm{IR}}, \sigma_{\mathrm{IR}}^2)$. In other words, we have

$$\mathrm{E}(\bar{Y}_{\mathrm{IR}}) = \mu_{\mathrm{IR}}, \quad \mathrm{E}(s_{\mathrm{IR}}^2) = \sigma_{\mathrm{IR}}^2. \tag{7}$$

The AR model in molecular clock dating, also known as the geometric Brownian (GBM) model, assumes that the lineage-specific rate follows a geometric Brownian motion with a drift parameter of zero. It is thus parameterized by two parameters, μ_{AR}, the log-transformed rate at the root, and σ_{AR}^2, which determines the volatility of the rate changes over time. Accordingly, it can be shown that the log-transformed rate under AR model after time t follows $\mathcal{N}\left(\mu_{\mathrm{AR}} - \frac{1}{2}t\sigma_{\mathrm{AR}}^2, t\sigma_{\mathrm{AR}}^2\right)$ (Rannala and Yang 2007; Panchaksaram et al. 2025). Let $C = (c_{ij})_{n\times n}$ denote the branch-wise covariance matrix where n is the number of branches in a rooted tree (note that this is different from what is so-called phylogenetic covariance matrix which is related to only the tips [Fig. S17]). Further, the log-transformed rates Y_{AR} follow the following multivariate distribution

$$Y_{\mathrm{AR}} \sim \mathcal{N}\left(\left(\mu_{\mathrm{AR}} - \frac{1}{2}\sigma_{\mathrm{AR}}^2 c_{11}, \dots, \mu_{\mathrm{AR}} - \frac{1}{2}\sigma_{\mathrm{AR}}^2 c_{nn}\right), \sigma_{\mathrm{AR}}^2 C\right).$$

Thus, it is easy to see that

$$\mathrm{E}\left(Y_{i,\mathrm{AR}}\right) = \mu_{\mathrm{AR}} - \frac{1}{2}\sigma_{\mathrm{AR}}^2 c_{ii}. \tag{8}$$

The expectation of the sample variance s_{AR}^2 of log-transformed rate Y_{AR} across all n branches is calculated as

$$\mathrm{E}(s_{AR}^2) = \frac{1}{n-1}\mathrm{E}\left\{\sum\nolimits_i \left(Y_{i,\mathrm{AR}} - \frac{1}{n}\sum\nolimits_j Y_{j,\mathrm{AR}}\right)^2\right\}. \tag{9}$$

Expanding the right-hand side, we obtain

$$\mathrm{E}\left[\sum_{i=1}^{n}\left(Y_{i,\mathrm{AR}} - \frac{1}{n}\sum_{j=1}^{n} Y_{j,\mathrm{AR}}\right)^2\right]$$

$$= \sum_{i=1}^{n}\mathrm{E}\left(Y_{i,\mathrm{AR}}^2\right) - \frac{1}{n}\sum_{i=1}^{n}\left(\sum_{j=1}^{n} 2\mathrm{E}\left(Y_{i,\mathrm{AR}}Y_{j,\mathrm{AR}}\right) - \frac{1}{n}\sum_{j=1}^{n}\sum_{k=1}^{n}\mathrm{E}\left(Y_{j,\mathrm{AR}}Y_{k,\mathrm{AR}}\right)\right)$$

$$= \sum_{i=1}^{n}\mathrm{E}\left(Y_{i,\mathrm{AR}}^2\right) - \frac{1}{n}\sum_{i=1}^{n}\sum_{j=1}^{n}\mathrm{E}\left(Y_{i,\mathrm{AR}}Y_{j,\mathrm{AR}}\right). \tag{10}$$

Further, note the following relationship

$$\mathrm{E}\left(Y_{i,\mathrm{AR}}^2\right) = \mathrm{Var}\left(Y_{i,\mathrm{AR}}\right) + \left[\mathrm{E}\left(Y_{i,\mathrm{AR}}\right)\right]^2 = c_{ii}\sigma_{\mathrm{AR}}^2 + \left(\mu_{\mathrm{AR}} - \frac{1}{2}\sigma_{\mathrm{AR}}^2 c_{ii}\right)^2,$$

$$\mathrm{E}\left(Y_{i,\mathrm{AR}}Y_{j,\mathrm{AR}}\right) = \mathrm{Cov}\left(Y_{i,\mathrm{AR}}, Y_{j,\mathrm{AR}}\right) + \mathrm{E}\left(Y_{i,\mathrm{AR}}\right)\mathrm{E}\left(Y_{j,\mathrm{AR}}\right)$$

$$= \sigma_{\mathrm{AR}}^2 c_{ij} + \left(\mu_{\mathrm{AR}} - \frac{1}{2}\sigma_{\mathrm{AR}}^2 c_{ii}\right)\left(\mu_{\mathrm{AR}} - \frac{1}{2}\sigma_{\mathrm{AR}}^2 c_{jj}\right).$$

Introducing the above into Eq. (10), we have

$$\sum_{i=1}^{n}\mathrm{E}\left(Y_{i,\mathrm{AR}}^2\right) - \frac{1}{n}\sum_{i=1}^{n}\sum_{j=1}^{n}\mathrm{E}\left(Y_{i,\mathrm{AR}}Y_{j,\mathrm{AR}}\right)$$

$$= \sum\nolimits_i \left[c_{ii}\sigma_{\mathrm{AR}}^2 + \left(\mu_{\mathrm{AR}} - \frac{1}{2}c_{ii}\sigma_{\mathrm{AR}}^2\right)^2\right] - \frac{1}{n}\sum\nolimits_{i,j}\left[c_{ij}\sigma_{\mathrm{AR}}^2 + \left(\mu_{\mathrm{AR}} - \frac{1}{2}c_{ii}\sigma_{\mathrm{AR}}^2\right)\left(\mu_{\mathrm{AR}} - \frac{1}{2}c_{jj}\sigma_{\mathrm{AR}}^2\right)\right]$$

$$= \underbrace{\sigma_{\mathrm{AR}}^2 \left[\sum_i c_{ii} - \frac{1}{n}\sum_{i,j} c_{ij}\right]}_{\text{Term 1}}$$

$$+ \underbrace{\left[\sum_i \left(\mu_{\mathrm{AR}} - \frac{1}{2}c_{ii}\sigma_{\mathrm{AR}}^2\right)^2 - \frac{1}{n}\sum_{i,j}\left(\mu_{\mathrm{AR}} - \frac{1}{2}c_{ii}\sigma_{\mathrm{AR}}^2\right)\left(\mu_{\mathrm{AR}} - \frac{1}{2}c_{jj}\sigma_{\mathrm{AR}}^2\right)\right]}_{\text{Term 2}}.$$

Substituting $\sum_i c_{ii} = \mathrm{tr}(C)$ and $\sum_{i,j} c_{ij} = \frac{1}{n}\mathbf{1}'C\mathbf{1}$ into Term 1, we can rewrite it as

$$\sigma_{\mathrm{AR}}^2 \left[\sum_i c_{ii} - \frac{1}{n}\sum_{i,j} c_{ij}\right] = \sigma_{\mathrm{AR}}^2 \left[\mathrm{tr}(\mathbf{C}) - \frac{1}{n}\mathbf{1}'C\mathbf{1}\right].$$

