Molecular clocks without rocks: new solutions for old problems

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Abstract

Molecular data have been used to date species divergences ever since they were described as "documents of evolutionary history" in the 1960s. Yet, an inadequate fossil record and discordance between gene trees and species trees are persistently problematic. We examine how, by accommodating gene tree discordance and by scaling branch lengths to absolute time using mutation rate and generation time, multispecies coalescent (MSC) methods can potentially overcome these challenges. We find that time estimates can differ — in some cases, substantially — depending on whether MSC methods or traditional phylogenetic methods that apply concatenation are used, and whether the tree is calibrated with pedigree-based mutation rates or with fossils. We discuss the advantages and shortcomings of both approaches and provide practical guidance for data analysis when using these methods.

Divergence Time Estimation

Zukerkandl and Pauling [1] were the first to posit that genetic distances between organisms could be converted to absolute geological times, describing genomes as "documents of evolutionary history." The most commonly used **molecular clock** methods (See Glossary) estimate absolute times from genetic distances by calibrating the **species tree** with fossil data, assuming either a constant rate of evolution among lineages (the molecular clock) or variable rates (**relaxed clock models**) [2-4]). Recently, the **multispecies coalescent (MSC)** [5] is on the ascent as a method for estimating divergence times [6, 7] due at least in part to potential freedom from **fossil calibrations** [8, 9]. However, conflicts can arise in empirical studies between **traditional phylogenetic clock models** and MSC methods, raising the question of which method is more reliable for placing evolutionary events in a temporal context. Here, we examine the fundamental assumptions and analytical details of these two general methodological classes: 1) Traditional phylogenetic clock models that use **concatenation** of genetic **loci** and 2) MSC models that explicitly model **gene tree discordance** due to **incomplete lineage sorting (ILS)**. Both approaches can be used without fossil calibrations where *a priori* information on absolute rates of evolution are available, but some features of the MSC are ideal for estimating species divergence times by leveraging external *de novo* **mutation rate (μ)** estimates, typically measured from pedigree trios (Fig. 1; Key Figure). We conclude by describing conditions that influence the suitability of the two approaches and offer recommendations for the proper application of both.

The Allure of the Molecular Clock

Clock models and their applications have had enormous impacts on our understanding of the history of life on earth, including the timing of life history transitions [10], global ecological change in response to climate oscillations [11], the ancient origins of orders such as Lepidoptera [12], and even the origin of life or the last universal common ancestor (LUCA) after the Moon-forming impact [13]. Calibration of the molecular clock has historically been performed using fossil ages [14] or geological events [15] though only a tiny fraction of phylogenetic lineages have reliable fossil records for appropriate calibration [16]. Thus, the lack of a detailed fossil record for many groups is a major constraint for investigating the evolutionary history of those lineages. For instance, both plant [17] and animal fossils [18] are difficult to characterize in tropical rainforests where available rock formations for fossilization are typically absent [19]. In some groups, such as grasses, calibrations based on phytolith microfossils are contentious because of ambiguous diagnostic characters that compromise accurate phylogenetic placement [20]. And for many groups, such as the glass frogs [21], fossils are entirely absent.

Thus, for the analysis of most clades across the tree of life, investigators must depend on fossil calibrations that are phylogenetically distant from the organisms of interest. As phylogenetic distance increases, the complexities of modeling rate variation among lineages also increases given the now extensive evidence that molecular rates change frequently across phylogeny. Finally, a growing body of literature suggests that by ignoring genetic polymorphism in ancestral species, divergence times may be systematically biased [6, 7, 9].

Relaxed Clock Models

When a calibration point can be placed with confidence within a given clade and close to its **most recent common ancestor (MRCA)**, it is possible to estimate **per-year substitution rates**. Using that rate, an investigator can then infer divergence times for other nodes in the phylogeny that do not have fossil calibrations. This assumes, however, that all lineages share a single rate of evolution: i.e., that there is a **strict molecular clock**. While this is not an unreasonable assumption for closely related species, the strict clock is typically violated when

more distantly related species are included [22]. Such violations can arise not only from differences in the molecular mechanisms that generate mutations [23], but also from variation in life history traits [24, 25]. For example, great apes have lower substitution rates compared to Old World and New World monkeys (the hominoid slowdown hypothesis [26]), a phenomenon that can largely be explained by differences in **generation time** among species [27]. Similar observations have been made in plants by comparing woody and herbaceous species [28, 29].

