Deconstructing age estimates for angiosperms

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ABSTRACT

Estimates of the age of angiosperms from molecular phylogenies vary considerably. As in all estimates of evolutionary timescales from phylogenies, generating these estimates requires assumptions about the *rate* that molecular sequences are evolving (using clock models) and the *time* duration of the branches in a phylogeny (using fossil calibrations and branching processes). Often, it is difficult to demonstrate that these assumptions reflect current knowledge of molecular evolution or the fossil record. In this study we re-estimate the age of angiosperms using a minimal set of assumptions, therefore avoiding many of the assumptions inherent to other methods. The age estimates we generate are similar for each of the four datasets analysed, ranging from 130 to 400 Ma, but are far less precise than in previous studies. We demonstrate that this reduction in precision results from making less stringent assumptions about both rate and time, and that the analysed molecular dataset has very little effect on age estimates.

Key words: angiosperms, time-calibrated-phylogeny, genomics, fossils

1. INTRODUCTION

The age of angiosperms has been debated extensively for decades. Arguments have centred on whether the sudden emergence of diverse angiosperm fossils in the early Cretaceous is evidence for the origin and diversification of angiosperms in the early Cretaceous (e.g. Scott et al. 1960; Doyle 1969), or whether angiosperms originated far earlier but a significant number of fossils only began being deposited during the Cretaceous (e.g. Axelrod 1954). More recent studies that estimate time-calibrated molecular phylogenies (phylogenies with branch lengths scaled to absolute time) have contributed to this debate by giving age estimates for the angiosperm crown node (which corresponds to the most recent common ancestor of all extant angiosperms) that range from the early Cretaceous to the Triassic (Magallón et al. 2015; Salomo et al. 2017).

Understanding the age of angiosperms is important. Angiosperms dominate virtually every terrestrial ecosystem on the planet and encompass over 90% of

terrestrial plant diversity. The rise of angiosperms to becoming such a dominant part of the earth's biota led to major climatic changes (Holborn et al. 2018) and substantially affected evolutionary diversification across the tree of life (Condamine et al. 2020; Benton et al. 2022). Knowledge of when angiosperms evolved is central to understanding how angiosperms became such an important component of life on earth.

Age estimates for the angiosperm crown node from time-calibrated molecular phylogenies are based on assumptions about the rate that molecular sequences are evolving (substitution rate) along each branch and the time duration for each branch (Fig. 1; Table 1). These assumptions are necessary because the primary source of data underpinning time-calibrated-phylogenies – molecular sequence data – is not directly informative about time. Instead, only the total number of molecular substitutions for each branch in the phylogeny can be estimated directly from this data (Fig. 1a) (Britton 2005). The number of substitutions is itself a product of the substitution rate and time duration for each branch (Fig. 1a). Therefore, if no assumptions were made that restrict the rates and times for each branch, an infinite combination of rates and times could explain the total number of substitutions on any given branch.

Assumptions used in previous studies are based on explicit models of the evolutionary process. For substitution rates, "clock models" are used which specify the pattern of among-branch-substitution-rate-variation (Fig. 1b-c; Table 1), whilst a branching process is also often used in which the time duration of branches is underpinned by a constant rate of speciation and extinction (Fig. 1d; Table 1). Alongside this, time constraints are implemented as fossil calibrations, which are often specified such that clade ages must correspond relatively closely to the oldest fossil for that clade (Fig. 1e; Table 1).

