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A practical guide to molecular dating

Guide pratique de la datation moléculaire

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ABSTRACT

Molecular dating has now become a common tool for many biologists and considerable methodological improvements have been made over the last few years. However, the practice of estimating divergence times using molecular data is highly variable among researchers and it is not straightforward for a newcomer to the field to know how to start. Here I provide a brief overview of the current state-of-the-art of molecular dating practice. I review some of the important choices that must be made when conducting a divergence time analysis, including how to select and use calibrations and which relaxed clock model and program to use, with a focus on some practical aspects. I then provide some guidelines for the interpretation of results and briefly review some alternatives to molecular dating for obtaining divergence times. Last, I present some promising developments for the future of the field, related to the improvement of the calibration process.

Mots clés :
Contrainte d'âge
Intervalle de confiance
Calibration fossile
Datation moléculaire
Horloge relâchée
Incertitude

RÉSUMÉ

La datation moléculaire est devenue un outil commun pour de nombreux biologistes et des progrès méthodologiques considérables ont été apportés ces dernières années. Cependant, l'estimation des temps de divergence à partir de données moléculaires demeure très variable dans sa pratique et sa mise en œuvre est délicate pour le novice. Cet article propose une revue concise de l'état de l'art de la pratique de la datation moléculaire. Plusieurs décisions importantes sont nécessaires, notamment la sélection et l'implémentation des calibrations, ainsi que le choix d'un modèle d'horloge relâchée et le logiciel pour l'analyse. Après une discussion de certains aspects pratiques de la conduite de ces analyses, plusieurs règles sont proposées pour l'interprétation des résultats. Les alternatives possibles pour obtenir des temps de divergence sont également discutées. Enfin, plusieurs développements prometteurs pour l'avenir de la discipline sont soulignés, en particulier dans le cadre de l'amélioration du processus de calibration.

1. Introduction

Estimating divergence times among species or lineages using molecular sequence data, commonly referred to as molecular dating, has now become a commonplace tool for

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many evolutionary biologists and ecologists. Although the idea was laid more than 50 years ago, the field of molecular dating has now become more mature, largely thanks to considerable methodological improvements brought over the last 15 years. However, many questions remain and significant improvements and discoveries are still being made on various aspects of the method. Yet, the practice of molecular dating varies considerably in quality. Numerous reviews have been written on the topic (Donoghue and Benton, 2007; Forest, 2009; Kumar, 2005; Laurin, 2012; Magallón, 2004; Pulquério and Nichols, 2007; Renner, 2005; Rutschmann, 2006). In this paper, I do not intend to provide yet another review of the field or a hands-on tutorial based on a specific example. Instead, my aim is to provide practical guidelines to design, conduct, and interpret a molecular dating experiment according to the state-of-the-art, citing extensive reviews and key studies where appropriate.

The idea of molecular dating was originally proposed in a paper by Zuckerkandl and Pauling (1962), who suggested that the divergence time between two species could be measured by the number of differences between two molecular sequences (in their case, protein sequences). This was centered on the assumption of a molecular clock, whereby the rate of molecular evolution remains constant through time. Although this was initially envisioned as an alternative and independent method to using the fossil record, it is widely accepted today that fossils should be used to calibrate the molecular clock (except in special cases such as virus phylogenies when virus sequences can be sampled through recent time). Indeed, molecular sequence divergences can only provide a relative time scale. Calibration from another source of information is always required in order to convert relative into absolute divergence times. At about the same time when technological progress allowed biologists to routinely obtain molecular sequences and reconstruct phylogenies using them, important methodological advances were made in molecular dating methods. These advances largely focused on relaxing the assumption of a molecular clock. Indeed, in many studies, this assumption appeared to be contradicted by lineages with molecules evolving particularly fast or slow. Therefore, methods were developed that allowed molecular rates to vary through time and across lineages (Rutschmann, 2006; Sanderson et al., 2004). These so-called *relaxed clock* methods are now used preferentially by most researchers. Finally, increased attention has been given recently in improving calibration of molecular dating studies. It is now commonly accepted that not only one, but instead multiple fossil calibrations are required for accurate estimation of divergence times (Ho and Phillips, 2009; Magallón et al., 2013; Sauquet et al., 2012). New standards are being proposed for documenting and justifying these calibrations as well as implementing them in the statistical framework of Bayesian relaxed clocks.

In this paper, we will first ask whether a molecular dating study is needed or not to answer a particular biological question. We will then go through the various stages of designing a molecular dating analysis, from choosing genes, taxa, and calibrations to selecting a relaxed clock model and software to compute the analysis. After giving some practical advice about the analysis itself, I will suggest some guidelines for interpreting the results. Finally, we will look at some alternative ways to obtain divergence times quickly, and I will briefly outline some exciting developments to expect in the field over the next few years.

2. To date or not to date: is molecular dating essential for my study?

First, it is essential to state that paleontological dating is an alternative to molecular dating only for very few branches of the Tree of Life and for a restricted set of applications. Indeed, even for the most fossil-rich taxa with well-known phylogenetic relationships to extant taxa, a fossil date cannot be given for every single divergence in a given phylogeny (except in exceptional circumstances). Molecular dating, calibrated with multiple fossils, must thus be seen as a reasonable alternative to obtain divergence time estimates for all nodes of a phylogeny, when such information is required to conduct further analyses.

Molecular dating has been used to answer a wide range of questions. Many of these applications fall in the following categories.

2.1. Biogeography

In order to compare the evolutionary history of a group with global processes of the Earth, an absolute time scale is required. For example, molecular dating studies have severely challenged the commonly assumed role of vicariance (due to plate tectonics) in shaping the biogeographic distribution of many clades (for reviews, see Crisp et al., 2011; Renner, 2005). Furthermore, current probabilistic approaches to reconstructing biogeographic history typically rely on dated trees, such as the dispersal-extinction-cladogenesis (DEC) model implemented in Lagrange (Ree and Smith, 2008). Another example where molecular dating studies have played an important role is whether past climate change had an influence on the diversification of large clades at a regional scale (Linder, 2003).

2.2. Diversification rates

Over the last few years, considerable interest has grown in estimating speciation and extinction rates from molecular phylogenies, with the continuous development of increasingly complex models accounting for variable rates through time and across lineages (Alfaro et al., 2009; FitzJohn et al., 2009; Morlon et al., 2011; Stadler, 2011). All of these methods require an absolute or relative time scale as a prerequisite.

2.3. Comparative methods

A very wide range of contemporary methods in comparative biology rely on dated trees, for instance to infer ancestral states or to test the correlation between two traits (for reviews, see O'Meara, 2012; Pagel, 1999). However, for many methods, nonultrametric trees obtained from phylogenetic analyses (i.e., phylograms, with branch lengths

proportional to the number of inferred substitutions) are also acceptable and commonly used.

2.4. Co-diversification

Molecular dates can be used to test whether two clades of interacting organisms, such as parasites and their hosts or plants and their pollinators, have diversified synchronously (Cardinal and Danforth, 2013; Cruaud et al., 2012; Ramírez et al., 2011).

2.5. Population history

Molecular dating methods have also been developed for and applied to infraspecific studies to address a wide range of topics, including applications to medicine such as the timing of emergence of HIV strains (Gilbert et al., 2007).