The clock can be "relaxed" by allowing for variable rates among branches on a phylogeny while maintaining computational tractability and statistical identifiability [2, 3, 30, 31]. The first relaxed clock methods that could leverage uncertainty across multiple calibrations were implemented with maximum likelihood and required *a priori* assumptions to partition branches into different rate groups (e.g., "local clocks" [32] or heuristic approaches [33, 34]). Recent **Bayesian methods** have incorporated uncertainty in calibrations and as well as in rates of evolution through the use of **prior distributions**. Different models of rate variation among branches are available, including autocorrelation among lineages [2, 35], uncorrelated rates [3, 30, 36], or a mixture of the two [37]. However, per-year substitution rates and divergence times are sensitive to prior distribution on node calibrations [38] and justifying informed node calibrations is not trivial [39]. Relaxed clock methods have recently been extended to account for uncertainty in fossil placement [40] by leveraging morphological data from both extant and fossil species [41-44]. These **total-evidence** [41] approaches include **tip-dating methods** that treat extinct fossil lineages as tips where fossil occurrence [40] or morphological characters from fossils [43] can calibrate rates of evolution to absolute time. They can also incorporate different speciation mechanisms that best suit an organismal group [45]. As with more traditional methods, however, these total-evidence tip-dating methods can only be applied to clades with an available fossil record [42] and therefore cannot solve the problem of poor or absent fossil records.

Tip-dating methods that use only molecular data [46, 47] offer one approach for overcoming an absence of fossil calibrations. These methods have been applied to viruses, where high substitution rates generate sufficient variation from contemporary samples to determine relative ages [48], as well as to cases wherein ancient DNA samples can calibrate the molecular clock such as for woolly mammoths [49] and humans [50]. Even so, **ancient DNA methods** are equally or even more restrictive than fossil-calibrated methods given that they can only be applied to a limited number of organisms for which well-preserved and relatively recent samples are available [51]. Most significantly, all of the above methods use **concatenation** of genetic loci**,** thereby making the fundamental assumption that the phylogenetic history of each locus matches the species tree. We here discuss how concatenation can be problematic, and how MSC methods overcome these problems.

The Multispecies Coalescent as a Backward Time Machine
Coalescent theory is a branch of population genetics that describes the genealogical histories of a sample of alleles in a population, going back from a sample of extant alleles to their most recent common ancestor [52]. Two alleles are said to coalesce when they share a common ancestor. The MSC is a simple extension of the single-population coalescent to multiple species [5] and accommodates the species phylogeny and the coalescent processes in both the extant and extinct species [53, 54]. The MSC jointly estimates **divergence times** and rates of evolution (Fig. 1) while explicitly modeling gene tree discordance due to incomplete lineage sorting (ILS, also known as deep coalescence). ILS occurs when sequences from different species fail to coalesce in their most recent ancestral species. The shorter the branch in coalescent units between two speciation events, the more likely is ILS to occur (Box 1). Short

coalescent branch lengths can be caused not only by small time intervals between speciation events, but also by large ancestral **effective population size**.

It is now well accepted that gene trees do not consistently match species trees [55]. Though this was initially considered to be a hindrance to the accurate reconstruction of phylogenies [56], investigators are increasingly aware that these heterogeneities provide valuable information about the timing and population dynamics of organismal lineages over their evolutionary history. Described as a "backward time machine" [57], the MSC treats the stochastic variation of the coalescent process over genes or genomic regions as a source of information rather than as "mistakes" or "conflicts", and is thus uniquely suited to harness the power of many loci from modern genomic data. Accordingly, the MSC is of increasing interest to investigators who seek to place divergence events in a temporal context. The MSC makes a number of simplifying assumptions including a lack of post-divergence **gene flow**, ILS as the only source of gene tree discordance, no recombination within loci, and a lack of selection. Where high amounts of gene flow among non-sister species are a concern, extensions to the MSC are available [58].