Age estimates based on these approaches have stimulated valuable debate about when and in what environment angiosperms evolved, and broader discussion of the nature of substitution rate variation and utility of the fossil record for understanding evolutionary timescales (Clark et al. 2011; Beaulieu et al. 2015; Magallón et al. 2015; Foster et al. 2017; Barba-Montoya 2018; Benton et al. 2022; Sauquet et al. 2022; He and Lamont 2022). Nonetheless, the varied age estimates from different studies are challenging to interpret and provide little clarity about the age of angiosperms (Sauquet et al. 2022). Alongside this, it is difficult to confidently

assert that existing approaches are consistent with current knowledge of evolutionary history or the fossil record: there is little explicit evidence that among-branch-substitution-rate variation corresponds to an autocorrelated clock model or an uncorrelated clock model (Fig. 1b and c); or that crown group Eudicots originated shortly before their appearance in the fossil record in the Cretaceous (as in Foster et al. 2017; Table 1) or perhaps much earlier (as in Magallòn et al. 2013; Table 1). Further, even though previous studies have experimented with a range of different clock models and interpretations of the fossil record, the scope of these experiments is limited. For example, all the studies in Table 1 use an uncorrelated lognormal clock model or an autocorrelated clock model. Instead, among-branch-substitution-rate-variation may be better characterised by other models such as those incorporating infrequent large and discrete substitution rate shifts (Drummond and Suchard 2011; Bellot and Renner 2014), or associations between substitution rates and either discrete or continuous traits (Smith and Donoghue 2008; Smith et al. 2010; Beaulieu et al. 2015)

Here, we perform a series of analyses where we estimate the age of the angiosperm crown node using a minimal set of assumptions. We avoid using any explicit model of the evolutionary process, or fossil calibrations that constrain clade age estimates such that they are close to the age of sampled fossils. By comparing the resulting age estimates to those of previous studies, we can better understand the effects of the assumptions used in these studies (Table 1) and the causes of the conflicting age estimates that they present.

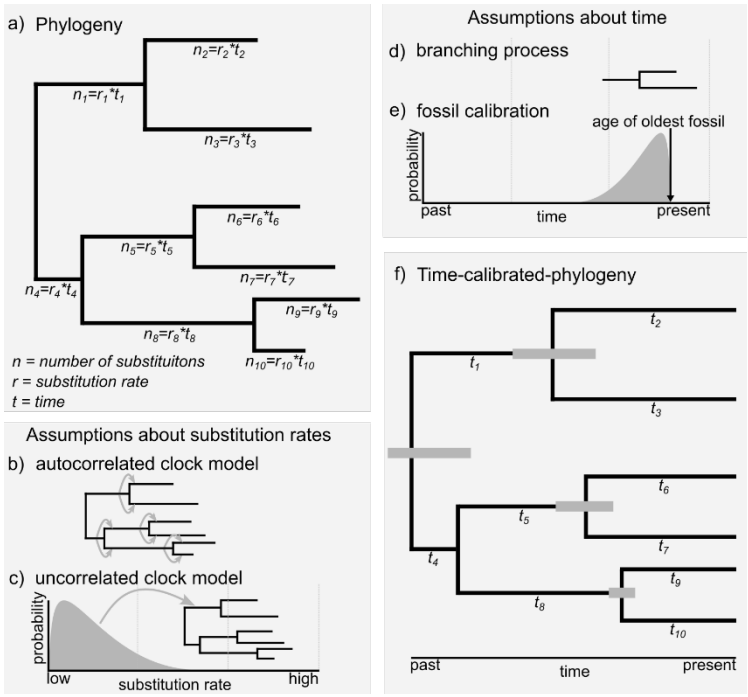

Figure 1. The relationship between a phylogeny, common assumptions about substitution rates and times, and a time-calibrated phylogeny. **a.** Phylogeny with branch lengths equal to the number of substitutions, a product of the substitution rate and time duration of each branch. **b-c.** Common assumptions about substitution rates: **b.** autocorrelated clock model where rates are inherited between ancestral and descendant branches, **c.** uncorrelated clock model where rates for all branches belong to a single lognormal distribution. **d-e.** Common assumptions about time: **d.** branching process which typically has constant speciation and extinction rates, **e.** probability distribution for a fossil calibration that describes how much older than its oldest fossil a clade is assumed to be (different distributions can be used). **f.** Time-calibrated phylogeny that can be inferred when the assumptions in **b-e.** are used. Each node has an associated confidence interval for its age.