For Term 2, noting that terms involving μ_{AR} cancels out, we have

$$\sum_i \left(\mu_{\mathrm{AR}} - \frac{1}{2}c_{ii}\sigma_{\mathrm{AR}}^2\right)^2 - \frac{1}{n}\sum_{i,j}\left(\mu_{\mathrm{AR}} - \frac{1}{2}c_{ii}\sigma_{\mathrm{AR}}^2\right)\left(\mu_{\mathrm{AR}} - \frac{1}{2}c_{jj}\sigma_{\mathrm{AR}}^2\right)$$

$$= \left(n\mu_{\mathrm{AR}}^2 - \mu_{\mathrm{AR}}\sigma_{\mathrm{AR}}^2\mathrm{tr}(C) + \frac{1}{4}\sum_i c_{ii}^2\sigma_{\mathrm{AR}}^4\right) + \left(-n\mu_{\mathrm{AR}}^2 + \mu_{\mathrm{AR}}\sigma_{\mathrm{AR}}^2\mathrm{tr}(C) - \frac{1}{4n}\sum_{i,j} c_{ii}c_{jj}\sigma_{\mathrm{AR}}^4\right)$$

$$= \frac{\sigma_{\mathrm{AR}}^4}{4}\left[\sum_i c_{ii}^2 - \frac{1}{n}\left(\mathrm{tr}(C)\right)^2\right].$$

Hence,

$$\mathrm{E}(s_{\mathrm{AR}}^2) = \frac{\sigma_{\mathrm{AR}}^2}{n-1}\left[\left(\mathrm{tr}(C) - \frac{1}{n}\mathbf{1}'C\mathbf{1}\right) + \frac{\sigma_{\mathrm{AR}}^2}{4}\left(\sum_i c_{ii}^2 - \frac{1}{n}\left(\mathrm{tr}(C)\right)^2\right)\right].$$

Because $\mathrm{E}(s_{\mathrm{IR}}^2) = \sigma_{\mathrm{IR}}^2$, equating $\mathrm{E}(s_{\mathrm{IR}}^2)$ with $\mathrm{E}(s_{\mathrm{AR}}^2)$ and rearranging terms gives the quadratic equation for σ_{AR}^2

$$\frac{1}{4}\left[\sum_i c_{ii}^2 - \frac{1}{n}\left(\mathrm{tr}(C)\right)^2\right]\sigma_{\mathrm{AR}}^4 + \left[\mathrm{tr}(C) - \frac{1}{n}\mathbf{1}'C\mathbf{1}\right]\sigma_{\mathrm{AR}}^2 - (n-1)\sigma_{\mathrm{IR}}^2 = 0. \tag{11}$$

Discarding the negative root, along with solving Eq. (8), we ultimately get

$$\hat{\sigma}_{\mathrm{AR}}^2 = \frac{2(n-1)\sigma_{\mathrm{IR}}^2}{\sqrt{a^2 + 4b(n-1)\sigma_{\mathrm{IR}}^2} + a}, \quad \hat{\mu}_{\mathrm{AR}} = \mu_{\mathrm{IR}} + \frac{\hat{\sigma}_{\mathrm{AR}}^2\mathrm{tr}(C)}{2n} \tag{12}$$

where $a = \mathrm{tr}(C) - \frac{1}{n}\mathbf{1}'C\mathbf{1}$, $b = \frac{1}{4}\left[\sum_i c_{ii}^2 - \frac{1}{n}\left(\mathrm{tr}(C)\right)^2\right]$, and $\hat{\sigma}_{\mathrm{AR}}^2$ and $\hat{\mu}_{\mathrm{AR}}$ are the values of the parameters for the AR model that satisfy Eq. (6) given the parameters for the IR model (σ_{IR}^2 and μ_{IR}).

Calibrations used in molecular dating analysis on simulated datasets

Molecular dating analysis were conducted using MCMCtree v4.10.7 (see also Note S3) on phylogenies simulated with procedures described above under varying calibration strategies: a root-only calibration, or root calibration plus one internal calibration, chosen as the node at the median age of all nodes in the true timetree, or two internal calibrations which targeted the 1/3 and 2/3 age quantiles. Time priors for calibrated nodes were set to be uniform within the interval [true_age - (true_age/5), true_age + (true_age/5)], with soft bounds (MCMCtree's default: 2.5% probability of age outside bounds). Other information about MCMCtree analysis can be found in Note S3. The five calibration schemes mainly used in the present study are *root_only*: a single bounded calibration (both upper and lower bounds); *single_min*: bounded calibration at the root and one minimum-bounded internal node; *single_interval*: bounded calibration at both the root and an internal node; *two_min*: bounded calibration at the root and two minimum-bounded internal nodes; *two_intervals*: bounded calibration at both the root and two internal nodes.

Comparing the performance of different substitution models on simulated datasets

We evaluated the performance of various substitution models mainly using the relative difference (reldiff) that compared the posterior mean divergence times and substitution rates estimated by MCMCtree (using each model) to the true values used in simulations. Relative difference: calculated as $mean\left(\frac{|b_i^m - b_i^{true}|}{\max(b_i^m, b_i^{true})}\right)$, where b_i^m and b_i^{true} denote the posterior mean divergence

648 times or branch-specific substitution rates inferred by MCMCtree, and those used in simulation
649 (true values), respectively.
650
651 **Empirical data analysis**
652 Genomes of Microsporidia and Rickettsiales were downloaded from MicrosporadiaDB
653 (Aurrecoechea et al. 2011) and GenBank (last accessed July 2024), respectively. Sequences of
654 five additional Proteobacteria as the outgroup were retrieved from our prior study (Wang and
655 Luo 2025). TreeCluster v1.0.4 (Balaban et al. 2019) was used to select representative taxa with a
656 cutoff of phylogenetic depth (which takes phylogenetic distance and topology into account to
657 cluster taxa into subclades) of 0.5 and 2.5 respectively for Microsporidia (yielding 27 members)
658 and Rickettsiales (22 members). Genes used to reconstruct phylogeny and molecular dating for
659 Microsporidia were based on 27 orthologs identified by OrthoFinder v3.0.1 (Emms and Kelly
660 2019) present in all members allowing ≤2 copies per genome, while for Rickettsiales we adopted
661 the mitochondrial endosymbiosis-based strategy (Wang and Luo 2021) by using 24
662 mitochondria-encoded genes conserved across Alphaproteobacteria (Wang and Wu 2015) (see
663 Data availability). For both datasets, we partitioned the alignments into one, two, or three subsets
664 by fitting the logarithm of the average substitution rates estimated by CODEML into a Gaussian
665 mixture model. We selected the best-fitting substitution models for each partition under both site-
666 homogeneous and site-heterogeneous models according to AIC (Note S3). Calibration
667 information along with alternatives is available in Data S6.
668

Data availability

phyloHessian is available at https://github.com/evolbeginner/phyloHessianWrapper. Other codes and the scripts (Goto et al. 2010) generating them, are made available at https://figshare.com/s/96bc117f18051f9642ce.