Although absolute divergence times might be interesting in themselves, most researchers will need them for a specific purpose as outlined above. If the clade studied has a rich fossil record and only a few nodes need to be scaled in time, paleontological dating might be sufficient (Magallón and Sanderson, 2001; Marjanović and Laurin, 2007). If time-scaled trees are needed for comparative analyses without specific attention to absolute time, molecular dating might or might not be necessary. The risk is to increase error compared to using molecular branch lengths only. The gain is to dissociate rates of molecular and morphological evolution, in situations of strong molecular rate variation, where long branches do not necessarily imply long times, and short branches do not necessarily reflect short times. However, if the assumption that molecular and morphological rates are linked can be justified to be biologically sound, then molecular dating can be avoided in comparative analyses. In studies of biogeography, diversification rates, or co-diversification requiring all divergence times to be known in absolute time, molecular dating analyses appear necessary, but results should be interpreted with caution depending on the quality of the data available for estimating divergence times (see below).

3. Step 1: assembling a molecular data set

The same general rules appear to apply to molecular phylogenetic reconstruction and molecular dating: one gene and a few taxa are, in theory, sufficient, but significant increases in both precision and accuracy can be gained by using as many genes and taxa as possible. Likewise, it is recommended to choose a substitution model and partitioning scheme representing a good trade-off between a realistic representation of the molecular evolutionary process and the number of parameters. Recent studies have demonstrated that all of these parameters can have an impact on estimated divergence times (Brandley et al., 2011; Goodall-Copestake et al., 2009; Magallón et al., 2013; Phillips, 2009; Schenk and Hufford, 2010). All molecular dating methods allow the tree topology to be fixed according to the results of a previous phylogenetic analysis; thus it is possible to use a different sample of genes and taxa, but in practice most researchers use the same data set for both the phylogenetic and the molecular dating analyses.

One potentially important issue to consider when assembling a data set for molecular dating is the detrimental effect missing data can, in some circumstances, have on phylogenetic inference and branch length estimation (Lemmon et al., 2009; Roure et al., 2013; Wiens and Morrill, 2011). Molecular supermatrices with extensive missing data are routinely being used to estimate divergence times, without any apparent bias, but this is an area where further research is needed (Wiens and Morrill, 2011).

4. Step 2: deciding on calibrations

Solid evidence has now accumulated showing that calibration has a significant, sometimes drastic impact on estimated divergence times (Goodall-Copestake et al., 2009; Ho et al., 2008; Inoue et al., 2010; Magallón et al., 2013; Meredith et al., 2011; Ruane et al., 2011; Sauquet et al., 2012). Therefore, calibration is a critical step in any molecular dating study and needs to be thoroughly justified and documented. Rather than fixing the age of a particular node to a set date, calibration is nowadays most commonly applied in the form of age constraints: minimum age constraints, maximum age constraints, or more elaborate prior distributions as discussed below.

4.1. Sources of calibration

There are four main kinds of calibration for molecular dating analyses.

4.1.1. Rate of substitution

This would be the most straightforward source and has been used in a number of studies (for reviews, see Ho and Lo, 2013; Weir and Schluter, 2008). However, the absolute rate of substitution is hardly ever known prior to the analysis. Instead, molecular dating analyses (calibrated with other sources of information) are now commonly used to estimate absolute rates of substitution.

4.1.2. Fossil record

Fossils are by and large the most widely used source of calibration. In general, fossils only provide minimum age constraints. The greatest challenge is to link fossil taxa to the phylogeny of extant taxa represented in the molecular data set (see below). The greatest problem is that appropriate fossils are lacking for many groups of organisms, which therefore require alternative sources of calibration.

4.1.3. Geology and biogeography

Geological events such as the separation of continents by plate tectonics or the emergence of volcanic islands can be and have been used to calibrate molecular dating analyses, especially in groups with a poor fossil record. However, geological calibration requires strong a priori assumptions on the role of geology in shaping the biogeographic distribution of the clades concerned. These assumptions should therefore be stated very clearly. In addition, to avoid circularity, geological calibration should be avoided when the molecular dating analysis is conducted specifically to test a biogeographical hypothesis.

4.1.4. Secondary calibration

Dates obtained in a previous molecular dating analysis are commonly used as a last-resort calibration when no other sources are available. This is possible when at least one node in the focus phylogeny (e.g., the root node) has been included and dated in another study (e.g., a higher-level analysis calibrated with the fossil record). This creates various problems, in particular the increased error and uncertainty in estimated ages (Graur and Martin, 2004). When using secondary calibration, special attention should be given to the quality of the original analysis and its original calibration scheme. In addition, given evidence that a single secondary calibration is often insufficient to replicate estimated ages (Sauquet et al., 2012), the simultaneous use of multiple secondary calibrations would seem preferable.

4.2. Fossil calibration

Fossil calibration often involves three sources of uncertainty, which should be properly acknowledged.

4.2.1. Phylogenetic uncertainty

The exact position of a fossil taxon on a given phylogeny of extant taxa is seldom known, except for a few very well studied taxa with a dense fossil record such as mammals (Fig. 1A). Often, at best the fossil has a number of derived characters of a given clade that allow us to hypothesize that it branched off some time after this clade diverged from its extant sister group. This apomorphy-based approach is very widely used in the molecular dating community and allows one to state that the age of the stem node of that clade must be at least as old as the fossil (minimum age constraint). To use a fossil to constrain the age of the crown node of a clade, one must be able to argue that the fossil has the derived characters (synapomorphies) of one of the lineages within that clade. The distinction between stem and crown nodes is essential for calibration (for clear explanations and illustrations on this difference, see Benton and Donoghue, 2007; Doyle and Donoghue, 1993; Forest, 2009; Ho and Phillips, 2009; Magallón, 2004; Renner, 2005). However, the apomorphy-based approach has important limitations. First, one can never be sure that an apomorphy shared between a fossil and an extant taxon is not, in fact, a convergence, even if it evolved only once in the extant phylogeny. Second, this approach gives a considerable weight to one or a handful of characters. Last, it should be noted that synapomorphies used to defend fossil relationships in molecular dating analyses are not commonly spelled out explicitly and have seldom been identified as such with a formal approach (i.e., with an explicit extant phylogeny and character matrix). The obvious alternative would be to conduct a phylogenetic analysis that includes the fossil as a terminal taxon and this remains the preferred, but seldom used approach for justifying a fossil calibration. Phylogenetic analyses of fossil taxa require a morphological data set that includes both fossil and extant taxa. In addition, it is highly recommended to include molecular data in such analyses so that the overall tree obtained is compatible with the molecular tree used for divergence time estimation. Two approaches have been used for this integration: either constraining the relationships among extant taxa to follow a previously obtained molecular tree (molecular backbone or scaffold approach), or combining molecular and morphological data in the same analysis (total evidence approach) (for a review, see Hermsen and Hendricks, 2008). Even in such cases, multiple, equally parsimonious or likely positions are often obtained for each fossil. One solution has then been to constrain the most recent common ancestor of all these positions to be at least as old as the fossil (Sauquet et al., 2009). This may result in poorly informative calibrations and an alternative, time-consuming approach would be to conduct as many molecular dating analyses as there are positions of a fossil on the phylogeny (Rutschmann et al., 2007). Finally, in many cases, only an intuitive assignment to extant taxa, similar to using an overall similarity criterion, is available from the literature in which the descriptions of the fossil are provided. Such assignments are not necessarily incorrect, but must be acknowledged as risky from a phylogenetic standpoint. Ideally, if such fossils are to be considered in molecular dating analyses, they should be clearly flagged as such and the analyses should be conducted without and with these fossils for comparison (Sauquet et al., 2012).

