Letter

Puzzling rocks and complicated clocks: how to optimize molecular dating approaches in historical phytogeography

In recent decades, molecular dating has become an important component of phytogeographic studies because the establishment of an evolutionary timescale enables the testing of hypotheses about the distribution and evolution of plants across time and space (Renner, 2005; Crisp *et al.*, 2011; Ho, 2014). Based on molecular sequences and fossil calibration points, divergence times between living species can be estimated using a molecular clock. This approach was first proposed by Zuckerkandl & Pauling (1962) and assumes that the genetic distance between molecular sequences increases linearly from time of divergence between any pair of species. Great efforts have been made in recent years to improve molecular dating approaches for timing divergence events (Box 1). Despite these advances, there remain various problems with the acquisition (and interpretation) of age estimates of lineage diversification by means of molecular dating approaches (Pulquério & Nichols, 2007; Sauquet *et al.*, 2012). In this regard, Wilf & Escapa (2015) recently pointed out that a number of newly discovered fossils provide evidence that previously published molecular dating estimates have underestimated lineage divergence times, which, in turn, has greatly affected ideas about how the breakup of the Gondwana supercontinent (from *c.* 180 to 30 Ma) shaped biogeographic patterns in the Southern Hemisphere (Sanmartín & Ronquist, 2004). They argued that the use of 'megabiased clocks' has resulted in the underestimation of divergence dates (younger bias) and wrongly led to the formulation of a 'Green Web' hypothesis that dispersal (over oceanic barriers), rather than vicariance due to the breakup of Gondwana, played a major role in the diversification of plant genera and families. Consequently, they concluded that 'fossils and geochronology provide the only rigorous, enduring temporal framework for evolutionary radiations'. Although Wilf & Escapa (2015) showed that '. . . the (fossil) record from Patagonia convincingly demonstrates that Gondwanan history remains fundamental to the evolutionary radiations, distributions, survival, and conservation of Southern Hemisphere plants and plant associations . . .', their views about the value of molecular dating are, in our opinion, partial and misleading.

First, they argued that 'molecular dates usually cannot be tested adequately with fossils', since molecular dates that are too old 'cannot be falsified because the required fossils never existed', whereas 'if they are younger than comparable fossils they are still not wrong because they represent minimum ages' (Wilf & Escapa, 2015). However, this apparent 'logic' may reflect shortcomings in a molecular clock method that calibrates nodes using a fixed age constraint, but will not apply if a Bayesian relaxed molecular clock method is used that integrates multiple fossil calibrations and calibration node age priors. When integrating fossil age information into molecular phylogenies, any calibration node age prior that comprises a minimum age constraint will prevent molecular dates being 'younger than comparable fossils' known at the time. Similarly, applying maximum age constraints will avoid 'truly too old molecular dates' from being obtained. Clearly, discrepancies between the fossil record and molecular dates must be considered in all phylogenetic studies, in order to strengthen the rigor of molecular dating approaches (Parham *et al.*, 2012; Sauquet, 2013).

. . . Wilf & Escapa (2015) were right to draw attention to the potential younger biases in traditional 'node dating' approaches that were introduced via unjustified but routine fossil calibrating protocols (a list of ways to reduce these potential biases is provided in Table 1) . . .

Second, Wilf & Escapa (2015) based their molecular dates on mean values. Although this is a common approach, it results in inappropriate interpretation and should be avoided (Sauquet, 2013). Instead, interpretation must take account of the uncertainty of dates (e.g. credible intervals of Bayesian estimates) as this facilitates statistically sound testing of different hypotheses for comparing age estimates from different studies, between molecular dates and the fossil record, and especially between geological events and molecular dates (Crisp *et al.*, 2011).