Table 1. Summary of key assumptions used in studies estimating the age of the angiosperm crown node since 2010

Study	Assumptions					
	Time					Rate
	Spermatophyte crown node constraint (Ma)	Angiosperm crown node constraint (Ma)	Eudicot stem node constraint (Ma)	Eudicot crown node constraint (Ma)	Branching process	
Barba-Montoya et al. (2018)	308.1-365.6*	125.9-247.3*	-	119.6 (min only)	Yes	Uncorrelated clock model (lognormal distribution)
Beaulieu et al. (2015)	-	-	-	125-135.2*	Yes	Uncorrelated clock model (lognormal distribution)
Clark et al. (2011)	306-366.8*	124-248.4*	124-248.4*	-	Yes	Uncorrelated clock model (lognormal distribution)
Foster et al. (2017)	350 (max only)*	138.7-350*	-	126.7 (max only)*	Yes	Uncorrelated clock model (lognormal distribution)
He and Lamont (2022)	-	-	-	-	Yes	Uncorrelated clock model (lognormal distribution)

Li et al. (2019)	350 (max only)	132.9 (min only)	-	125 (min only)	No	Autocorrelated clock model
Magallón et al. (2013)	318-417.9*	136-178.7*	136-157.3*	125-164.3*	Yes	Uncorrelated clock model (lognormal distribution)
Magallón et al. (2015)	314-350	136-139.35	125-164.3*	-	Yes	Uncorrelated clock model (lognormal distribution)
Morris et al. (2018)	308.1-365.6*	125-247.2*	119.6-128.6*	119.6-128.6*	Yes	Uncorrelated clock model (lognormal distribution)
Murat et al. (2017)	-	-	-	125 (min only)	Yes	Uncorrelated clock model (lognormal distribution)
Nei et al. (2020)	306.2-366.8*	124-248.4*	124-248.4*	-	Yes	Uncorrelated clock model (lognormal distribution)
Ramírez-Barahona et al. (2020) CC	-	154.2 (max only)	127.2-154.2	110.8-154.2	Yes	Uncorrelated clock model (lognormal distribution)
Ramírez-Barahona et al. (2020) UC	-	247 (max only)	127.2-247	110.8-247	Yes	Uncorrelated clock model (lognormal distribution)
Ramírez-Barahona et al. (2020) RC	-	-	127.2-154.2	110.8-154.2	Yes	Uncorrelated clock model (lognormal distribution)
Salomo et al. (2017)	323-400	-	125-152.7*	-	Yes	Uncorrelated clock model (lognormal distribution)
Smith et al. (2010)	-	-	-	-	Yes	Uncorrelated clock model (lognormal distribution)
Zeng et al. (2014)	290-310	-	-	125-129.0*	Yes	Uncorrelated clock model (lognormal distribution)
Zhang et al. (2020)	308-314.8*	-	124-133.6*	-	Yes	Autocorrelated clock model

*calibrations that specify a small probability (<=5%) that the node age can be greater than the maximum constraint indicated

2. METHODS

To estimate the age of the angiosperm crown node with a minimal set of assumptions we used *exTREEmaTIME v.1.0* (Carruthers and Scotland 2022) which was implemented in R v.4.2.1 (R core team 2022). This method does not use a clock model or a branching process or probability distributions as fossil calibrations. Instead, an input tree with molecular branch lengths (equal to the number of substitutions) (Fig. 2a) is analysed alongside specified minimum and maximum substitution rates, and minimum and/or maximum time constraints at one or several nodes. These rate and time constraints are specified such that they represent minimum and maximum feasible values. As such, the range they incorporate is typically broad and encompasses the range incorporated by the rate and time assumptions of other methods.