4.2.2. Sampling uncertainty

Even if the exact branching position of a fossil were known with precision, the time lapse between its divergence from the extant taxon tree and the time when the specimen lived and died is often unknown(Fig. 1B). This observation further advocates the use of minimum ages rather than fixed ages in fossil calibration. Interesting approaches have been and are being developed to estimate and bracket these sampling gaps (Didier et al., 2012; Heath, 2012; Marshall, 2008; Tavaré et al., 2002), but these require new assumptions and their applicability will depend on the quality of the fossil record.

4.2.3. Absolute age uncertainty

Although geostratigraphy is constantly improving and many deposits can now be dated with precision, the exact age of most fossils remains unknown within a given stratigraphic range (Fig. 1C). A common approach, compatible with the use of safe minimum ages is to use the upper (i.e., most recent) limit of the oldest geologic stage in which the taxon has been confirmed. In any case, the age and stratigraphy of a fossil deposit may have changed since the fossil was last described or reviewed. It therefore appears important to check such data in recent literature before setting the age of a calibration. Finally, global geologic time scales have been refined through time, implying that the accepted boundaries of two geological stages might not have been the same, for instance, in 1999 and 2004. Therefore, the geologic time scale of reference used in the conversion of stratigraphic ages into absolute ages should be specified.

For further reading on the questions and problems associated with fossil calibration, a number of recent papers have dealt with the topic in greater detail (Benton and Donoghue, 2007; Donoghue and Benton, 2007; Gandolfo

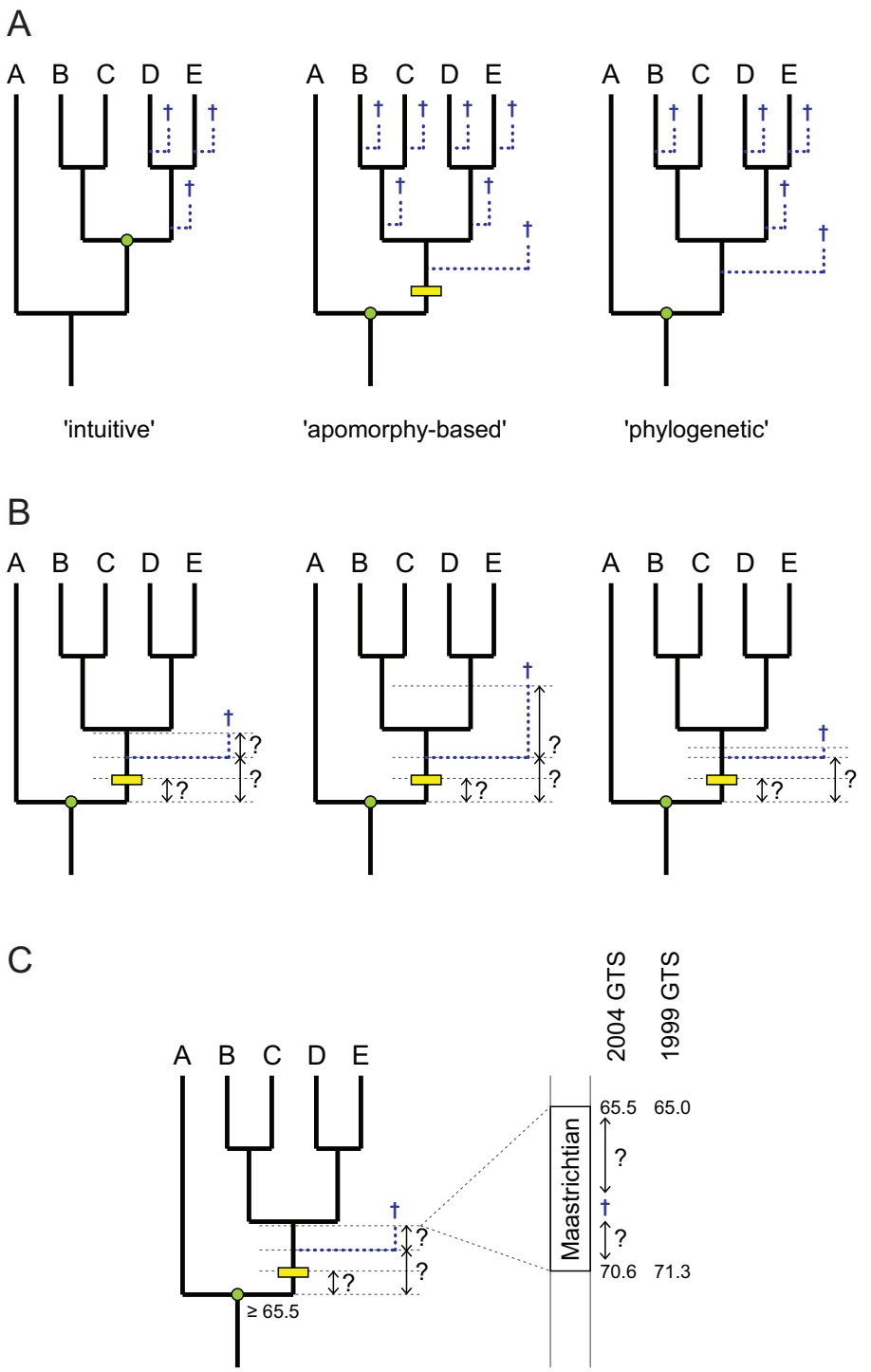

Fig. 1. Important aspects of fossil calibration. In this hypothetical example, A, B, C, D, and E are five extant taxa, whose phylogenetic relationships are assumed to be known, and the dagger represents a fossil taxon. The node calibrated (usually in the form of a minimum age constraint) is indicated by a plain circle. A. Phylogenetic uncertainty: the exact branching point of the fossil on the extant phylogeny is often uncertain. A common approach is to calibrate the most recent common ancestor of all possible branching positions. For the apomorphy-based approach, this is the stem node of the extant clade that shares a synapomorphy with the fossil. B. Sampling uncertainty: the time elapsed between the divergence of the fossil lineage from the extant phylogeny and its oldest occurrence in the fossil record is often uncertain. This is why most fossils can only be used as minimum age constraints. C. Absolute age uncertainty: the exact age of a fossil is often unknown. When it occurs within a known stratigraphic interval, a common approach is to use the youngest boundary of this interval as a minimum age constraint. However, the absolute age of this boundary depends on the Geologic Time Scale (GTS) used as a reference.

et al., 2008; Magallón, 2004; Parham et al., 2012; Sauquet et al., 2012).