Accounting for the Coalescent Process
Traditional phylogenetic clock models equate species divergence (i.e., "split times") to sequence divergence. This is problematic given that sequence divergence will always predate speciation events in the absence of gene flow [59, 60] (Fig. 2). In contrast, coalescent methods explicitly accommodate the differences between the two and directly estimate *species* divergence times which are generally the evolutionary events of interest (Fig. 3; Box 2). Moreover, when fossil calibrations are used, divergence time estimates can be strongly affected, with the direction of the bias depending on the placement of the most precise calibrations. If these calibrations are placed on young nodes within a phylogeny, divergence times will be underestimated across the entire phylogeny, while if calibrations are placed on ancient nodes, the ages of young nodes are likely to be overestimated. Accordingly, for phylogenies with complex mixtures of fossil calibrations, both underestimation and overestimation of divergence times may occur across the phylogeny – regardless of the analytic method applied.

Traditional phylogenetic analysis of concatenated sequences assumes that a single tree topology with one set of divergence times underlies the multilocus sequence data, irrespective of how rate variation is modeled among sites, loci, or branches. Gene tree discordance due to ILS then appears as additional substitutions on branches in the species phylogeny [6, 61], leading to overestimation of species divergence times when ILS is not accounted for [9]. In line with these theoretical expectations, Stange et al. [7] showed that in cases of high gene tree discordance, concatenation methods will overestimate ages of young nodes when ancient nodes are constrained. Similarly, Fang et al. [62] found that recent species divergences were correctly estimated to be more recent when using MSC methods. Simulations generally suggest that the MSC can improve divergence time estimates when gene tree discordance is high [7, 9], while comparable performance should be expected between concatenation and MSC methods when gene tree discordance is low (Fig. 3).

Although empirical studies using MSC approaches have thus far focused on recent species divergences (1-10 MYA) [7, 62, 63], the effects of discordant gene trees should also impact divergence time estimates for older divergences where the coalescent branch length is short and ILS is high [9]. These patterns are expected for rapid radiations that occurred deep in evolutionary history, such as placental mammals [64], passerine birds [65], and lepidopterans [12]. Divergence time estimates for these groups are important for interpreting species biogeography and trait evolution, and as computational efficiency and resources continue to improve, the evolutionary history of these groups should be re-evaluated with MSC models that

also leverage fossil calibrations. In angiosperms, reconciliation of molecular dates with those interpreted from the fossil record has been the topic of vigorous debate even though molecular data have largely been restricted to chloroplast sequences, which represent a single **gene tree** [66-69]. As large multilocus nuclear datasets become increasingly available for plants [70], the benefits of fossil-calibrated MSC methods could be realized.

The Coalescent Time Unit

Because the average coalescence time between two randomly sampled sequences from a diploid population is $2N$ generations, it is convenient to scale branch lengths in the species tree in coalescent units, that is, to use $T = t/(2N)$ where t is the number of generations until the coalescent event. T can also be rescaled by mutations and represented as $\tau = \mu t$, where μ is the per-generation mutation rate, so that $T = \tau/(\theta/2)$. Here $\theta = 4N\mu$ is the population-scaled mutation rate; a fundamental parameter in population genetic models which represents the average number of mutations per site between two sequences randomly sampled from the population.

MSC programs like StarBEAST2 [6] and BPP [5, 71] use multilocus sequence alignments to estimate species trees as well as parameters in the MSC model including species divergence times (τ) and population sizes (BPP estimates θ and StarBEAST2 estimates $N\mu$), both measured by the expected number of mutations per site. If fossil calibrations or mutation rates are available to calibrate the tree, they can be used to convert genetic distance to absolute times and absolute rates. When a per-generation mutation rate is available, generation times are also necessary (Fig. 1) to convert to divergence times in years. This approach assumes that the per-generation mutation rate and generation time are constant throughout the species tree, which is a reasonable assumption for analyses of closely related species for which genetic divergences likely satisfy a strict clock [72, 73].

de novo Mutation Rate Estimates Provide Freedom from the Fossil Record

In order to estimate absolute divergence times in the absence of fossil calibrations, direct estimates of the mutation rate estimates are needed. Recently, whole-genome sequencing data from **pedigree trios** have been used to estimate the **de novo mutation rate** for many animals [74-78] and parent-progeny pairs in plants [79]. Recent examples of divergence time estimation based on mutation rates and **coalescent age estimates** include the age of human migration events [80] and of domestication histories among agriculturally important species [81-83].