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818 **Figure legends**

819 **Figure 1.** Comparison of the accuracy of divergence time and branch-specific substitution rate
820 estimation under substitution models LG+G and LG+C60+G by simulation. (A, B) Each plot
821 contains 30 dots representing the mean relative difference (compared to the true values used in
822 simulation) based on MCMCtree molecular clock dating with the corresponding calibration
823 strategy on 30 simulated datasets. For each simulation, 30 timetrees each containing 20 tips are
824 simulated using a birth-death process with a mean substitution rate 0.25 substitutions/site/Gyr
825 across four different root ages ranging from 1.0 to 4.0 Ga. Sequence alignments are generated
826 with LG+C60+G4{1.0} (+G4{1.0} indicates $\alpha = 1$ in a four-category discrete Gamma-
827 distributed across-site relative rates) under AR (left-hand side) and IR (right-hand side) clock
828 models respectively. *P*-values are obtained from a paired Wilcoxon signed-rank test. The box
829 plots (median, interquartile range, 1.5×IQR whiskers) illustrate the relative differences in time
830 (A) and rate (B) estimates compared to true values. (C) A schematic figure showing the five
831 calibration schemes used for molecular clock analyses on simulated data. *root_only*: a single
832 bounded calibration (both upper and lower bounds); *single_min*: bounded calibration at the root
833 and one minimum-bounded internal node; *single_interval*: bounded calibration at both the root
834 and an internal node; *two_min*: bounded calibration at the root and two minimum-bounded
835 internal nodes; *two_intervals*: bounded calibration at both the root and two internal nodes.

836

837 **Figure 2.** Investigating the robustness of divergence time estimation to alternative settings in
838 simulation. (A) Sequence simulated under substitution model LG+C60+G4{0.5} (*Gamma_0.5*;
839 the parameter alpha in the discrete Gamma distribution equal to 0.5), thus more rate
840 heterogeneity across sites than the focal analysis (LG+C60+G4{1.0}). (B) Timetree simulated

under a flat time prior (*flat_time_prior*). (C) Timetree simulated under a molecular clock with larger across-branch rate variance (*var_lograte_0.38*). The plots on the left and right correspond to analyses using the AR and IR clock models, respectively. More detailed information about the above alternative simulation schemes can be found in Note S1. Calibration strategies and other settings are the same as those used in Fig. 1.

Figure 3. Evaluation of the impacts of calibration and model misspecification on molecular clock dating. Accuracy of divergence time estimation (relative difference) is shown as the *y*-axis. (A, B) The lower and upper time bounds of all calibrations are shifted forward (A) and backward (B) in time by 20% (f0.2, b0.2) or 50% (f0.5, b0.5) of their true values, respectively. Three calibration schemes are considered: (i) a root-only calibration (*root_only*), (ii) the root plus one fully calibrated internal node (*single_interval*), and (iii) the root plus two fully calibrated internal nodes (*two_intervals*), each with time ranges of the true value ±20% or ±50%. (C) Assessment of the accuracy of divergence time estimation under alternative substitution models used in simulation (LG+C60+G used in most other analyses): LG+G, LG+G+I, LG+R, LG+C20+G, LG+C60+G+I. Boxplots compare the accuracy obtained by the true model (purple) and by LG+C60+G (blue). Each plot contains 30 dots representing the mean relative difference based on MCMCtree molecular clock dating with the corresponding calibration strategy on 30 simulated datasets. For each simulation, 30 timetrees each containing 20 tips are simulated using a birth-death process with a mean substitution rate 0.25 substitutions/site/Gyr across four different root ages ranging from 1.0 to 4.0 Ga. The plots on the left and right correspond to analyses using the AR and IR clock models, respectively. Calibration strategies are the same as those used in Fig. 1.

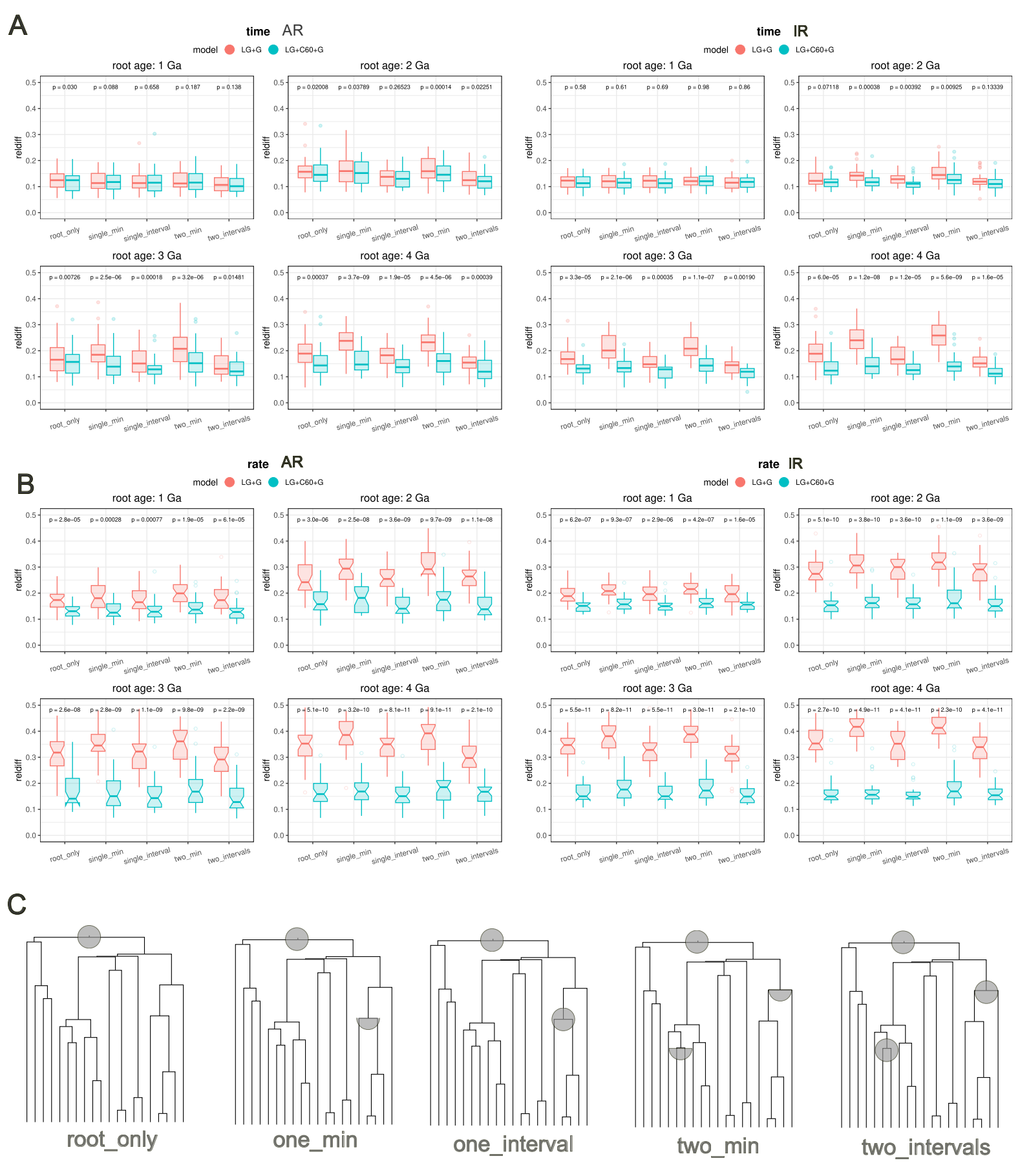
Figure 4. Evaluation of the approximation of PMSF and bootstrapped Hessian on molecular clock analysis by simulation. Phylogenies are simulated under the same strategy as the focal dataset. The sequence alignment is simulated under LG+C60+G{1.0}. The bootstrapped Hessian matrix is calculated using the software bs_inBV. The *y*-axis indicates the relative accuracy of the posterior mean of the divergence times (A) or substitution rates (B) compared to that obtained by the first MCMC run under LG+C60+G (note that this is different from other figures where the baseline in comparison is the true simulated times and rates). Each plot contains 30 dots representing the mean relative difference based on MCMCtree molecular clock dating with the corresponding calibration strategy on 30 simulated datasets. For each simulation, 30 timetrees each containing 20 tips are simulated using a birth-death process with a mean substitution rate 0.25 substitutions/site/Gyr across four different root ages ranging from 1.0 to 4.0 Ga. *LG+C60+G_run2*: the second independent MCMC run of MCMCtree under LG+C60+G. *LG+C60+G+PMSF*: PMSF approximation with LG+C60+G. *LG+C60+G+bs_inBV*: bootstrapped Hessian under LG+C60+G. *LG+C60+G+PMSF+bs_inBV:* bootstrapped Hessian under LG+C60+G+PMSF. Calibration strategies are the same as those used in Fig. 1.