Minimum and maximum possible node ages that are consistent with these inputs are then calculated (Fig. 2b-c). To calculate minimum ages, each branch

length in the input tree is divided by the maximum specified substitution rate (giving branch lengths in units of time). Where relevant, these branches are subsequently extended backwards in time by the minimum amount required to satisfy minimum time constraints and tip sampling times (Fig. 2b). To calculate maximum ages, branch lengths in the input tree are divided by the minimum specified substitution rate. These branches may also undergo subsequent adjustment to satisfy time constraints and sampling times (Fig. 2c). Note that these adjustments to the time duration of branches must not cause violation of the specified minimum or maximum substitution rates - for example, if the terminal branch leading to A in Fig. 2b required extension to such an extent that the necessary substitution rate would have to fall below the minimum specified substitution rate. In such a case, *exTREEmaTIME* cannot run to completion.

Although *exTREEmaTIME* is critically dependent on assumptions (like every method for estimating time-calibrated-phylogenies), by using assumptions designed solely to represent minimum and maximum feasible substitution rates and times, and calculating minimum and maximum node ages that are consistent with these, it can provide a basis for evaluating the implications of the more specific assumptions of other methods. However, a consequence of this is that outputs from *exTREEmaTIME* should not be interpreted as a most probable reconstruction of evolutionary history. Such an endeavour would instead require probabilistic models for among-branch-substitution-rate-variation, and potentially, probability distributions for fossil calibrations.

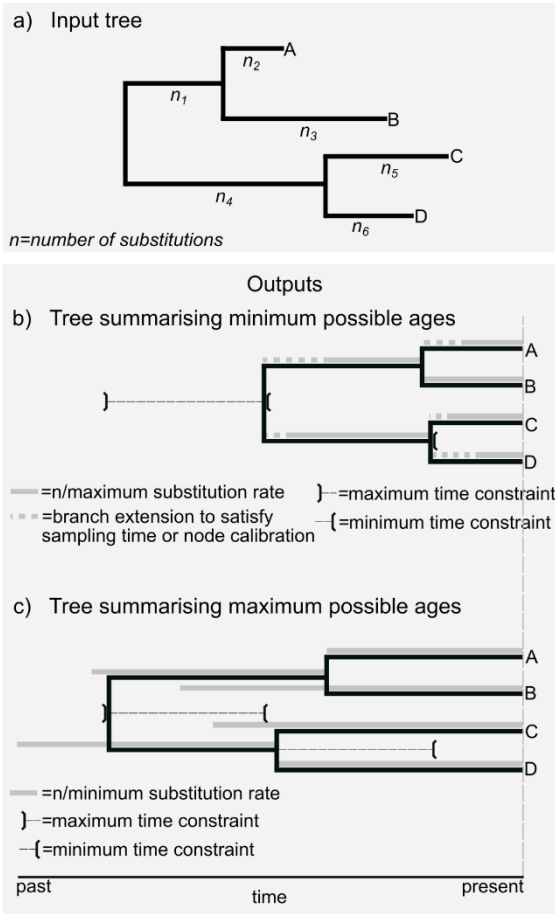

Figure 2. A visual example of how *exTREEmaTIME* works. **a.** Input tree with molecular branch lengths equal to the number of substitutions. **b.** Calculation of minimum possible ages. Branch lengths in the input tree are divided by the minimum specified substitution rate, with the result of this calculation shown by the solid grey bar next to each branch. Given that all tips are sampled at the present and minimum time constraints must be satisfied, some branches are subsequently extended, with this branch extension indicated by the dashed grey line next to these branches. **c.** Calculation of maximum possible ages. Branch lengths in the input tree are divided by the minimum specified substitution rate, with the result of this calculation shown by the solid grey bar next to each branch. Given that all tips are sampled at the present and maximum time constraints must be satisfied, some branches are subsequently shortened, as indicated by branches in the tree that are shorter than the grey bars. Note that **b.** and **c.** have the same time constraints. The minimum time constraints are of primary importance when calculating minimum possible ages, and the maximum time constraints are of primary importance when calculating maximum possible ages.