To estimate a *de novo* mutation rate, the father, mother, and offspring from a pedigree trio are sequenced and aligned to a reference genome. Variants detected in the child that are distinct from both the mother and father and do not match the reference are considered *de novo* mutations. Because the number of sequencing errors are more than an order of magnitude greater than the number of true mutations, strict filtering criteria in computational analysis must be applied to the called variants to avoid false positives. Also, mutations cannot be identified at all sites because of variable sequencing read depth and alignment uncertainty in repetitive regions. Thus, the number of **callable sites** needs to be estimated as the denominator to accurately estimate μ [76]. Ideally, the final estimate of μ is averaged over multiple pedigrees, as any single pedigree will yield few mutations. Best practices for reducing false positives and false negatives for inferred mutations are still being developed [84].

The availability of a reference genome can be a critical limitation for estimating *de novo* mutation rates in non-model organisms. Although high-quality reference genomes are anticipated for most Eukaryotic lineages in the near future [85], there will ultimately be barriers for some groups. In the absence of direct estimates of μ for a species of interest, distributions of

μ can be developed based on studies of related organisms [72]. Generation time estimates must be considered as well given that mutation rates from pedigree studies are scaled by generation to recover absolute divergence times (Fig. 1).

Discrepancies between Concatenation and MSC methods for Divergence Time Estimates
Though empirical examples are as yet few, discrepancies between divergence dates estimated by fossil-calibrated concatenation and mutation rate-calibrated MSC methods are emerging (e.g., Fig 4). For the closely related species pair of human and chimpanzees, the mutation rate-calibrated MSC [9] and concatenated time estimation give similar results. Fossil-calibrated concatenation and fossil-calibrated MSC methods place the divergence between 5.7 and 10 MYA, typically near the center of the calibration density at 7.5 MYA [9, 86]. A mutation rate-calibrated MSC analysis that assumed the human mutation rate for both species recovered a posterior mean of 8.2 MYA [9]. Divergence time estimates calibrated directly with mutation rates but not using the MSC are also similar, but only after considering the difference between species and sequence divergence. In one such study, **pairwise sequence divergence** between chimp and human (t_{Seq}; Fig. 2) yielded a divergence time of 12.1 MYA assuming the human mutation rate [27], though subtracting $2N_{HC}$ (the effective population size for the human-chimpanzee common ancestor) yields a divergence time of 7.9 MYA. Thus, per-generation mutation rates can be used to estimate divergence times from concatenated data too, but the difference between species divergence and sequence divergence needs to be accommodated by some calculation of population size estimate (Fig. 2).

The sensitivity of these methods to the mutation rate estimate is keen. For example, when the human mutation rate was applied unilaterally across a primate phylogeny, a divergence time between Old world monkeys (*Macaca mulatta*) and humans of 62 MYA was recovered [87], in stark contrast to the 35 MYA age estimate indicated by fossil evidence [86]. The discrepancy is likely explained by a slower mutation rate in humans compared to Old World monkeys [88] and indicates that caution is needed when applying pedigree-based mutation rates to divergence time estimation, especially across large phylogenies. While one possible reason for discrepancies across long times scales is that purifying selection may lead to lower substitution rates compared to mutation rates [89], as observed in mutation accumulation lines with *Arabidopsis thaliana* [90], the discrepancy in this case was in the opposite direction. Thus, given the small number of empirical examples at present, it is difficult to generalize the causes of disparities between substitution and mutation rates at present.

In one such empirical example, MSC methods produce significantly more recent age estimates than fossil-calibrated concatenation methods for mouse lemurs (genus *Microcebus*). Whereas a mutation rate-calibrated MSC analysis yields a MRCA for the genus of 1.5 MYA [63], previous analyses using fossil-calibrated concatenation methods yielded estimates of ~10 MYA [86, 91]. Though this discrepancy could, in part, be the consequence of a falsely elevated pedigree-based mutation rate estimate [91], the discrepancy would still be pronounced even if the true rate is only half of the estimated rate. Conversely, for the fossil-calibrated divergence time estimate using concatenation, phylogenetically distant, external calibrations [86, 91] were used by necessity given that there is a complete dearth of fossils within the lemuriform clade. As described above, the fossil-calibrated concatenation estimate is thus likely to overestimate divergence times for young nodes given the dependence on older fossil calibrations deeper in the phylogeny (Fig. 3; [9]). This is similar to cases of Stange et al. [7] and Fang et al. [62] where MSC methods using geological calibrations resulted in more recent divergence times compared with those found with concatenation – even when using the same calibrations. In summary, it is important to note that the differences in time estimates between the MSC and phylogenetic

concatenation methods may be complex, depending on biases of mutation rate estimates and on the relative placement of calibrations within the phylogeny.