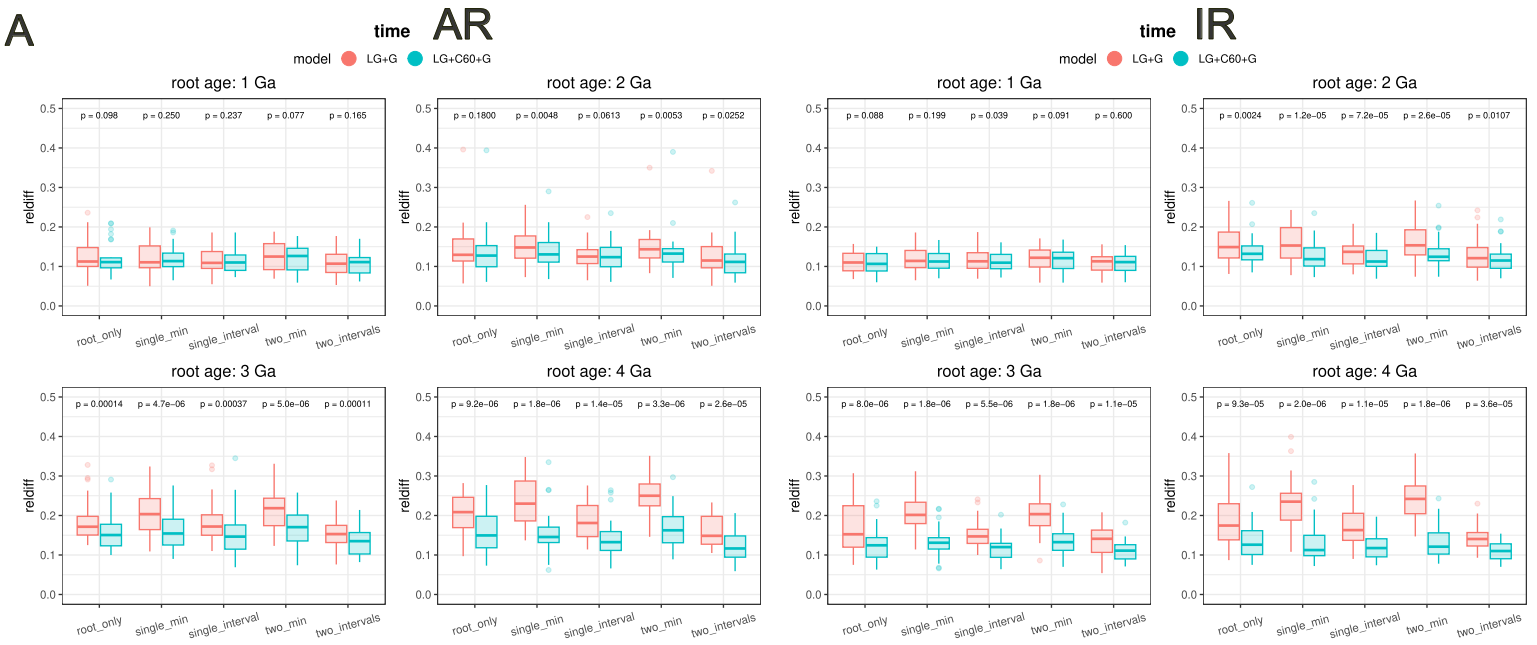
Figure 5. Evolutionary timeline and substitution rates estimated by MCMCtree under different substitution models for Microsporidia. A uniform calibration prior between 0.5 and 0.9 Ga was applied to the root (see Note S2), with soft bounds extending 2.5% beyond each time bound. Branch colors indicate the logarithm of the posterior mean substitution rate (estimated by MCMCtree), ranging from blue (low) to red (high). (B) Posterior divergence times and branch-specific substitution rates estimated by the mixture substitution model (*y*-axis) versus those

886 estimated by the homogeneous substitution model (*x*-axis) under the AR or IR clock models. The
887 bars indicate the 95% HPD.
888
889 **Figure 6.** Evolutionary timeline and substitution rates estimated by MCMCtree under different
890 substitution models for Rickettsiales. This is based the mitochondrial endosymbiosis dating
891 strategy (Wang and Luo 2021), using eukaryotic fossils to calibrate Rickettsiales evolution,
892 based on the evolutionary scenario that mitochondria originated from a lineage closely related to
893 α-Proteobacteria. (A) The estimated evolutionary timeline of Rickettsiales under different
894 substitution (the best-fitting homogeneous or mixture) and clock (IR or AR) models. All
895 calibrated nodes are denoted by a circle. In addition to the calibration at the root, all four
896 calibrations are placed within eukaryotes in the clade of mitochondria (see Note S2). Branch
897 colors indicate the logarithm of the posterior mean substitution rate estimated by MCMCtree,
898 ranging from blue (low) to red (high). (B) Posterior divergence times and branch-specific
899 substitution rates estimated by the mixture substitution model (*y*-axis) versus those estimated by
900 the homogeneous substitution model (*x*-axis) under the AR or IR clock models. The bars indicate
901 the 95% HPD.
902

Table 1. Reduction of the relative differences in substitution rate estimates, calculated as as $\frac{\text{reldiff}_{\text{LG+G}} - \text{reldiff}_{\text{LG+C60+G}}}{\text{reldiff}_{\text{LG+G}}}$, under LG+G versus LG+C60+G under different root ages and calibration strategies.

Calibration strategy	**AR (root age)**				**IR (root age)**			
	1.0 Ga	2.0 Ga	3.0 Ga	4.0 Ga	1.0 Ga	2.0 Ga	3.0 Ga	4.0 Ga
root_only	26%	35%	47%	51%	23%	44%	52%	55%
single_min	27%	38%	53%	56%	24%	46%	52%	59%
single_interval	23%	41%	51%	55%	24%	44%	49%	55%
two_min	28%	42%	50%	55%	24%	45%	52%	57%
two_intervals	28%	42%	50%	48%	21%	42%	50%	51%
Average	26%	40%	50%	53%	23%	44%	51%	55%