4.3. Finding a suitable maximum age constraint

A problematic aspect of conducting molecular dating analyses calibrated with the fossil record is that a set of minimum age constraints alone is not sufficient for the analysis to converge on a unique or restricted range of solutions (Ho and Phillips, 2009). Therefore, it is often necessary to add a maximum age constraint on at least one node in the tree, which will effectively restrict the range of solutions for the entire tree. This is often applied to the root node. A maximum age constraint could be the oldest biologically sound age of a clade, argued either from the fossil record or from previous molecular dating analyses. To justify a maximum age based on the fossil record is difficult because many lineages have long sampling gaps (or ghost lineages), which are difficult to quantify, and absence from the fossil record does not mean that the lineage was not there. A case is more easily made when the fossil record is rich, well sampled, and globally distributed, and the clade in question has unmistakably diagnostic features. This is the case, for instance, of tricolpate pollen, diagnostic of the eudicot clade of angiosperms, which appears at the Barremian/Aptian boundary but is completely absent from any older deposits. This has been used extensively to justify a maximum age constraint of 125 million years on crown eudicots in many studies of angiosperm divergence times (e.g., Magallón and Castillo, 2009). Finally, it should be noted that although a single maximum bound is in principle sufficient, multiple maximum bounds would in fact be desirable in order to best inform molecular rate variation across the tree.

4.4. Choosing probability distributions on fossil calibration

Penalized likelihood dating (implemented in r8s; Sanderson, 2002, 2003) and various other dating methods only allow strict minimum or maximum age constraints for calibration. However, a rising number of dating methods are implemented in Bayesian frameworks that allow more sophisticated age constraints to be applied, in the form of prior distributions (for reviews, see Ho, 2007; Ho and Phillips, 2009). These priors, such as the lognormal and the exponential priors, allow the user to enforce a strict minimum age (offset value) while specifying that the age of the node is unlikely to be much older (diminishing tail of probability) than the set minimum age (for an exponential prior) or median age (for a lognormal prior). These properties can be particularly useful when modeling the sampling gap discussed above. However, the current practice of using these priors in molecular dating analyses is somewhat debatable. Indeed, in addition to a strict minimum age, the exponential prior requires one additional parameter (mean), and the lognormal prior requires two parameters (mean and standard deviation). These parameters are often set quite arbitrarily (or using the defaults of the software) and how exactly this is preferable to uniform priors (the equivalent of simple strict minimum or maximum age constraints) remains uncertain. All of the priors cited above (uniform, lognormal, exponential) share hard minimum bounds, allowing the enforcement of strict minimum ages. However, additional priors implemented in some (but not all) Bayesian relaxed clocks also allow the specification of soft minimum or maximum bounds (Ho and Phillips, 2009; Yang and Rannala, 2006). These properties can be interesting when one wishes to use minimum or maximum age constraints without being absolutely certain that these are correct.

5. Step 3: choosing a relaxed clock method

When choosing a relaxed clock dating method and software, both practical and theoretical considerations are important. From a practical point of view, contemporary dating methods fall into two general categories: post-tree dating methods (i.e., methods that take a reconstructed phylogram as input and then stretch it based on a clock model and set of calibrations) and simultaneous tree reconstruction and dating methods (i.e., methods that take an alignment of sequences as input and test ultrametric trees as the search goes). The former tend to be very fast, while the latter, usually implemented in Bayesian frameworks, tend to be much slower. This difference becomes even more pronounced as the number of taxa increases. Simultaneous tree and dating methods have, however, several advantages: confidence intervals on estimated ages are obtained at the same time as mean or point estimates; the tree need not be fixed and phylogenetic uncertainty is taken into account when estimating divergence times (to the extent that Bayesian methods can measure phylogenetic uncertainty); and these methods are, in general, more transparent and flexible in the relaxed clock model implemented.

From a theoretical point of view, a central question is to choose between an *autocorrelated* and an *uncorrelated* relaxed clock model. Autocorrelated clocks assume that the rates of substitution between parents and descendants are correlated (but still vary), while uncorrelated clocks do not make this assumption. The former appear to be biologically

Fig. 1. Aspects importants de la calibration fossile. Dans cet exemple hypothétique, A, B, C, D et E sont cinq taxons actuels, dont les relations phylogénétiques sont supposées connues, et la croix représente un taxon fossile. Le nœud calibré (généralement sous la forme d'une contrainte d'âge minimum) est indiqué par un disque. A. Incertitude phylogénétique : le point exact de divergence du fossile sur la phylogénie des taxons actuels est souvent incertain. Dans ce cas, le nœud que l'on calibre est généralement l'ancêtre commun le plus récent de toutes les positions possibles. Pour une approche basée sur le partage d'apomorphie, cela revient à calibrer le nœud de divergence du clade actuel (portant l'apomorphie) avec son groupe-frère. B. Incertitude d'échantillonnage : le temps écoulé entre la divergence du fossile de l'arbre des taxons actuels et son occurrence la plus ancienne est souvent incertain. C'est la raison pour laquelle la plupart des fossiles ne peuvent être utilisés que sous la forme de contraintes d'âge minimum. C. Incertitude d'âge absolu : l'âge exact du fossile est souvent inconnu dans un intervalle stratigraphique donné. Dans ce cas, il est fréquent d'utiliser la limite supérieure de cet intervalle comme contrainte d'âge minimum. Cependant, l'âge absolu de cette limite dépend de l'échelle de temps géologique de référence.

sound in some cases, while the latter allow for significant jumps in rates over the tree, which may also be biologically reasonable in some instances (for a review of this topic, see Ho, 2009). However, it is important to note that even in uncorrelated clocks, rates over the tree are not completely independent as they are drawn from a prior distribution set by the user. A situation with no specification of how rates across the tree relate to one another (i.e., with a complete dissociation of branch lengths and time) would lead to an intractable problem with an infinite number of solutions. In practice, it is a good idea to try out different relaxed clocks and compare results. In addition, most Bayesian dating frameworks now allow users to test a variety of relaxed clocks, compare them, and choose the one with the best fit to the data (Lepage et al., 2007; Li and Drummond, 2012; Ronquist et al., 2012b). Finally, there is now clear evidence that the inference of molecular rate variation across the tree can be influenced by the number and distribution of fossil calibrations (Meredith et al., 2011; Sauquet et al., 2012).

These issues and the methods available have been extensively reviewed (Rutschmann, 2006; Sanderson et al., 2004; Welch and Bromham, 2005). In practice, r8s and BEAST (see below) have been the most popular dating programs recently, but a growing number of interesting alternatives are now available and might quickly become common practice in the field. These include PhyloBayes (Lartillot et al., 2009), the MCMCTree function of recent versions of PAML (Yang, 2007), treePL (Smith and O'Meara, 2012), and MrBayes 3.2 (Ronquist et al., 2012b).

6. Step 4: performing the analysis

In this section, I outline a few technical aspects one might not have anticipated when setting up an analysis. For more practical details, I encourage the reader to follow the tutorials and explore the manuals, which are very well documented in both cases. I have chosen r8s and BEAST because of their popularity and my previous experience with both programs. However, I would also recommend the user to try MrBayes, which now implements interesting alternatives to the relaxed clock models offered by BEAST (Ronquist et al., 2012a, 2012b).

6.1. Molecular dating with r8s (penalized likelihood)

The program r8s (http://loco.biosci.arizona.edu/r8s/) is a post-tree dating software with autocorrelated relaxed clock models. r8s was originally written by Sanderson (1997) to implement nonparametric rate smoothing (NPRS), an autocorrelated relaxed clock. NPRS was very popular in the early 2000s and has been used in many molecular dating studies, but has since been replaced by *penalized likelihood* (PL), another autocorrelated relaxed clock (Sanderson, 2002, 2003). Penalized likelihood is an interesting trade-off between a strict molecular clock and an entirely uncorrelated clock, with a smoothing value determining the level of autocorrelation. The optimal smoothing value depends on each data set and is typically determined during a cross-validation step prior to the estimation of divergence times. Cross-validation can take a few minutes to a few hours to complete, depending on the number of taxa, whereas divergence time estimation is virtually instantaneous. For very large trees (e.g., 10,000 taxa), a modified, much faster version of PL is now available in the program treePL (Smith and O'Meara, 2012).