2.1 Main time-calibrated-phylogeny estimation

We performed the main analyses according to the principles of *exTREEmaTIME*, which is to use a minimal set of assumptions about substitution rates and times. We based these analyses on a selection of the molecular datasets from the studies in Table 1: Morris et al. (2018) and Nei et al. (2020) which are respectively based on plastome and nuclear genomic datasets for all land plants and closely related algae; Li et al. (2019), which is based on a plastome dataset for flowering plants; and Ramírez-Barahona et al. (2020), which is based on a densely sampled chloroplast and nuclear marker dataset for seed plants.

2.1.1 Specifying time constraints

For our analyses based on Morris et al. (2018) and Nei et al. (2020), we used three maximum time constraints: 3.5Ga at the root node corresponding to the age of the oldest fossil ever recorded (Westall et al. 2001); 1.042Ga at the embryophyte crown node corresponding to the age of the oldest sediments in which land plants could potentially occur (Strother et al. 2011); and 458.4Ma at the tracheophyte crown node (Steemans et al. 2009). This third maximum time constraint is likely to have the biggest effect on angiosperm crown node age estimates given the tracheophyte crown node is comparatively close to the angiosperm crown node. It is justified on the basis that this time constraint corresponds to the lower boundary of the late Ordovician, the period during which Tracheophyte megafossils and microfossils both begin to appear in the fossil record. Given such fossils represent evidence of the origination of the tracheophyte *stem* lineage, the use of a 458.4Ma maximum time constraint at the tracheophyte crown node is relatively conservative. Nevertheless, it cannot be justified unequivocally and the true age is potentially older than this constraint. Aside from this, the other two maximum time constraints can be justified fairly conclusively, and that is why they were used.

For our analyses based on Li et al. (2019) and Ramírez-Barahona et al. (2020), we used a single maximum time constraint at the root node in each case. For Ramírez-Barahona et al. (2020) (where the root node was the spermatophyte crown node), this was the maximum age estimate for the spermatophyte crown node from the *exTREEmaTIME* analysis based on Nei et al. (2020). For Li et al. (2019), this was the maximum age estimate for the angiosperm crown node from the *exTREEmaTIME* analysis based on Ramírez-Barahona et al. (2020). Key time constraints from all analyses are summarised in Table 2.

In this set of analyses, we do not experiment with alternative maximum time constraints, as has often been the case in previous studies (e.g. Morris et al. 2018; Ramírez-Barahona et al. 2020). This is because the purpose of these analyses is to incorporate minimal assumptions about time (and substitution rates), with the maximum time constraints that we use therefore representing the maximum feasible ages for respective nodes. By contrast, younger maximum time constraints, including time constraints implemented as specific probability distributions, would represent a more precise assumption about the relationship between fossil ages and clade ages. This would contradict the purpose of these analyses.

In all cases, minimum time constraints were based on the oldest known fossils for represented clades. All minimum time constraints are summarised in Table S1.

2.1.2 Specifying minimum and maximum substitution rates

To specify minimum and maximum substitution rates we used the *SetAutoRates* function (option 2) in *exTREEmaTIME* (Carruthers and Scotland 2022). This function first divides the mean root to tip distance in the input tree by the maximum possible age of the root node (derived from the time constraints) for the minimum substitution rate, and the minimum possible age of the root node for the maximum substitution rate. This initial step approximates the mean substitution rate across the tree according to the maximum and minimum time constraints at the root node, but it does not adequately account for rate differences among branches. Therefore, these initial values are respectively multiplied and divided by the largest proportional change in substitution rate between terminal branches in the input tree (as determined by branch length comparison). This second step generates the minimum and maximum substitution rates used by *exTREEmaTIME*. Given that *exTREEmaTIME* cannot run to completion if the minimum and maximum substitution rates are incompatible with the time constraints and input tree, *SetAutoRates* sequentially trials substitution rates encompassing a broader range (by respectively multiplying and dividing the minimum and maximum substitution rates by a scaler) until the analysis can run to completion. This additional step was not necessary in the main analyses performed here.