A
time AR
model LG+G LG+C60+G
root age: 1 Ga
p = 0.030 p = 0.088 p = 0.658 p = 0.187 p = 0.138
root age: 2 Ga
p = 0.02008 p = 0.03789 p = 0.26523 p = 0.00014 p = 0.02251
root age: 3 Ga
p = 0.00726 p = 2.5e−06 p = 0.00018 p = 3.2e−06 p = 0.01481
root age: 4 Ga
p = 0.00037 p = 3.7e−09 p = 1.9e−05 p = 4.5e−06 p = 0.00039
time IR
model LG+G LG+C60+G
root age: 1 Ga
p = 0.58 p = 0.61 p = 0.69 p = 0.98 p = 0.86
root age: 2 Ga
p = 0.07118 p = 0.00038 p = 0.00392 p = 0.00925 p = 0.13339
root age: 3 Ga
p = 3.3e−05 p = 2.1e−06 p = 0.00035 p = 1.1e−07 p = 0.00190
root age: 4 Ga
p = 6.0e−05 p = 1.2e−08 p = 1.2e−05 p = 5.6e−09 p = 1.6e−05
reldiff
root_only single_min single_interval two_min two_intervals
B
rate AR
model LG+G LG+C60+G
root age: 1 Ga
p = 2.8e−05 p = 0.00028 p = 0.00077 p = 1.9e−05 p = 6.1e−05
root age: 2 Ga
p = 3.0e−06 p = 2.5e−08 p = 3.6e−09 p = 9.7e−09 p = 1.1e−08
root age: 3 Ga
p = 2.6e−08 p = 2.8e−09 p = 1.1e−09 p = 9.8e−09 p = 2.2e−09
root age: 4 Ga
p = 5.1e−10 p = 3.2e−10 p = 8.1e−11 p = 9.1e−11 p = 2.1e−10
rate IR
model LG+G LG+C60+G
root age: 1 Ga
p = 6.2e−07 p = 9.3e−07 p = 2.9e−06 p = 4.2e−07 p = 1.6e−05
root age: 2 Ga
p = 5.1e−10 p = 3.8e−10 p = 3.6e−10 p = 1.1e−09 p = 3.6e−09
root age: 3 Ga
p = 5.5e−11 p = 8.2e−11 p = 5.5e−11 p = 3.0e−11 p = 2.1e−10
root age: 4 Ga
p = 2.7e−10 p = 4.9e−11 p = 4.1e−11 p = 2.3e−10 p = 4.1e−11
reldiff
root_only single_min single_interval two_min two_intervals
C
root_only
one_min
one_interval
two_min
two_intervals


A
time
AR
model LG+G LG+C60+G
root age: 1 Ga
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root age: 2 Ga
p = 0.1800 p = 0.0048 p = 0.0613 p = 0.0053 p = 0.0252
root age: 3 Ga
p = 0.00014 p = 4.7e−06 p = 0.00037 p = 5.0e−06 p = 0.00011
root age: 4 Ga
p = 9.2e−06 p = 1.8e−06 p = 1.4e−05 p = 3.3e−06 p = 2.6e−05
time
IR
model LG+G LG+C60+G
root age: 1 Ga
p = 0.088 p = 0.199 p = 0.039 p = 0.091 p = 0.600
root age: 2 Ga
p = 0.0024 p = 1.2e−05 p = 7.2e−05 p = 2.6e−05 p = 0.0107
root age: 3 Ga
p = 8.0e−06 p = 1.8e−06 p = 5.5e−06 p = 1.8e−06 p = 1.1e−05
root age: 4 Ga
p = 9.3e−05 p = 2.0e−06 p = 1.1e−05 p = 1.8e−06 p = 3.6e−05
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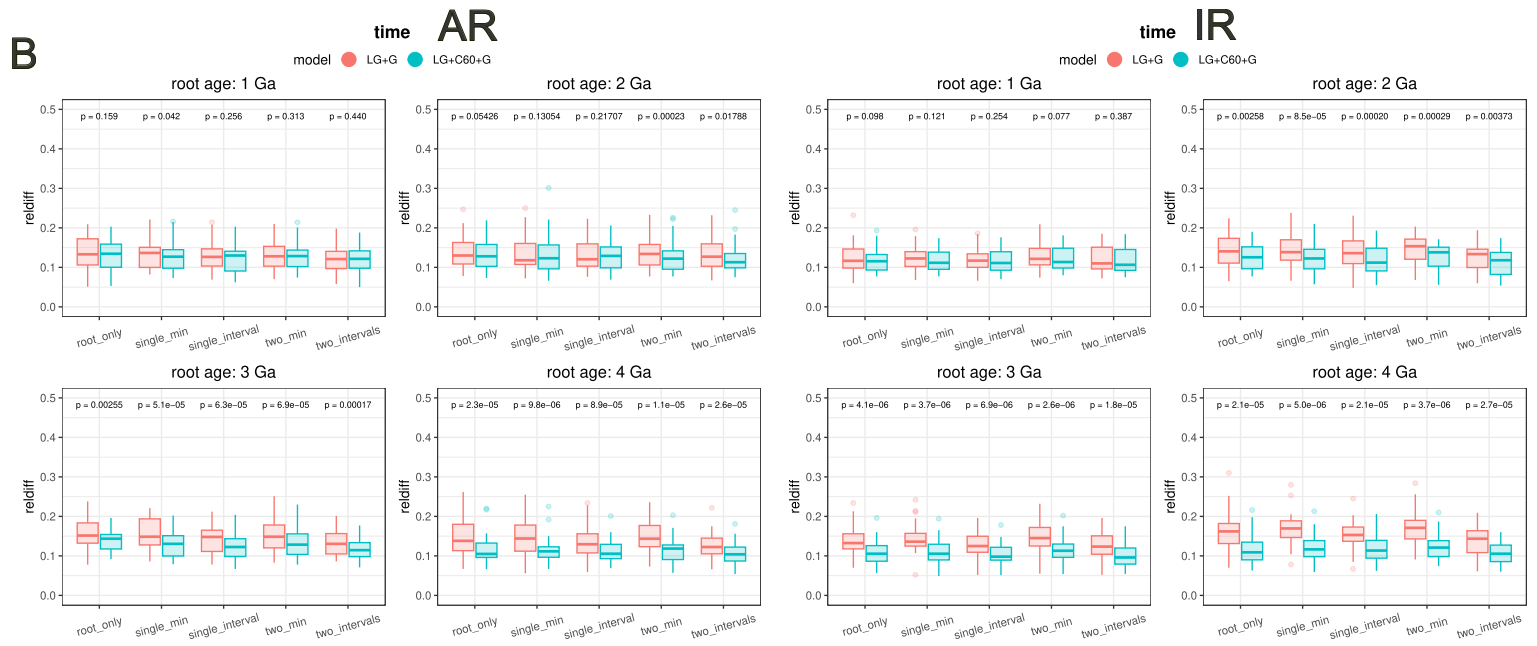
root_only single_min single_interval two_min two_intervals