The input file is a NEXUS file where a phylogram in Newick format has been pasted from a previous phylogenetic analysis. The choice of a particular phylogram for the dating analysis can be important. The best-scoring tree from a maximum likelihood analysis such as RAxML (Stamatakis, 2006) is an option. The majority-rule consensus tree from a Bayesian analysis such as MrBayes is another one, but polytomies can have a confounding effect on accurate divergence time estimation. Finally, it is not recommended to use a maximum parsimony phylogram as the input tree because parsimony branch lengths typically require additional assumptions. If a maximum parsimony topology is still chosen, branch lengths can be optimized according to an explicit model of molecular evolution in software such as PAUP* (Swofford, 2002). It is important that the input phylogram is rooted correctly and that the most external outgroup lineage is pruned prior to the analysis (either by manually editing the phylogram or by using the prune command of r8s). This is because phylogenetic reconstruction programs work on unrooted trees and are not able to split the outgroup branch length correctly without additional information. In addition, zero-length (or near-zero-length) terminal branches must be pruned prior to the analysis (prune command) and the internal ones must be collapsed (collapse command). Calibrations are set easily in the NEXUS file with the constrain command either in the form of minimum or maximum age constraints. The nodes for calibration must have been previously defined with the mrca command by providing at least two terminal taxa, of which the most recent common ancestor is the node of interest.

One important shortcoming of r8s is that it does not provide confidence intervals on estimated ages, but instead point estimates. However, it is quite easy to obtain confidence intervals by running the analysis on a collection of bootstrapped trees, varying in branch length but not topology. This collection must be generated in another program such as RAxML or PAUP, with the molecular sequence data and the fixed tree topology as input.

6.2. Molecular dating with BEAST (uncorrelated lognormal clock)

The program BEAST (http://beast.bio.ed.ac.uk/) is a simultaneous tree reconstruction and dating software with uncorrelated relaxed clock models. BEAST allows a wide diversity of analyses on rooted timetrees in a Bayesian framework (Drummond and Rambaut, 2007; Drummond et al., 2012). Although BEAST can also be used to reconstruct phylogenies, it became popular for molecular dating due to its implementation of the *uncorrelated lognormal* (UCLN) relaxed clock proposed by Drummond et al. (2006). At the time, the UCLN offered a unique uncorrelated alternative to the surge of autocorrelated relaxed clocks, although uncorrelated clocks are now also available in other packages (e.g.,

PhyloBayes and MCMCTree; Lartillot et al., 2009; Yang, 2007).

Running BEAST involves several steps, each requiring a different program (but all are distributed as part of the same package). First, the user configures the analysis in a graphical interface called BEAUTi. The input is a molecular sequence alignment. The user is invited to set up data partitions, assign a specific model of substitution to each partition, specify calibrations in the form of prior distributions, and adjust various parameters of the MCMC analysis. The output of BEAUTi is an XML file (very different from a NEXUS file), which is the input for BEAST and can also be edited by hand for further adjustments. Then, the Bayesian analysis is run in BEAST and can take a long time to complete (a few hours up to a few months), depending on the number of taxa, the size of the alignment and number of partitions, the number of generations of the MCMC chain, and the power of your computer. For this reason, many users tend to run BEAST on a multicore server or a remote cluster facility such as CIPRES (Miller et al., 2010). As with other Bayesian phylogenetic software, it is strongly recommended to run and combine multiple independent searches and it is also necessary to check for convergence, for instance by using Tracer (Rambaut and Drummond, 2007). The main outputs of BEAST are a parameter log file (useful, in particular, to check convergence) and a tree file. Log and tree files from multiple runs can be combined and resampled using LogCombiner. Finally, TreeAnnotator is used to summarize the sample of post-burnin trees (chronograms) as a single tree (chronogram) annotated with various relevant statistics, such as mean age and 95% confidence intervals for each node, branch support in the form of posterior probabilities, and estimated rates of substitution for each branch. These metadata can typically be read by a tree viewer such as FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

In spite of the excellent tutorials and manual, the user if often faced with difficult choices, partly because BEAST can perform so many different analyses. First, BEAST does not require an outgroup to be specified and can potentially root the trees simply based on the pattern of branch lengths (i.e., some rooted trees have a better likelihood than others). However, it is possible to specify an outgroup by defining the ingroup as a taxon and constraining this taxon to be monophyletic (easily done in BEAUTi). Second, the fact that BEAST reconstructs the phylogeny at the same time as it estimates divergence times can be an advantage as much as a problem. It is clearly an advantage because, unlike r8s, BEAST accounts for phylogenetic uncertainty in divergence time estimation. It can be a problem because calibration, as outlined earlier, is conditional on a given phylogeny. Indeed, the exact position of fossils may depend on the relationships among extant taxa. A solution is to calibrate only nodes with very strong support in previous phylogenetic analyses and to check, after the BEAST analysis, that these nodes are still receiving strong support, which is generally the case. An alternative is to enforce the presence of the calibrating node by constraining the corresponding group to be monophyletic, or to specify a stem rather than a crown node calibration if appropriate (e.g., if a fossil can be justified to be more closely related to a clade than any other taxon in the phylogeny, but the sister group of this clade is uncertain). Third, the user must choose and parameterize a prior distribution for each calibration, a difficult decision that should always be justified in the methods (see above). Fourth, BEAST requires a tree prior to be set, which will affect the likelihood of the distribution of the nodes across the tree. Typically, for analyses above the population level, a simple birth process (Yule) prior has been used in most molecular dating analyses, although a birth-and-death prior is also an option that has been used by various authors (e.g., Nagalingum et al., 2011). This is a difficult choice because a constant speciation and extinction rate, assumed in both priors, may not be realistic for some analyses, especially at large taxonomic scales (Alfaro et al., 2009). Although this is an area of current concern, especially among the diversification rate community, it has been argued that this choice may not have a strong impact on the analysis after all (Couvreur et al., 2010).

Finally, an important decision must be made when choosing the topology and node ages to be represented in the summary tree generated by TreeAnnotator. Contrary to MrBayes, the philosophy of BEAST is to choose a single tree sampled from the prior rather than build a consensus. The most commonly used rule for selecting a particular tree is to choose the one with the highest product of clade posterior probabilities, termed the *maximum clade credibility* tree. However, even when choosing this tree for topology, one still has to decide whether to set its node ages to the exact ages in the sampled tree or to the mean or median ages of the nodes calculated across the posterior collection of trees. This decision will depend on the context and have a different impact, whether the tree is only plotted (e.g., for a figure) or it is used in post-dating analyses (e.g., diversification rate or ancestral state reconstruction). Median ages are generally more appropriate than mean ages because estimates of divergence times are lognormally distributed (Morrison, 2008).

7. Step 5: interpreting the results

Once an absolute timescale has been obtained through a molecular dating analysis, a few important rules are to bear in mind when interpreting and comparing the estimated ages to other studies or to other sources of evidence.