Although subject to uncertainty and a degree of arbitrariness, the range of substitution rates calculated by this approach typically encompasses the range of substitution rates from other methods, as well as the correct substitution rate value when evaluated with simulated datasets (Carruthers and Scotland 2022). The minimum and maximum substitution rates used in each analysis are shown in Table 2.

2.1.3 Input trees used

For our analyses based on the molecular datasets of Morris et al. (2018), Li et al. (2019), and Ramírez-Barahona et al. (2020), the input tree was the phylogeny with molecular branch lengths from each of these studies. For our analysis based on the molecular dataset of Nei et al. (2020), an input tree was estimated in RAxML (Stamatakis 2014) with a GTR+GAMMA model, and with the alignment of the 1st and 2nd codon positions that was provided with this study.

2.2 Estimating time-calibrated-phylogenies using the same fossil calibrations as previous studies

Debate has centred on the role of fossil calibrations in influencing age estimates in time-calibrated phylogenies, and their importance relative to the sequence data and clock model. Recently, discussion has emphasised the importance of fossil calibrations (Magallón et al. 2013; Ramírez-Barahona et al. 2020). To understand the role of fossil calibrations for age estimates of the angiosperm crown node, we performed additional analyses in *exTREEmaTIME* where we used exactly the same fossil calibrations as previous studies. We could therefore determine the extent to which potential differences in age estimates in this study relative to other studies result from how *exTREEmaTIME* accounts for among-branch-substitution-rate-variation relative to other methods, or whether it results from the use of different fossil calibrations.

We performed these analyses with Li et al. (2019), and the RC, UC, and CC calibration strategies from Ramírez-Barahona et al. (2020). RC, UC, and CC all use 177 unique minimum time constraints throughout angiosperms (Table S1), with each strategy differing by the maximum time constraints used. CC uses a maximum time constraint of 154.2Ma at the angiosperm crown node, but no maximum time constraint on any other node; UC uses a maximum time constraint of 247Ma at the angiosperm crown node, but no maximum time constraint on any other node; and RC uses a maximum time constraint of 154.2 for all nodes, except the angiosperm crown node, for which there is no maximum.

We did not perform these additional analyses with Morris et al. (2018) or Nei et al. (2020) because these studies use “soft bounds” that cannot be replicated in *exTREEmaTIME*. Aside from the different fossil calibrations, this second set of analyses were performed as in the main analyses (see Table 2 for further details). Note that for Li et al. (2019), *SetAutoRates* was required to perform one round of the scaling step that increases the range between the minimum and maximum substitution rate. This resulted in the minimum rate used by *exTREEmaTIME* being half of the rate calculated in the initial *SetAutoRates* step. Code and phylogenies for all analyses performed can be found here: https://github.com/TomCarr/angiosperm_age_estimates.

Table 2. Summary of assumptions in analyses performed here.

	Time	

Study					Substitution Rate (myr^{-1})
		Spermatophyte crown node (Ma)	Angiosperm crown node (Ma)	Eudicot stem node (Ma)	
Main	Li et al. (2019)	-	423.3 (max only)	127.2 (min only)	$1.5e^{-4} – 5.7e^{-3}$
	Morris et al. (2018)	-	-	127.2 (min only)	$2.1e^{-5} – 4.8e^{-3}$
	Nei et al. (2020)	-	-	127.2 (min only)	$1.6e^{-5} – 1.5e^{-3}$
	Ramírez-Barahona et al. (2020)	306.2-423.3	-	127.2 (min only)	$6.8e^{-5} – 2.6e^{-2}$
Same fossil calibrations as previous studies	Li et al. (2019)	350 (max only)	132.9 (min only)	125 (min only)	$7.2e^{-5} – 1.1e^{-2}$
	Ramírez-Barahona et al. (2020) (CC)	-	154.2 (max only)	127.2 (min only)	$7.6e^{-5} – 2.6e^{-2}$
	Ramírez-Barahona et al. (2020) (UC)	-	247 (max only)	127.2 (min only)	$7.6e^{-5} – 2.6e^{-2}$
	Ramírez-Barahona et al. (2020) (RC)	-	-	127.2-154.2	$7.6e^{-5} – 2.6e^{-2}$