B
time
AR
model LG+G LG+C60+G
root age: 1 Ga
p = 0.159 p = 0.042 p = 0.256 p = 0.313 p = 0.440
root age: 2 Ga
p = 0.05426 p = 0.13054 p = 0.21707 p = 0.00023 p = 0.01788
root age: 3 Ga
p = 0.00255 p = 5.1e−05 p = 6.3e−05 p = 6.9e−05 p = 0.00017
root age: 4 Ga
p = 2.3e−05 p = 9.8e−06 p = 8.9e−05 p = 1.1e−05 p = 2.6e−05
time
IR
model LG+G LG+C60+G
root age: 1 Ga
p = 0.098 p = 0.121 p = 0.254 p = 0.077 p = 0.387
root age: 2 Ga
p = 0.00258 p = 8.5e−05 p = 0.00020 p = 0.00029 p = 0.00373
root age: 3 Ga
p = 4.1e−06 p = 3.7e−06 p = 6.9e−06 p = 2.6e−06 p = 1.8e−05
root age: 4 Ga
p = 2.1e−05 p = 5.0e−06 p = 2.1e−05 p = 3.7e−06 p = 2.7e−05
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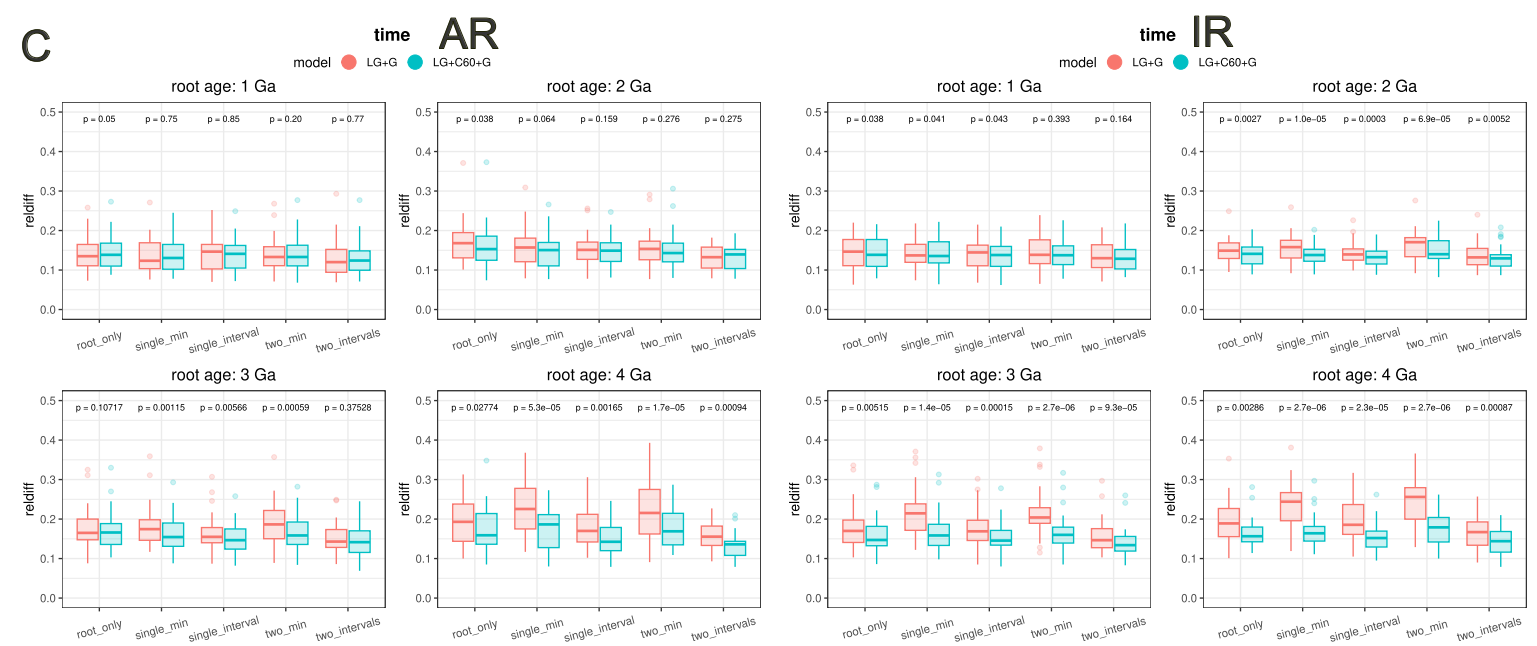
root_only single_min single_interval two_min two_intervals