7.1. Confidence intervals

It is essential to always report confidence intervals on estimated ages, rather than mean ages or point estimates obtained from a single analysis. This is important because molecular dating is not precise, even when calibrated with multiple fossils (Sauquet et al., 2012; Yang and Rannala, 2006). Reporting mean estimates to two decimal places without confidence intervals may lead to the impression of illusory precision, which is sometimes misinterpreted by readers not familiar with the field. Likewise, it would be better to compare confidence intervals on the estimated ages of a given node, when comparing different studies or different methods. If confidence intervals do not overlap, then a significant contradiction may be concluded among

two analyses, a conclusion that is harder to reach when only mean ages differ.

7.2. Comparing different methods

When comparing results obtained with different methods, for instance different relaxed clocks, it is important to remember the assumptions of each method. In particular, it remains difficult, if not impossible, to assess whether an autocorrelated or an uncorrelated relaxed clock model better suits a given phylogeny. Bayes Factor comparison is a possibility to perform this assessment in some Bayesian frameworks that allow different models to be tested on the same data (e.g., BEAST, MrBayes, or PhyloBayes). However, the computation of Bayes Factors in itself is an area of current active research that has probably not reached maturity yet (Baele et al., 2013; Xie et al., 2011). In addition, fossil calibrations appear to have a strong impact on best-fit relaxed clock model selection and it is possible that different relaxed clocks best fit different parts of the tree (Ronquist et al., 2012a).

7.3. Comparing estimated ages with previous studies

When comparing a molecular dating analysis to previous studies, an important, potentially overlooked rule is to compare the same nodes. Indeed, many researchers have an interest in estimating the crown age of a particular taxon, and it is such ages that are commonly reported in tables of molecular dating papers. However, whether different studies are in fact reporting the age of the same node for a given taxon is highly conditional on taxonomic sampling. Studies focused on a particular taxon will often have included a wide, nonrandom sample of taxa designed to include all of the potentially basal lineages in the phylogeny, and therefore are likely to have the actual crown node of this taxon represented in their study. However, in large-scale studies (e.g., all flowering plants) it is common that the basalmost lineage of a taxon (e.g., an order) has not been included in the sample and therefore that the crown-group age reported for this taxon is in fact that of a subclade (Magallón and Castillo, 2009).

An additional source potentially interfering with direct comparisons of absolute ages obtained in different studies is the variation through time of the geological time scales themselves. As outlined earlier, if the latest time scale has been used in an analysis to convert stratigraphic ages into absolute ages for calibration, it is possible that the same analysis with the same fossils but a different time scale would have led to different results. However, this difference is likely to be thin in comparison to the wide confidence intervals generally obtained and may thus not be so problematic in practice.

7.4. Comparing estimated ages with the fossil record

A frequent problem with molecular dating results is that they often seem to contradict the fossil record. This contradiction can take two forms. Either the estimated crown-group age of a clade is much older than its oldest known fossils, as was found in many studies of angiosperms (Bell et al., 2005; Magallón, 2010; Magallón and Sanderson, 2005; Smith et al., 2010) or bilaterian animals (Douzery et al., 2004; Peterson et al., 2004), or the estimated ages, usually for subclades of the ingroup, are much younger than the fossil record suggests. These discrepancies have led to a large body of debate and discussion in the literature, which have pointed at the limitations of both the fossil record and molecular dating methods (Donoghue and Benton, 2007; Graur and Martin, 2004; Pulquério and Nichols, 2007). First, it should be stated that such differences can be very interesting as a stimulus for further discovery and methodological improvement if one accepts the limitations of both approaches. Much older molecular ages may indicate that a clade has remained very low in standing diversity for a long time in its early history or was living in an environment that did not favor fossilization. Much younger molecular ages may, on the other hand, signify a failure of the relaxed clock model to represent accurately molecular rate variation without including more fossil calibrations, or that the presumed older fossils (not included as calibrations) belong instead to different clades or represent extinct stem lineages of the clade they were thought to be nested in. Either way, it is important to state that if one is very confident in the fossil record and not ready to accept a contradiction from molecular dating, then one should include the information from these fossils as additional age constraints in the analysis. For this reason, molecular dating should perhaps be named *molecular and paleontological dating* instead, better reflecting its truly hybrid nature, and any discrepancies between molecular and fossil ages should be regarded as a conflict between the fossils used for calibration and the rest of the fossil record. However, other sources of variation in molecular dates, such as substitution and relaxed clock models, may also contribute to these discrepancies. Finally, a recurrent theme and argument in these comparisons is that molecular ages are always underestimated and should only be taken as minimum ages, since the fossils only provide minimum ages. While this was true of earlier dating analyses using fixed calibration points, it would be incorrect to generalize this to contemporary molecular dating practice because, as stated earlier, some form of maximum age constraint is almost always needed in the analysis (either in the form of a hard maximum constraint, or in the form of priors with a diminishing tail of probability towards older ages).

8. Dating a clade without molecular dating: options and limits

Depending on the reasons why one might want an age estimate for a node of interest, there are alternative options to running a new molecular dating analysis. The first one, already discussed earlier, is paleontological dating (for a review, see Laurin, 2012). Interestingly, the widespread use of fossils in molecular dating studies has been an incentive for developing new statistical models for the fossil record (Didier et al., 2012; Marshall, 2008; Tavaré et al., 2002), which should further improve paleontological dating on its own. However, these alternatives are only relevant for fossil-rich taxa or analyses at very broad taxonomic scales. An interesting resource for doing

this with vertebrates is the online Date-a-Clade service (http://www.fossilrecord.net/dateaclade/). The Paleobiology Database (http://paleodb.org/) may also be regarded as a helpful resource to explore the fossil record of a clade, however it was not designed for this purpose nor as a source of calibrations and great caution should be taken with the taxonomic assignments provided in the database.

The second option, used by some researchers, is to use an online service that compiles ages estimated in previous molecular dating analyses. One of the most visible such services is the TimeTree database (http://www.timetree.org/; Hedges et al., 2006; Kumar and Hedges, 2011), which allows the user to enter two taxa and obtain a divergence time estimate, calculated as the mean of all mean estimates obtained for this node in published dated trees that are in the database. While such a service can be useful for providing a rough indication of how old a split is, the ages provided should be taken with great care for several reasons: the confidence intervals are not reported nor taken into account; not all relevant studies are included in the database; the studies included vary widely in quality, methods, calibrations, and their results may not be comparable (see above). An interesting experimental alternative is the DateLife service (http://datelife.org/), but the source database of timetrees is still in construction.

The third option is to use a program that combines a tree without branch lengths (such as a supertree) and a set of estimated ages for specific nodes obtained in a previous analysis, and outputs an ultrametric tree by distributing evenly the nodes of unknown age between two dated nodes. This is implemented in the bladj function of the Phylocom program (Webb et al., 2008), often in conjunction with the Phylomatic service (http://phylodiversity.net/phylomatic/) to provide the supertree for a given list of taxa, and has been used extensively by evolutionary ecologists (especially for studying the phylogenetic assembly of plant communities). This is an interesting and time-saving alternative to obtain approximate branch times and use them in further analyses, in comparison to the much longer assembly of original data and their molecular dating analysis. Nevertheless, users should bear in mind the many limitations and potential biases of such an approach, in particular the lack of a real model to extrapolate branch times in undated parts of the tree. In addition, particular caution should be paid to the source used for the dated nodes. Indeed, by default Phylocom comes with a table of ages from the Wikström et al. (2001) molecular dating study of angiosperms, which is now clearly regarded as outdated and incorrect. Thus, when using such fast alternatives to real molecular dating, the user should make sure to use the latest reliable dating study of the clade considered as a source for the dated nodes (e.g., for angiosperms use Magallón and Castillo, 2009).