A New Frontier in Divergence Time Estimation
Divergence time estimates can fundamentally affect interpretations of trait evolution, biogeography, and the processes that underlie species radiations. Thus, the stakes for evolutionary studies are high. As an important step forward, future studies that leverage genomic data and fossil calibrations should consider comparing traditional phylogenetic clock models and the MSC to evaluate the effects of ILS on divergence time estimation. We further recommend that uncertainty in both mutation rates and generation times should be explicitly incorporated in analyses wherein coalescent units are converted to absolute time [63, 72]. This can be easily done by drawing mutation rates and generation times from prior distributions rather than relying on point estimates, given that variation in inferred mutation rates can be high among pedigrees [84], and mutation rates may change over time [27]. Moreover, estimating generation times can be problematic, especially for perennial plants given the lack of clear segregation in the germ line. The impact of the number, quality, and placement of fossil calibrations – as well as model choice on divergence time estimation using traditional phylogenetic concatenation methods – has been extensively studied [10, 38, 67, 69, 86]. Conversely, the careful evaluation of MSC methods for divergence time estimation is still in its infancy. We therefore predict that future studies that directly compare the two approaches are likely to identify as yet unrecognized though critical considerations for accurate divergence time analysis.

Concluding Remarks
We conclude by noting that despite its advantages, the MSC method involves a heavy computational burden and may not always be feasible for divergence time estimation on large phylogenies [92-94]. In such cases, traditional phylogenetic clock analyses that use concatenation may be the most practical approach [3, 30]. In particular, approximate likelihood calculation appears useful in estimating divergence times for large phylogenies or for very long alignments [86]. These models should not be seriously biased when divergence times are old (Fig. 2) and ILS is low (Fig. 3). But given the prevalence of ILS across the tree of life, the applications of the MSC for divergence time estimation in both shallow and **deep phylogenies** will be of increasing interest and importance (see Outstanding Questions). It remains to be seen to what degree divergence time estimates will agree when both traditional phylogenetic clock models and mutation-rate calibrated MSC methods are applied within the same study systems.

Glossary

Ancient DNA Methods: Sequence data is obtained from the remains of ancient specimens. The DNA is typically damaged and fragmented by absolute time and by exposure to damaging agents such as heat, oxidation, and UV irradiation.

Bayesian Methods: Bayes theorem is used to approximate the maximum likelihood estimates of a model and its parameters by sampling many estimates with proposal distributions and commonly implemented with a Markov chain Monte Carlo algorithm. Prior distributions are used to constrain the search space of parameters and may include *a priori* expectations for parameter estimates but are often left vague. Bayesian methods are used as a matter of computational convenience when maximum likelihood optimization is intractable.

Callable Sites: The number of sites where a *de novo* mutation should be detectable.

Concatenation: Multiple loci are treated as a single nonrecombining locus with a single underlying topology.

Coalescent: The stochastic process of lineage joining when one traces the genealogical history of a sample of sequences from a population backwards in time.

Coalescent Age Estimate: The divergence time for two sequences based on sampling theory and measured in the expected number of generations.

Coalescent Time Unit: The expected coalescent time for a pair of sequences, which is $2N$ generations for a diploid species with population size N.

***de novo* Mutation Rate**: The spontaneous germline mutation rate revealed in comparisons of whole genomes from both parents and their progeny (aka, pedigree trios).

Deep Phylogenies: Phylogenies that contain species with high sequence divergence. In such cases, substitutional saturation or "multiple hits," long-branch attraction, gene duplication and loss, and model misspecification can result in gene tree discordance. ILS can still be a substantial source of conflict between gene trees and species trees in deep phylogenies.

Divergence Times: The expected absolute age at which two species became isolated from each other.

Effective Population Size: The number of individuals that would produce the observed rate of genetic drift in an idealized Fisher-Wright population model.

Fossil Calibrations: Fossil evidence from morphological characters that can constrain the age of the crown group of a clade (e.g., with a hard minimum and a soft maximum).