C
time
AR
model LG+G LG+C60+G
root age: 1 Ga
p = 0.05 p = 0.75 p = 0.85 p = 0.20 p = 0.77
root age: 2 Ga
p = 0.038 p = 0.064 p = 0.159 p = 0.276 p = 0.275
root age: 3 Ga
p = 0.10717 p = 0.00115 p = 0.00566 p = 0.00059 p = 0.37528
root age: 4 Ga
p = 0.02774 p = 5.3e−05 p = 0.00165 p = 1.7e−05 p = 0.00094
time
IR
model LG+G LG+C60+G
root age: 1 Ga
p = 0.038 p = 0.041 p = 0.043 p = 0.393 p = 0.164
root age: 2 Ga
p = 0.0027 p = 1.0e−05 p = 0.0003 p = 6.9e−05 p = 0.0052
root age: 3 Ga
p = 0.00515 p = 1.4e−05 p = 0.00015 p = 2.7e−06 p = 9.3e−05
root age: 4 Ga
p = 0.00286 p = 2.7e−06 p = 2.3e−05 p = 2.7e−06 p = 0.00087
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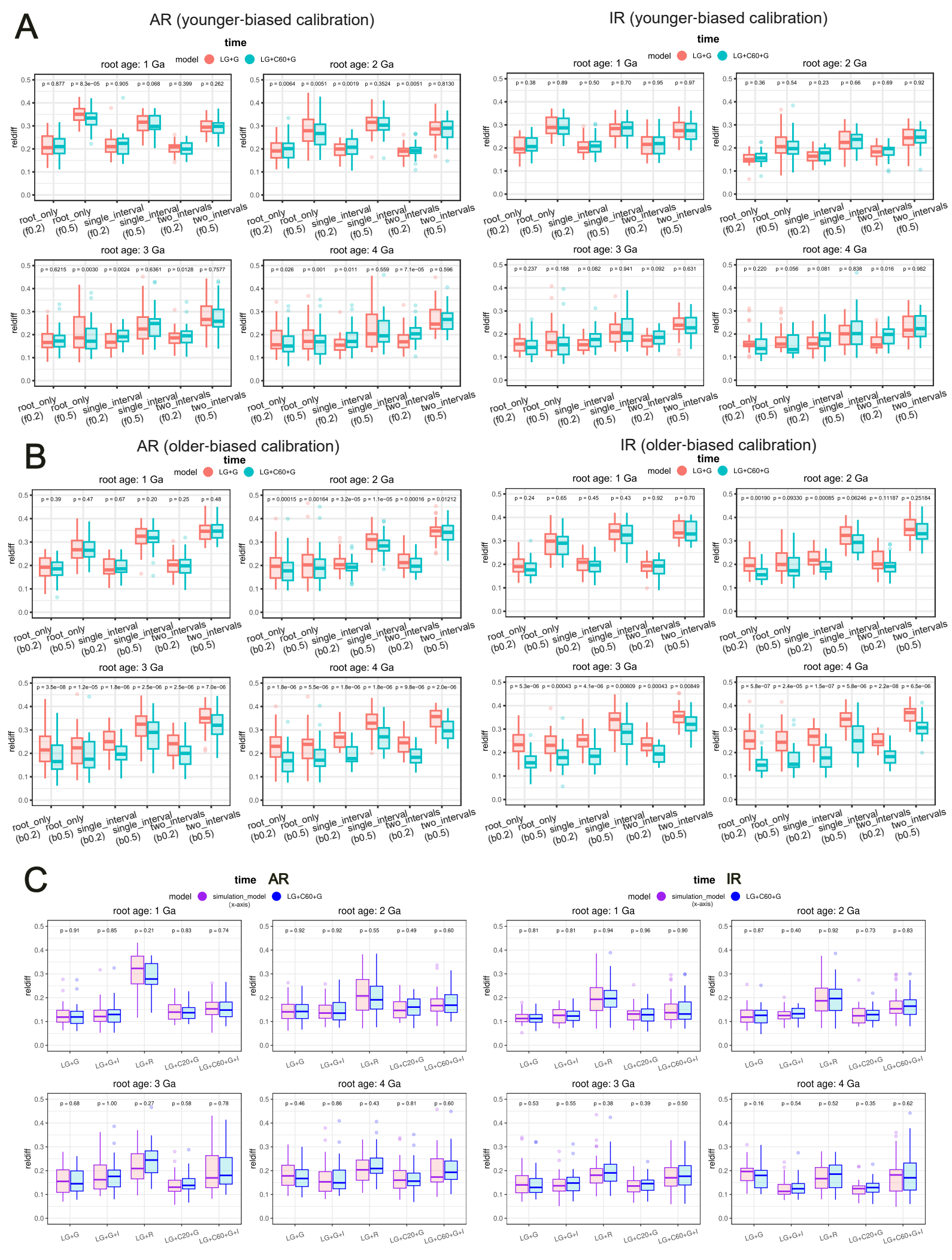
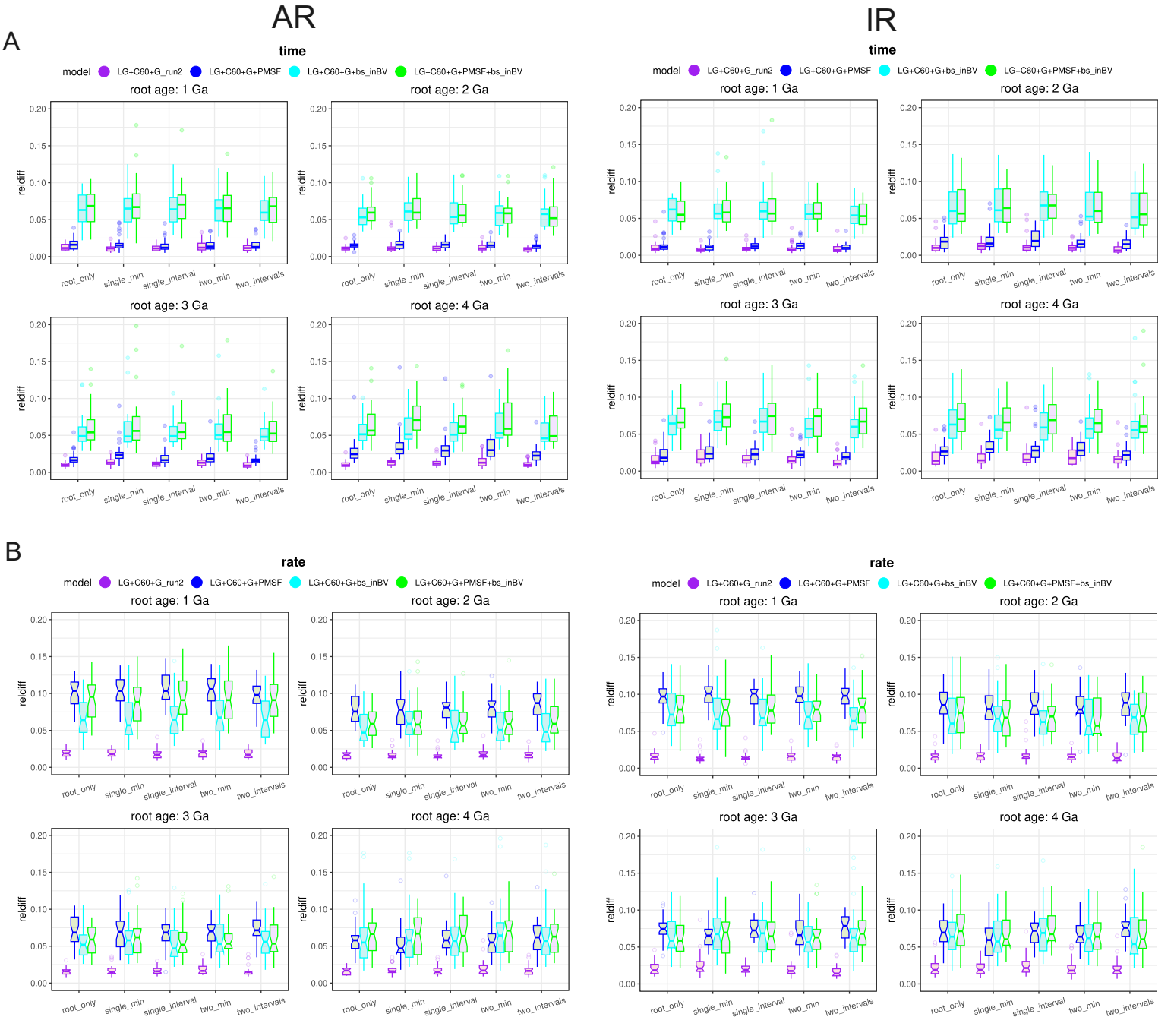

A
AR (younger-biased calibration)
IR (younger-biased calibration)
B
AR (older-biased calibration)
IR (older-biased calibration)
C
AR
IR
time
model
LG+G
LG+C60+G
simulation_model
(x-axis)
root age: 1 Ga
root age: 2 Ga
root age: 3 Ga
root age: 4 Ga
reldiff
root_only (f0.2)
root_only (f0.5)
single_interval (f0.2)
single_interval (f0.5)
two_intervals (f0.2)
two_intervals (f0.5)
root_only (b0.2)
root_only (b0.5)
LG+G
LG+G+I
LG+R
LG+C20+G
LG+C60+G+I


AR
IR
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B
time
rate
model
LG+C60+G_run2
LG+C60+G+PMSF
LG+C60+G+bs_inBV
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root age: 1 Ga
root age: 2 Ga
root age: 3 Ga
root age: 4 Ga
reldiff
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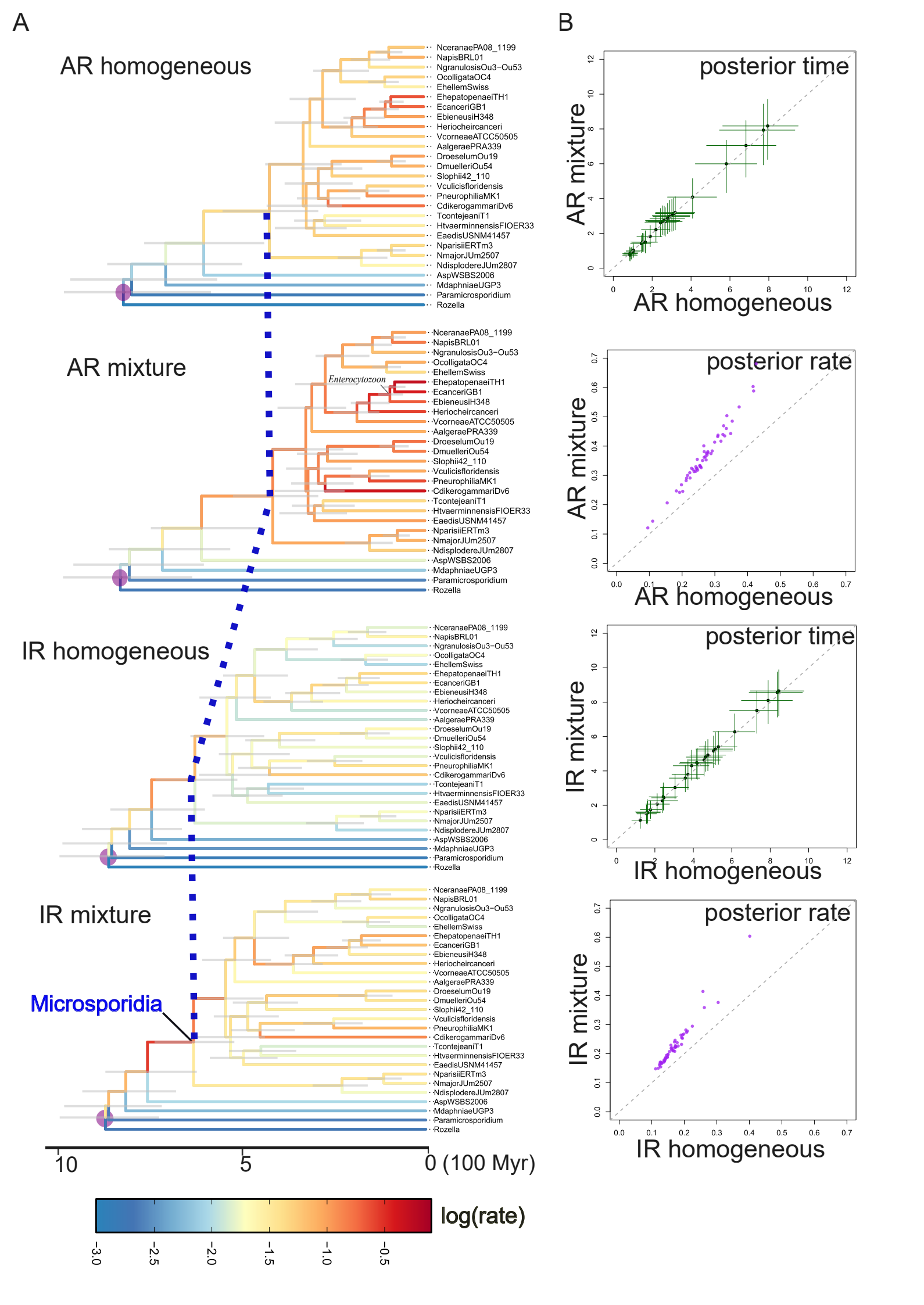
0.10
0.15
0.20
root_only
single_min
single_interval
two_min
two_intervals