Third, much of the evidence presented by Wilf & Escapa (2015) in support of their argument of a 'megabiased clock' is flawed. In fact, for 19 plant lineages, they compared fossil age with mean molecular age (rather than credible interval). The credible interval of estimated molecular age was compatible with the oldest fossils of the respective groups (*Todea*, *Acompyle*, *Athrotaxis*, *Papuacedrus*, *Bailiancarpus*, and *Ripogonum*), whereas a younger bias existed for estimated molecular dates relative to fossil age in nine cases (*Agathis*, *Dacrycarpus*, *Tripylocarpa*, *Monimiophyllum*, *Raiguenrayun*, *Gymnostoma*, *Paracacioxylon*, *Eucalyptus*, *Orites*). In the nine latter cases, however, older fossils of these groups were reported after those molecular dating studies had been published. These examples of 'megabiased clocks' will be invalid if these fossils

Box 1 Improvements made in recent years to molecular dating approaches for timing divergence events

1 *Advancement of molecular phylogeny*: In the past decade, advances in sequencing technologies have resulted in the rapid accumulation of molecular data and increased access to genomic-scale data. Various tree-building methods have been developed to process large datasets; these have led to more accurate resolution of phylogenetic relationships and genetic distances between living taxa (Yang & Rannala, 2012).

2 *Increasing variety of molecular clock models*: Various molecular clock models, such as local molecular clocks (Li & Tanimura, 1987; Yoder & Yang, 2000), random local molecular clocks (Drummond & Suchard, 2010; Bellot & Renner, 2014), and especially relaxed molecular clock methods (Sanderson, 2002; Thorne & Kishino, 2002; Drummond *et al.*, 2006; Drummond & Rambaut, 2007; Rannala & Yang, 2007; Yang, 2007), have been developed to accommodate divergence rate variation among lineages (Renner, 2005; Brown & Yang, 2010; Ho & Duchene, 2014).

3 *Better strategies to integrate fossil ages*: There have been advances in how fossil calibration information is integrated into phylogenetic trees, such as using a multiple calibration points strategy (Soltis *et al.*, 2002), 'node dating' methods (e.g. soft maximum constraints, lognormal distribution, exponential distribution, for a review see Ho & Phillips, 2009; and hyperprior, Heath, 2012), 'total evidence' methods (Pyron, 2011; Ronquist *et al.*, 2012a), and the 'fossilized birth–death' method (Heath *et al.*, 2014).

4 *Upgrading techniques to extract fossil information*: Improvements in radioisotopic dating of 'rocks' (Burgess *et al.*, 2014) and methods of obtaining and identifying fossil remains (Bomfleur *et al.*, 2014).

5 *Enlarging coverage of the fossil record*: An increasing paleontological record across different geological strata and geographic regions (including previously poorly investigated regions, such as South America and mainland eastern Asia) has led to a better understanding of the evolutionary and biogeographic histories of organisms (Wilf *et al.*, 2013).

are simply integrated into a Bayesian molecular dating analysis as minimum age calibrations. Concerning this point, we consider that, because the fossil record is constantly updated, it makes sense that molecular dates whose estimation relies on fossil data should be updated when new fossils become available. It does not seem justified to use up-to-date fossil data to criticize molecular dates, when the latter were based on the less complete fossil record that was available at the time of node age estimation.

A further point is that informative traits of fossils are frequently missing or ambiguous, and convergent evolution among extinct and living lineages is common. Therefore, inappropriate age constraints related to the phylogenetic placement and evolutionary meaning of controversial fossils must also be considered as a source of any discrepancies between molecular dates and fossil ages. In this respect, exploring the effect of 'outlier' (wrongly-placed) fossil calibrations is a crucial step in generating reliable estimates of the diversification timescale of organisms (Warnock *et al.*, 2015). We argue that the four cases (*Kurtziana* spp. and three *Araucaria* spp.) cited in Wilf & Escapa (2015) where presumed fossil calibrations are significantly older than molecular dates may represent a set of 'outlier' fossil calibrations. In Araucariaceae, empirical studies have suggested that adoption of 'outlier' fossil calibrations (e.g. assigning stem fossils to crown nodes) will lead to unrealistic age estimates for other conifer lineages (Kranitz *et al.*, 2014) or a significant change in the evolutionary rate between stem and crown Araucariaceae lineages (Biffin *et al.*, 2010). Meanwhile, there are also abundant examples where molecular dates are older than the unequivocal fossil record, such as the crown age of angiosperms (Smith *et al.*, 2010; Zeng *et al.*, 2014), land plants (Smith *et al.*, 2010; Magallón *et al.*, 2013), and the crown age of mammals (Meredith *et al.*, 2011; Dos Reis *et al.*, 2014a), among others. Therefore, caution should be employed when integrating ambiguous fossils as minimum age constraints, or integrating unambiguous fossils as maximum age constraints into molecular dating (see also later).