9. The future of molecular dating

An important area of future improvement of the molecular dating methodology will continue to focus on new models of relaxed clocks and their relative fits to the data (Guindon, 2013; Li and Drummond, 2012; Paradis, 2013). In addition, exciting new pipelines and platforms are being developed that will eventually allow nonspecialist users to obtain comparable divergence time estimates for groups of interest in a much more satisfying and robust way than some of the alternative approaches discussed above (e.g., the SUPERSMART project: http://www.supersmart-project.org/; A. Antonelli et al., in prep.). However, two other particular significant advances are being made now that have the potential of profoundly changing the field of molecular dating in the next few years. Both are related to calibration and presented briefly below.

9.1. Improving calibration practices

Given the recently identified critical role of calibration and yet the presently very poor state of its practice and justification, it is not surprising that several different papers have independently come to the same conclusion that an improvement of this aspect of the field is urgently needed (Gandolfo et al., 2008; Parham et al., 2012; Sauquet et al., 2012). Although each of these papers has proposed guidelines for this improvement, centered around a much more extensive and explicit documentation of the calibrations, one group has gone further by also creating a global Fossil Calibration database (Ksepka et al., 2011). This initiative is particularly interesting because it is associated with a new publication model, whereby articles focused entirely on justifying calibrations will be submitted to a new Fossil Calibrations section of the open access journal Paleontologia Electronica, peer-reviewed, and linked to the entries in the database.

9.2. Total evidence dating

Since fossils can be included as terminal taxa in phylogenetic analyses, they could in principle be included as terminal taxa in molecular dating analyses too, as long as morphological data are combined with molecular data to inform the fossil relationships. Thus, instead of being taken into account in the form of age constraints on nodes of the extant phylogeny, fossils would be part of the whole analysis, without prior hypotheses on their relationships, and with the age of the fossils themselves effectively calibrating and constraining the analysis. This approach, termed *total evidence dating* (in contrast with traditional node-calibration dating), has recently been tested for the first time in two different Bayesian frameworks. Pyron (2011) implemented it in BEAST to test the temporal origin of Lissamphibia, while Ronquist et al. (2012a) used MrBayes to obtain divergence times in Hymenoptera. More recently, the approach was also used in BEAST for spiders (Wood et al., 2013) and many more examples are likely to come soon. In addition, Lee et al. (2009) implemented an earlier, two-step version of this approach in a case study on lizards. Total evidence dating is particularly attractive because it allows to explicitly take fossil phylogenetic placement uncertainty into account in divergence time estimation. In addition, this approach enforces the phylogenetic analysis of fossils, which is widely recognized as desirable but easily bypassed in traditional calibration

analyses, and because the age of the fossils is taken into account in this phylogenetic analysis, it may potentially lead to more accurate or realistic results than traditional, time-free total evidence analysis (e.g., a very old fossil is unlikely to be sister to an extant terminal taxon). However, the approach is not straightforward. It requires a morphological matrix of extant and fossil taxa in addition to the molecular data set of extant taxa, its proper implementation requires many tests and adjustments (Ronquist et al., 2012a), and future methodological improvements are expected. In particular, an area that will need special attention is the modeling of morphological evolution and the level of correlation between morphological and molecular change. In both BEAST and MrBayes, the models used so far are reversible Markov models (Lewis, 2001), which assume that rates of morphological evolution are constant through time and across lineages.

10. Conclusions

In this paper, I have emphasized that molecular dating is not trivial if one wants to obtain meaningful ages. The key to good practice is, in my opinion, to be aware of the assumptions and limitations of each methodological choice, and, perhaps even more importantly, to be explicit at each step. This is particularly true for calibration, which has a very strong impact on estimated absolute ages. The assembly of large morphological data sets and the systematic phylogenetic analysis of fossil relationships will undoubtedly improve calibration quality. For groups without a fossil record, geological or secondary calibration are alternatives but results are to be interpreted even more carefully. In some cases, it might be better not to try estimating absolute divergence times at all and instead rely on a relative (uncalibrated) molecular time scale or on nonultrametric trees (with branch lengths proportional to molecular change). Finally, it is often better to obtain a very wide confidence interval on the age of a clade, reflecting the many sources of uncertainty inherent to molecular dating, than to obtain an arbitrarily precise age or narrow interval, or than not having an age estimate at all.

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References

Alfaro, M.E., Santini, F., Brock, C., Alamillo, H., Dornburg, A., Rabosky, D.L., Carnevale, G., Harmon, L.J., 2009. Nine exceptional radiations plus high turnover explain species diversity in jawed vertebrates. Proc. Natl Acad. Sci. U. S. A. 106, 13410–13414.

Baele, G., Li, W.L.S., Drummond, A.J., Suchard, M.A., Lemey, P., 2013. Accurate model selection of relaxed molecular clocks in Bayesian phylogenetics. Mol. Biol. Evol. 30, 239–243.

Bell, C.D., Soltis, D.E., Soltis, P.S., 2005. The age of the angiosperms: a molecular timescale without a clock. Evolution 59, 1245–1258.

Benton, M.J., Donoghue, P.C.J., 2007. Paleontological evidence to date the Tree of Life. Mol. Biol. Evol. 24, 26–53.

Brandley, M.C., Wang, Y., Guo, X., Montes de Oca, A.N., Feria-Ortiz, M., Hikida, T., Ota, H., 2011. Accommodating heterogenous rates of evolution in molecular divergence dating methods: an example using intercontinental dispersal of *Plestiodon* (Eumeces) *lizards*. Syst. Biol. 60, 3–15.

Cardinal, S., Danforth, B.N., 2013. Bees diversified in the age of eudicots. Proc. R. Soc. Lond. B Biol. Sci., 280.

Couvreur, T.L.P., Franzke, A., Al-Shehbaz, I.A., Bakker, F.T., Koch, M.A., Mummenhoff, K., 2010. Molecular phylogenetics, temporal diversification, and principles of evolution in the mustard family (Brassicaceae). Mol. Biol. Evol. 27, 55–71.

Crisp, M.D., Trewick, S.A., Cook, L.G., 2011. Hypothesis testing in biogeography. Trends Ecol. Evol. 26, 66–72.

Cruaud, A., Rønsted, N., Chantarasuwan, B., Chou, L.S., Clement, W.L., Couloux, A., Cousins, B., Genson, G., Harrison, R.D., Hanson, P.E., Hossaert-Mckey, M., Jabbour-Zahab, R., Jousselin, E., Kerdelhué, C., Kjellberg, F., Lopez-Vaamonde, C., Peebles, J., Peng, Y.-Q., Pereira, R.A.S., Schramm, T., Ubaidillah, R., Van Noort, S., Weiblen, G.D., Yang, D.-R., Yodpinyanee, A., Libeskind-Hadas, R., Cook, J.M., Rasplus, J.-Y., Savolainen, V., 2012. An extreme case of plant–insect codiversification: figs and fig-pollinating wasps. Syst. Biol. 61, 1029–1047.