A
AR homogeneous
AR mixture
IR homogeneous
IR mixture
Microsporidia
Enterocytozoon
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OcolligataOC4
EhellemSwiss
EhepatopenaeiTH1
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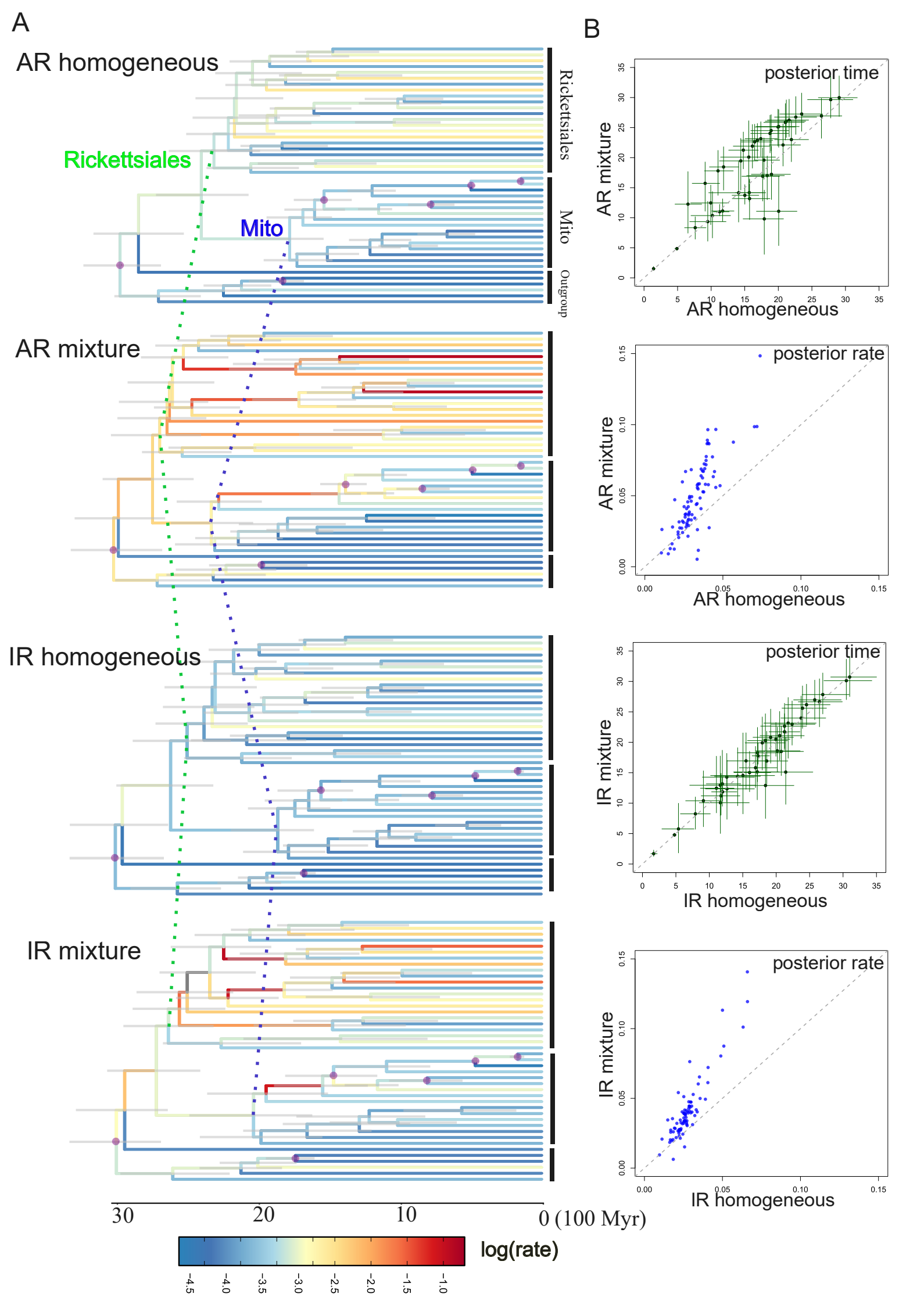
Heriocheircanceri
VcorneaeATCC50505
AalgeraePRA339
DroeselumOu19
DmuelleriOu54
Slophii42_110
Vculicisfloridensis
PneurophiliaMK1
CdikerogammariDv6
TcontejeaniT1
HtvaerminnensisFIOER33
EaedisUSNM41457
NparisiiERTm3
NmajorJUm2507
NdisplodereJUm2807
AspWSBS2006
MdaphniaeUGP3
Paramicrosporidium
Rozella
10
5
0 (100 Myr)
log(rate)
−3.0
−2.5
−2.0
−1.5
−1.0
−0.5
B
posterior time
AR mixture
AR homogeneous
posterior rate
AR mixture
AR homogeneous
posterior time
IR mixture
IR homogeneous
posterior rate
IR mixture
IR homogeneous


A
AR homogeneous
Rickettsiales
Mito
Rickettsiales
Mito
Outgroup
AR mixture
IR homogeneous
IR mixture
30
20
10
0 (100 Myr)
log(rate)
-4.5
-4.0
-3.5
-3.0
-2.5
-2.0
-1.5
-1.0
B
posterior time
AR mixture
AR homogeneous
posterior rate
AR mixture
AR homogeneous
posterior time
IR mixture
IR homogeneous
posterior rate
IR mixture
IR homogeneous