Generally speaking, potential errors (or biases) may be introduced into molecular dating in nearly all steps of the process, and it would be helpful to identify these errors and to propose strategies to reduce them. What then are the key factors likely to introduce age estimation errors into mainstream molecular dating approaches ('node dating', see later), such as Bayesian relaxed clocks (as implemented in the programs BEAST, MCMCtree and Multidivtime)? These factors can be divided into three main categories: the estimation of branch length and phylogenetic topology, clock models and priors, and most importantly, the uncertainty of fossil calibration (Inoue *et al.*, 2010; Dos Reis & Yang, 2013).

Until now, most molecular dating studies did not follow a systematic protocol to justify their fossil calibration scheme. As pointed out by Wilf & Escapa (2015) and others (Sauquet *et al.*, 2012), molecular dating is strongly dependent on the selection and placement of fossils in phylogenetic trees in order to calibrate the timescale of diversification. A problem arises with the selection of relatively ancient fossils in that traits that might link ancient fossils to living taxa are often likely to be absent or ambiguous. By contrast, it is easier to select younger fossils linked to extant lineages because better identification of synapomorphies or higher resolution of phylogenetic placement in the 'total evidence' tree are more likely for younger fossils. Sauquet *et al.* (2012) emphasized this point by describing the use of ancient and more recent fossils as 'early but risky' and 'safe but late', respectively, and further demonstrated that selecting younger fossils for molecular dating often leads to underestimation of the dates of lineage divergence.

Wilf & Escapa (2015) further emphasized that the conservative placement of fossils in a phylogenetic tree, such as at the stem rather than crown node, is likely to introduce a younger bias into molecular dating. In this respect, however, we argue that this is a two-way logic, if fossilized taxa cannot be assigned to crown nodes confidently, then they most likely represent extinct relatives of the stem lineage, in which case radical placement of these fossils on crown nodes may potentially introduce overestimation biases. Moreover, since each calibration point tends to have a greater influence on the age estimates of the closest nodes in a phylogeny, a deficiency in number and coverage of calibration points usually leads to younger estimates of node ages in other parts of the phylogeny (Duchêne *et al.*, 2014).

To reduce these errors in 'node dating', we strongly recommend that when choosing and placing fossils for calibration purposes, a strict and systematic protocol is followed, as outlined by Parham *et al.* (2012). This widely accepted protocol aims to justify why a certain set of fossils is employed for the calibration points, and to facilitate replication and assessment of age estimation results in the future.

Turning to other fossil calibration issues that can cause errors when commonly used models are employed for molecular dating (e.g. uncorrelated lognormal and exponential relaxed-clock models in BEAST), inappropriate parameter settings may be common, especially with regard to the uncertainty of ages of fossils that were adopted for calibration. Usually, this parameter is integrated into the analysis as a form of prior probability distribution (henceforth simply referred to as a 'prior') for a particular ancestral node age (see Ho & Phillips, 2009). Inappropriate setting of this parameter can potentially lead to biased estimates of node ages (Ho & Phillips, 2009; Heath, 2012). For example, use of an exponential prior or a lognormal prior assumes a rapidly declining probability of older ages, which may introduce a 'younger bias' to dating lineage divergence if the true node age is significantly older than the major part of the probability distribution for this prior (Clarke *et al.*, 2011). It has been proposed that many phylogenetic studies that reject the link between the Gondwana breakup and lineage divergence in the Southern Hemisphere possibly underestimate dates of lineage divergence only because particular calibrating node age priors have been inappropriately used in the Bayesian dating process (Heads, 2012). In addition to setting calibration priors, interactions between calibration priors at different nodes, and between the calibration prior and other priors in the clock model (such as tree priors) may also affect age estimations in Bayesian relaxed-clock dating (Inoue *et al.*, 2010; Heled & Drummond, 2012; Warnock *et al.*, 2015).