Gene Flow: Exchange of alleles between two populations.

Gene Tree: The evolutionary history of a short, nonrecombining segment of the genome

Gene Tree Discordance: Difference in gene tree topology from the species tree, possibly caused by deep coalescence.

Generation Time: The average time between two generations which is often quantified as the average age of parents at birth, averaged over individuals.

Lineage sorting: The process by which gene lineages become fixed within a species such that all alleles within that species coalesce to a single ancestral allele within the species lineage.

Incomplete Lineage Sorting (ILS): Failure for two sequences from two species to coalesce in the most recent common ancestral species, also known as deep coalescence.

Loci: Orthologous non-recombining sequences. Each locus corresponds to an independent gene tree.

Markov chain Monte Carlo (MCMC): a simulation approach for sampling from a target distribution such as the posterior distribution of parameters in Bayesian inference.

Molecular Clock: Hypothesis that the rate of molecular evolution is constant over time and among lineages.

Most Recent Common Ancestor (MRCA): The most recent node on a phylogeny from which all individuals in a clade of interest are derived.

Multispecies Coalescent (MSC): The extension of the coalescent process to multiple species which accommodates the species tree as well as the coalescent within populations.

Pairwise Sequence Divergence: The evolutionary distance between a pair of sequences measured as the expected number of substitutions per site.

Pedigree Trio: A child and the two parents for whom whole genomes are sequenced and compared to identify the new mutations in the child.

Per-year Substitution Rates: The number of substitutions per-site per-year that are obtained when calibrating a phylogeny to absolute time with information at nodes or tips.

Reciprocal Monophyly: All alleles within a species coalesce with each other before the first coalescence with an allele from another species.

Relaxed Clock Models: An extension of the strict clock model to allow changes in evolutionary rate over branches in a phylogeny.

Species Tree: The evolutionary history of species, which is often estimated from many individual gene trees or loci.

Strict Molecular Clock: A single rate of molecular evolution is enforced for all branches in a phylogeny.

Tip-Dating Methods: Rates of molecular evolution are calibrated to absolute time by known sampling dates of individuals, whether extant or extinct, at the tips of a phylogeny.

Total-Evidence: Morphological characters for extinct (fossil) and extant tips and rates of morphological evolution are used to infer species divergence times jointly with molecular data.

Traditional Phylogenetic Clock Models: Models for divergence time estimation that assume one tree and one set of divergence times for all loci.

Box Legends
Box 1 – Incomplete Lineage Sorting on a Rooted Three-Taxon Species Tree.
Box 2 – Differences between Bayesian methods for divergence time estimation and programs for implementing them.

Figure Legends
Figure 1 – Overview of the MSC model and its use for estimating absolute divergence times with external mutation rate data. *Input Data* – The MSC requires aligned orthologous sequence data as input. There can be many individual loci, and each locus is assumed to be non-recombining and not under selection. The MSC allows for multiple alleles per species per locus, and sampling multiple alleles can improve parameter estimates. Although joint estimation of the species tree and MSC parameters is possible, using a fixed species tree is computationally more efficient. *Estimate MSC Parameters by MCMC* – The MSC estimates model parameters with Bayesian **Markov chain Monte Carlo (MCMC)**. This requires a prior distribution for all model parameters including population sizes (θ), species divergence times (τ), and possibly rates among loci (r). The MSC estimates a gene tree for each locus ($1..n$) and the coalescent times on those trees. Gene tees can be incongruent with the species tree. The distribution of gene trees and their coalescent age estimates are used to jointly estimate θ and τ on the species tree. *Calibrate with Mutation Rate* – A per-generation mutation rate (μ) is obtained from independent pedigree-based studies and a generation time (g) is estimated from the distribution of parent ages at the time of birth for offspring. μ and g can then be used to obtain an absolute rate of evolution by multiplying τ by g/μ. μ can also be used to rearrange the expression for θ and obtain absolute population sizes (N).

Figure 2 – Overestimation of species divergence times from genetic distances. a) Species A and B diverged t_{AB} generations ago. Sequences A and B coalesced further back in time at t_{Seq} generations ago, with a mean $t_{Seq} - t_{AB}$ of $2N_{AB}$ generations. b) Sequences were simulated under the MSC for a pair of species with constant $N_{AB} = 10^5$ and $\mu = 1 \times 10^{-8}$ per site per generation. c) The relative expected overestimation of species divergence times by $2N_{AB}$ becomes smaller as t_{AB} becomes larger, because $2N_{AB}$ contributes only a small proportion of time to the overall divergence time estimate.