3. RESULTS AND DISCUSSION

Age estimates for the angiosperm crown node in previous studies (Fig. 3a) differ markedly. Confidence intervals range from the early Cretaceous to the early Carboniferous, and many do not overlap, suggesting the width of at least some confidence intervals are underestimated. Alongside these marked differences, every study except Magallón et al. (2015) excludes a crown node age in the Cretaceous, the period during which unequivocal crown group angiosperms first appear in the fossil record. By contrast, all age estimates for the angiosperm crown node from *exTREEmaTIME* (Fig 3b) are far broader, spanning the early Cretaceous (~130 Ma) to the Devonian (~400Ma). They are the widest set of confidence intervals ever generated for the age of this node and they incorporate all the confidence intervals from previous studies. Further, these confidence intervals are very similar for each of the four analyses which are based on entirely different datasets.

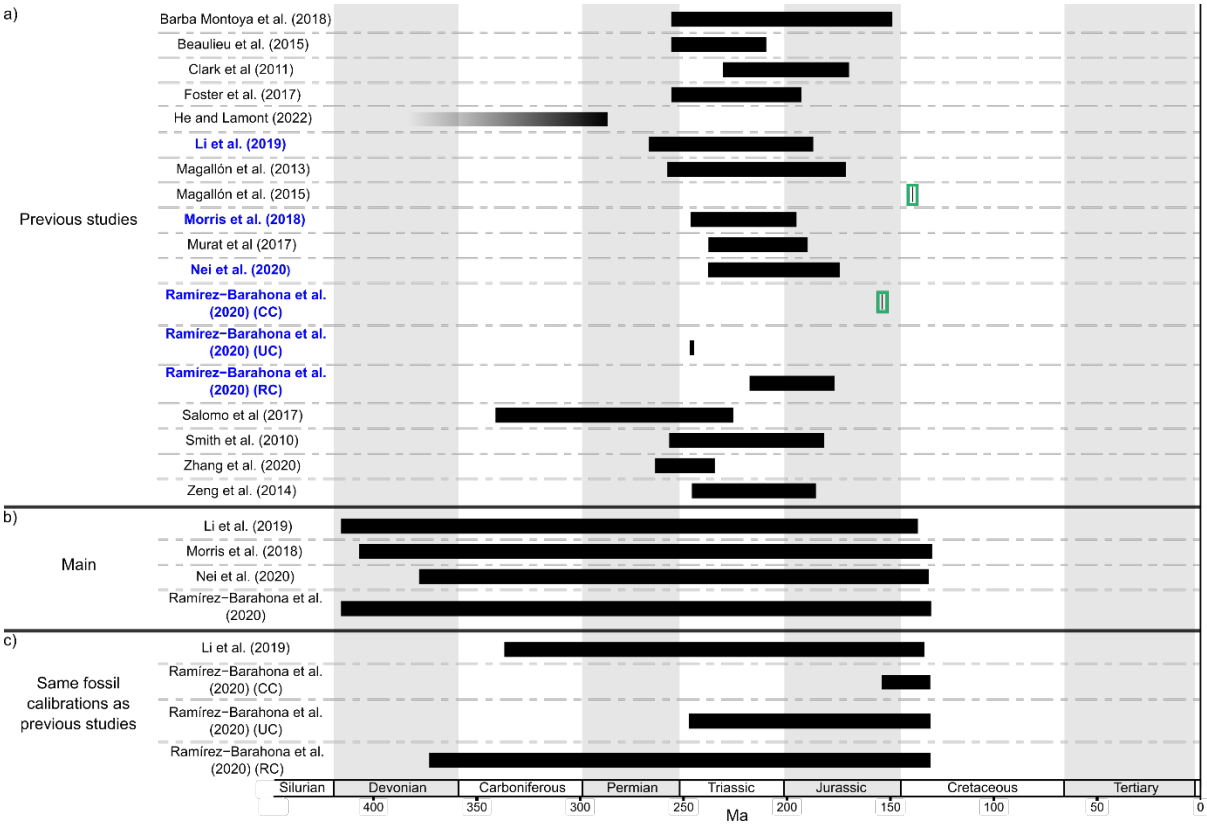

Figure 3. Summary of age estimates for the angiosperm crown node. The black bars indicate the confidence interval from each study. **a.** age estimates from studies undertaken since 2010 (the same studies as in Table 1). Note that for He and Lamont (2022) the error bar is for illustrative purposes only – the authors do not provide a formal confidence interval but suggest the angiosperm crown node is likely to be significantly older than 250Ma. The green boxes around the confidence intervals of Magallón et al. (2015) and Ramírez-Barahona et al. (2020) (CC) are for visual clarity given the narrowness of these confidence intervals. Studies shown in blue indicate those that we analysed in *exTREEmaTIME*. **b.** age estimates from the main analyses in *exTREEmaTIME*. **c.** age estimates from analyses in *exTREEmaTIME* where the same fossil calibrations are used as in previous studies.

The greater width of the confidence intervals results from the minimal assumptions that *exTREEmaTIME* makes (Table 2), unlike previous studies (Table 1) that incorporate clock models, branching processes, and more stringent time constraints based on the fossil record. Meanwhile, the confidence intervals are similar to each other because the minimal assumptions used by *exTREEmaTIME* are consistent in each analysis (Table 2). This contrasts to previous studies where the clock models, and in particular the time constraints, sometimes differ considerably (Table 1). The analyses in *exTREEmaTIME* also demonstrate that the molecular dataset used to estimate the underlying phylogeny and molecular branch lengths has little impact on the confidence interval: first, because radically different molecular datasets underpin each analysis in *exTREEmaTIME*; and second, because the same datasets underpin earlier studies which had far narrower