To overcome the problem described earlier, we recommend a strategy for assigning the appropriate calibration prior (for a summary of calibration priors or densities, see Ho & Phillips, 2009) to specific nodes (Warnock *et al.*, 2012). First, a minimum constraint (uniform prior) should be the first choice for all relatively shallow calibration nodes; if other priors are applied, justification should be provided based on a thorough survey of related fossils, and Bayesian priors should be evaluated with appropriate approaches (Nowak *et al.*, 2013; Norris *et al.*, 2015). For deeper nodes, choosing a conservative (hard) maximum constraint prior may lead to a 'younger bias', and therefore in most cases the use of a gamma hyperprior (Heath, 2012), a lognormal prior or a soft maximum constraint is preferable (Warnock *et al.*, 2012). In addition, it would always be helpful to compare and discuss age estimates from different calibration scenarios (Mao *et al.*, 2012; Sauquet *et al.*, 2012).

Molecular dating results may differ between different clock models (Duchêne *et al.*, 2014) and between different prior settings in the same model with the same set of fossil calibration points (Condamine *et al.*, 2015). Various molecular clock models relating to the heterogeneity of rate among lineages are now available, such as the strict clock, local multirate clock, discrete multirate clock, autocorrelated relaxed clock and uncorrelated relaxed clock, and empirical studies suggest that different clock models suit different situations (for a review, see Ho & Duchene, 2014). Empirical studies suggest that uncorrelated rate-relaxed clocks (as implemented in BEAST) usually produce older age estimates than penalized likelihood relaxed clocks (as implemented in R8S) (Mao *et al.*, 2010, 2012). In the same clock model, for example, the uncorrelated relaxed clock, a recent empirical study demonstrated that adoption of two branching process priors (Yule vs birth–death priors) resulted in strikingly different diversification timescales, with mean age estimates differing by a factor of three (Condamine *et al.*, 2015). We therefore recommend that sufficient justification should be provided when certain clock models and priors are selected, for example, according to likelihood-based or Bayesian-based criteria (Drummond & Suchard, 2010; Paradis, 2013; Ho & Duchene, 2014).

The estimation of branch length and phylogenetic topology may introduce errors into molecular dating analyses, but in recent years the increased feasibility of obtaining genome-scale data has progressively improved our ability to establish phylogenetic topology and genetic distance (Yang & Rannala, 2012). Nevertheless, challenges remain about how to integrate genomic data efficiently into molecular dating approaches (Ho, 2014). In one empirical example, when many loci were analyzed in molecular dating based on genome-scale data, the traditional oversimplified substitution rate prior may have dominated posterior age estimation, much more so than the Dirichlet prior (Dos Reis *et al.*, 2014b).