Figure 3 – Effects of ILS and the MSC on divergence time estimation. Data are from Table 2 of Angelis and dos Reis [9]. a) Three-taxon species tree used for simulation. All data were simulated under the MSC with the Jukes Cantor model of molecular evolution. A μ of 1×10^{-8} per site per generation and a generation time of ten years was used, and the species tree root (node r) had an age of 10 MYA or τ of 0.01. Data were simulated with 4 different population sizes (N) that were constant along the species tree. The root was calibrated with a gamma distribution as one might in a fossil-calibrated divergence time analysis. b) Divergence time estimates of node s when using concatenation (MCMCTREE) or the MSC (BPP). Because the calibration is placed on the older root node, the younger node is overestimated when N or ILS is high by concatenation but not MSC methods. Points represent posterior means and error bars are the 95% credible intervals.

Figure 4 – Illustration of the consequences of differences between divergence time estimates. Pedigree symbols represent mutation rate-calibrated divergence times and probability distributions represent traditional phylogenetic clock model estimates. a) The MRCA of Madagascar's mouse lemurs. The mutation rate-calibrated MSC estimate yields a mean divergence time of 1.5 MYA whereas a traditional phylogenetic clock model with fossil calibrations recovers a divergence time estimate of approximately 10 Ma. Though the position of

Madagascar relative to Africa is essentially the same at these two geological time points, Madagascar's climate would have been very similar to that of today at 1.5 MYA whereas it would have been much warmer and drier 10 MYA. b) The divergence between Old World monkeys and apes. A mutation rate-calibrated divergence time estimate (though not with the MSC) is 62 MYA whereas the traditional phylogenetic clock model yields a divergence time estimate of approximately 35 MYA. There are striking differences in both continental configuration and climate between these two time points. At 62 MYA, the earth was largely tropical and sea levels were markedly high, isolating Africa from the northern continents. At 35 MYA, Africa has shifted northward, making contact with the northern continents and Antarctica is partially glaciated indicating much cooler global temperatures. Global maps provided courtesy of the Deep Time Maps project.

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Highlights

- Molecular clock models using fossil calibrations have allowed investigators to estimate the age of speciation events.

- Theoretical and computational developments have relaxed the assumption of a molecular clock, thus improving the accuracy of divergence time estimation.

- Despite these advances, estimates can be biased when there is widespread incomplete lineage sorting (ILS).

- Increased understanding of gene tree heterogeneity has driven multispecies coalescent (MSC) methods to prominence, though the potential power of the MSC for divergence time estimation remains largely unexplored.

- Absolute times can be obtained by using mutation rates estimated from pedigrees, providing [some] freedom from the incomplete fossil record.

- Mutation-rate calibrated MSC methods and traditional phylogenetic clock-dating methods with fossil calibrations can yield strikingly different divergence times.

Outstanding Questions

- To what extent is among-lineage rate variation modeled by relaxed clock methods due to gene tree discordance from ILS?

- Have divergence times throughout the tree of life been systematically overestimated in clades that rely on external, and typically older, calibrations?

- Do divergence time estimates based on per-generation mutation rates and per-year substitution rates yield similar results, especially if substitution rates are estimated from presumably neutral regions of the genome such as third codon positions?

- Will MSC estimates that leverage fossil calibrations bring new insights to contentious age estimates such as the origins of placental mammals or angiosperms?

- Should effective population size variation among species be a concern for divergence time estimation studies using concatenation?

- Can mutation-rate calibrated MSC methods that account for variable rates and generation times among branches improve divergence time estimation for clades that have rapid life history transitions?

- How can we develop standard operating procedures for evaluating the strength of evidence for divergence time estimates from traditional phylogenetic analyses versus ages inferred from MSC methods that rely on mutation rate and generation time estimates?

- Are there alternative ways forward for estimating the absolute age of clades with poor or non-existent fossil representation?

- To what extent do the methods (MSC versus concatenation) and the calibrations (mutation rate versus fossils) impact divergence time estimates?