confidence intervals (Fig. 3a and b). Figure 3 also demonstrates that differences between analyses with *exTREEmaTIME* and previous studies result from a combination of assumptions about substitutions rates and time. This is because when *exTREEmaTIME* was used with the same fossil calibrations as previous studies, age estimates were considerably less precise than these previous studies (Fig. 3a and c). Given the same fossil calibrations are used, these differences are likely to result from the different assumptions that *exTREEmaTIME* makes about substitution rates compared to previous studies. However, the confidence intervals in Figure 2c are still more precise than the main *exTREEmaTIME* analyses (Fig. 3b) where a consistent set of fossil calibrations that made minimal assumptions about the relationship between clade ages and fossil ages was used. Therefore, the assumptions associated with fossil calibrations also have an important effect on age estimates.

It is evident that time-calibrated molecular phylogenies suggest that the age of the angiosperm crown node is very uncertain, with understanding of this uncertainty having accumulated gradually over previous decades as markedly different age estimates were published. Here, we show that this uncertainty can be represented in a single analysis, and that the variation and precision of estimates from previous studies results from the assumptions that they make and not the molecular datasets that they analyse. It is therefore important to use assumptions that reflect current knowledge of molecular evolution and the fossil record. Although challenging, methodological developments can facilitate this endeavour. This includes substitution rate models that explicitly incorporate biological variables that are associated with substitution rates, such as known relationships between rates and certain traits (Smith and Donoghue 2008; Lartillot and Poujol 2011; Beaulieu et al. 2015; Berv and Field 2018), and potentially new methods for defining realistic fossil calibrations (Claramunt 2022). In the future, such approaches may enable precise estimates for the angiosperm crown node that are based on justifiable assumptions. Focussing on justifying assumptions is not only relevant for the angiosperm crown node. Although this node is an iconic example of uncertainty in divergence time estimation, the issues that afflict age estimates for this node are not unique: sequence data is always uninformative about time, and all divergence time estimates are dependent on assumptions about rate and time that can be difficult to justify biologically.

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