Molecular dating approaches are constantly being updated (Ho, 2014; Ho & Duchene, 2014) and recent developments in Bayesian dating methods, for example, 'total evidence' (Ronquist *et al.*, 2012a) and 'fossilized birth–death' (Heath *et al.*, 2014) methods, may lead to further improvements in the accuracy of dating lineage diversification. In comparison to the traditional Bayesian methods of 'node dating' within a molecular phylogeny (e.g. Bayesian relaxed clocks that were implemented in the programs BEAST, MCMCTree, Multidivtime), 'total evidence' methods incorporate fossilized taxa into a molecular-and-morphological phylogeny as an extinct side branch (Pyron, 2011; Ronquist *et al.*, 2012a,b; Grimm *et al.*, 2015), while the 'fossilized birth–death' method considers living and fossil taxa together as part of the same macro-evolutionary process covering speciation, extinction and fossilization rates (Heath *et al.*, 2014). It is crucial to note that the 'fossilized birth–death' approach integrates all available fossilized taxa, and therefore avoids potential biased selection and placement of fossil calibrations (Heath *et al.*, 2014; Grimm *et al.*, 2015). There is no need for prior age densities to be applied to fossils as is the case for 'node dating' methods, nor is a morphological data matrix required as for 'total evidence' dating methods (Heath *et al.*, 2014; Grimm *et al.*, 2015). Hence, 'fossilized birth–death' is completely different from 'node dating' and 'total evidence' dating methods, both of which employ only a selected portion of fossils for calibration. These new developments might lead to improved accuracy of molecular dating, although their merits and shortcomings still require further evaluation (Arcila *et al.*, 2015; Grimm *et al.*, 2015). Time will tell whether their use will help to overcome some of the problems inherent in traditional Bayesian methods of 'node dating'.

Although suffering from a number of possible error-introducing factors (see Table 1), molecular dating approaches provide invaluable insights into the diversification and biogeographic histories of plants. Molecular-dated timescales of lineage divergence are a reliable complement to timescales based solely on fossils, if molecular dating is conducted carefully and age estimates are interpreted cautiously (Sauquet *et al.*, 2012; Sauquet, 2013;

Table 1 Comparisons between pairs of molecular dating (node dating) scenarios that showing common-seen bias-introducing effects of certain factors; note that in each line (excluding under-sampling), all parameters except the focal parameter (factor) are exactly the same

Scenario 1	Scenario 2	Age estimation for Focal lineage	References	Remarks
Favoring younger but reliable calibration fossils when assigning maximum age constraint	Applying 'older but risky' calibration with a proper prior (e.g. soft maximum constraint)	Most likely Scenario 1 (S1) < Scenario 2 (S2)	Sauquet *et al.* (2012)	The crown age of Angiosperm is dated to the early Cretaceous according to an unambiguous fossil record (Friis *et al.*, 2011) but molecular dating suggested a Triassic age (Smith *et al.*, 2010; Zeng *et al.*, 2014), which is in agreement with the recent finding of angiosperm-like pollen grains in the Middle Triassic (Hochuli & Feist-Burkhardt, 2013)
Applying priors that comprise a 'soft maximum age constraint' (e.g. a lognormal prior) to calibrate shallow nodes	Applying a minimum age constraint	Uncertain, MORE likely S1 < S2	Sauquet *et al.* (2012); Mao *et al.* (2012)	A conservative soft maximum age constraint will lead to younger age estimation if the 'true age' at a particular shallow node is older; fossils that are unambiguously assigned to shallow nodes are usually of conservative ages
Under-sampling of species in a particular lineage	Proportionately sampling across all lineages	Most likely S1 < S2	Linder *et al.* (2005)	Fossil calibrations (see later) and evolutionary rate variation, among others, are under-represented when a particular lineage is under-sampled (e.g. Sauquet *et al.*, 2012)
Assigning a minimum fossil age as the upper boundary of a stratigraphic epoch/stage	Applying an accurate radioisotopic age	Most likely S1 < S2	Parham *et al.* (2012)	For fossils found from the Cretaceous onwards, the age difference between the upper and lower bounds of an epoch or a stage could be as wide as 44.5 or 12.5 million yr (Cohen *et al.*, 2013)
Applying the penalized likelihood relaxed clock model (PLRS in R8S)	Applying the uncorrelated lognormal relaxed clock model (ULRC in BEAST)	For most nodes, S1 < S2	Mao *et al.* (2010)	Although credible intervals of these two methods overlapped with each other at each node, PLRS usually generates age estimates younger than ULRC
Poor fossil calibration coverage of certain lineages when more fossil calibrations are available	Fossil calibration coverage across lineages to the best of our knowledge	Most likely S1 < S2	Sauquet *et al.* (2012)	Applying the Fossilized birth–death method (Heath *et al.*, 2014), which considers all fossils related to focal lineage, will also avoid biases in S1
Secondary calibration with a single fixed age when fossil calibration is not available for the focal lineage	Calibrating with outgroup fossils under a proportionate sampling scheme	Uncertain, MORE likely S1 < S2	Sauquet *et al.* (2012)	Potentially a younger estimate for the calibrating node in the first round molecular dating (possibly due to under-sampling) and a single fixed calibration in the second round molecular dating, among others, are important factors in S1
Applying a Yule prior (branching process prior) in an ULRC model (in BEAST)	Applying a birth–death prior in an ULRC model (in BEAST)	Most likely S1 > S2	Condamine *et al.* (2015)	Condamine *et al.* (2015) found that crown node age estimates for S1 were approximately three times as old as those of S2 in Cycads; Bayesian-factor-based selection of these two priors should be used in any taxonomic group that experienced a high extinction rate
Using stem fossils to calibrate crown nodes	Using stem fossils to calibrate stem nodes	Most likely S1 > S2	Parham *et al.* (2012)	Obtaining a reliable phylogenetic position for fossil taxa following Parham *et al.* (2012)
Favoring older but risky calibration fossils when assigning minimum age constraints	Applying younger but reliable calibration fossils	Most likely S1 > S2	Sauquet *et al.* (2012)	Older but risky fossils probably represent extinct sister lineages of living lineages; an extreme case is *Athrotaxis ungeri*, if this fossilized taxa is employed to calibrate the crown node of living *Athrotaxis*, age estimates of this node will at least be four times as old as reported by Mao *et al.* (2012)
Integrating significantly older (outlier) fossil calibration	Molecular dating without outlier fossil calibration	Most likely S1 > S2	Mao *et al.* (2012)	Performing 'a priori evaluation of the intrinsic palaeontological, stratigraphic, geochronological and phylogenetic data' (Warnock *et al.*, 2015; p1; see also Parham *et al.*, 2012), and comparing dating results from both scenarios, so as to provide justification to follow either scenario with or without outlier fossil calibration(s); note that applying the Fossilized birth–death dating method (Heath *et al.*, 2014; Grimm *et al.*, 2015) will also avoid biases in S1

Hipsley & Müller, 2014). The alleged 'megabiased clock' issue identified by Wilf & Escapa (2015) referred to 19 cases where molecular dates are younger than corresponding fossil ages; these were mainly drawn from comparisons between outdated molecular dates and up-to-date fossil records. Except for a small number of controversial cases, molecular dates of most plant lineages will be compatible with fossil records by integrating up-to-date fossil calibrations. Nevertheless, Wilf & Escapa (2015) were right to draw attention to the potential younger biases in traditional 'node dating' approaches that were introduced via unjustified but routine fossil calibrating protocols (a list of ways to reduce these potential biases is provided in Table 1). Moreover, their systematic identification of key fossil taxa supporting the Gondwana vicariance hypothesis rather than the Green Web hypothesis for plant lineage diversification in the Southern Hemisphere is an important contribution to research on this topic. However, it is important not to 'throw the baby out with the bathwater' and dismiss the value of the molecular dating approach. It is our belief that closer cooperation between paleontologists and molecular phylogeneticists will steadily refine our understanding of the temporal framework of diversification and biogeographic history in both the Southern and Northern Hemispheres of the Earth.

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Author contributions

K.S.M. planned and designed the research. K.S.M. and Q.W. collected, compared and summarized data in previous publications. Q.W. and K.S.M. wrote the manuscript.

Qian Wang and Kang-Shan Mao

Key Laboratory for Bio-resources and Eco-environment of the Ministry of Education, College of Life Sciences, Sichuan University, Chengdu, 610064, China
(Author for correspondence: tel +86 28 85412053; email maokangshan@163.com)

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