Didier, G., Royer-carenzi, M., Laurin, M., 2012. The reconstructed evolutionary process with the fossil record. J. Theor. Biol. 315, 26–37.

Donoghue, P.C.J., Benton, M.J., 2007. Rocks and clocks: calibrating the Tree of Life using fossils and molecules. Trends Ecol. Evol. 22, 424–431.

Douzery, E.J.P., Snell, E., a Bapteste, E., Delsuc, F., Philippe, H., 2004. The timing of eukaryotic evolution: does a relaxed molecular clock reconcile proteins and fossils? Proc. Natl Acad. Sci. U. S. A. 101, 15386–15391.

Doyle, J.A., Donoghue, M.J., 1993. Phylogenies and angiosperm diversification. Paleobiology 19, 141–167.

Drummond, A.J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol. Biol. 7, 214.

Drummond, A.J., Ho, S.Y.W., Phillips, M.J., Rambaut, A., 2006. Relaxed phylogenetics and dating with confidence. PLoS Biol. 4, e88.

Drummond, A.J., Suchard, M.A., Xie, D., Rambaut, A., 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol. Biol. Evol. 29, 1969–1973.

FitzJohn, R.G., Maddison, W.P., Otto, S.P., 2009. Estimating trait-dependent speciation and extinction rates from incompletely resolved phylogenies. Syst. Biol. 58, 595–611.

Forest, F., 2009. Calibrating the Tree of Life: fossils, molecules and evolutionary timescales. Ann. Bot. (Lond) 104, 789–794.

Gandolfo, M.A., Nixon, K.C., Crepet, W.L., 2008. Selection of fossils for calibration of molecular dating models. Ann. Missouri Botanical Garden 95, 34–42.

Gilbert, M.T.P., Rambaut, A., Wlasiuk, G., Spira, T.J., Pitchenik, A.E., Worobey, M., 2007. The emergence of HIV/AIDS in the Americas and beyond. Proc. Natl Acad. Sci. U. S. A. 104, 18566–21857.

Goodall-Copestake, W.P., Harris, D.J., Hollingsworth, P.M., 2009. The origin of a mega-diverse genus: dating *Begonia* (Begoniaceae) using alternative datasets, calibrations and relaxed clock methods. Botanical J. Linnean Society 159, 363–380.

Graur, D., Martin, W., 2004. Reading the entrails of chickens: molecular timescales of evolution and the illusion of precision. Trends Genet. 20, 80–86.

Guindon, S., 2013. From trajectories to averages: an improved description of the heterogeneity of substitution rates along lineages. Syst. Biol. 62, 22–34.

Heath, T.A., 2012. A hierarchical Bayesian model for calibrating estimates of species divergence times. Syst. Biol. 61, 793–809.

Hedges, S.B., Dudley, J., Kumar, S., 2006. TimeTree: a public knowledge-base of divergence times among organisms. Bioinformatics 22, 2971–2972.

Hermsen, E.J., Hendricks, J.R., 2008. W(h)ither fossils? Studying morphological character evolution in the age of molecular sequences. Ann. Missouri Botanical Garden 95, 72–100.

Ho, S.Y.W., 2007. Calibrating molecular estimates of substitution rates and divergence times in birds. J. Avian Biol. 38, 409–414.

Ho, S.Y.W., 2009. An examination of phylogenetic models of substitution rate variation among lineages. Biol. Lett. 5, 421–424.

Ho, S.Y.W., Lo, N., 2013. The insect molecular clock. Aust. J. Entomol. 52, 101–105.

Ho, S.Y.W., Phillips, M.J., 2009. Accounting for calibration uncertainty in phylogenetic estimation of evolutionary divergence times. Syst. Biol. 58, 367–380.

Ho, S.Y.W., Saarma, U., Barnett, R., Haile, J., Shapiro, B., 2008. The effect of inappropriate calibration: three case studies in molecular ecology. PLoS One 3, e1615.

Inoue, J., Donoghue, P.C.J., Yang, Z., 2010. The impact of the representation of fossil calibrations on Bayesian estimation of species divergence times. Syst. Biol. 59, 74–89.
Ksepka, D.T., Benton, M.J., Carrano, M.T., Gandolfo, M., a Head, J.J., Hermsen, E.J., Joyce, W.G., Lamm, K.S., Patané, J.S.L., Phillips, M.J., Polly, P.D., Van Tuinen, M., Ware, J.L., Warnock, R.C.M., Parham, J.F., 2011. Synthesizing and databasing fossil calibrations: divergence dating and beyond. Biol. Lett. 7, 801–803.
Kumar, S., 2005. Molecular clocks: four decades of evolution. Nat. Rev. Genet. 6, 654–662.
Kumar, S., Hedges, S.B., 2011. TimeTree2: species divergence times on the iPhone. Bioinformatics 27, 2023–2024.
Lartillot, N., Lepage, T., Blanquart, S., 2009. PhyloBayes 3: a Bayesian software package for phylogenetic reconstruction and molecular dating. Bioinformatics 25, 2286–2288.
Laurin, M., 2012. Recent progress in paleontological methods for dating the Tree of Life. Front. Genet. 3, 130.
Lee, M.S.Y., Oliver, P.M., Hutchinson, M.N., 2009. Phylogenetic uncertainty and molecular clock calibrations: a case study of legless lizards (Pygopodidae, Gekkota). Mol. Phylogenet. Evol. 50, 661–666.
Lemmon, A.R., Brown, J.M., Stanger-Hall, K., Lemmon, E.M., 2009. The effect of ambiguous data on phylogenetic estimates obtained by maximum likelihood and Bayesian inference. Syst. Biol. 58, 130–145.
Lepage, T., Bryant, D., Philippe, H., Lartillot, N., 2007. A general comparison of relaxed molecular clock models. Mol. Biol. Evol. 24, 2669–2680.
Lewis, P.O., 2001. A likelihood approach to estimating phylogeny from discrete morphological character data. Syst. Biol. 50, 913–925.
Li, W.L.S., Drummond, A.J., 2012. Model averaging and Bayes Factor calculation of relaxed molecular clocks in Bayesian phylogenetics. Mol. Biol. Evol. 29, 751–761.
Linder, H.P., 2003. The radiation of the Cape flora, southern Africa. Biol. Rev. 78, 597–638.
Magallón, S.A., 2004. Dating lineages: molecular and paleontological approaches to the temporal framework of clades. Int. J. Plant Sci. 165, S7–S21.
Magallón, S., 2010. Using fossils to break long branches in molecular dating: a comparison of relaxed clocks applied to the origin of angiosperms. Syst. Biol. 59, 384–399.
Magallón, S., Castillo, A., 2009. Angiosperm diversification through time. Am. J. Bot. 96, 349–365.
Magallón, S., Sanderson, M.J., 2001. Absolute diversification rates in angiosperm clades. Evolution 55, 1762–1780.
Magallón, S.A., Sanderson, M.J., 2005. Angiosperm divergence times: the effect of genes, codon positions, and time constraints. Evolution 59, 1653–1670.
Magallón, S., Hilu, K.W., Quandt, D., 2013. Land plant evolutionary timeline: gene effects are secondary to fossil constraints in relaxed clock estimation of age and substitution rates. Am. J. Bot. 100, 556–573.
Marjanović, D., Laurin, M., 2007. Fossils, molecules, divergence times, and the origin of lissamphibians. Syst. Biol. 56, 369–388.
Marshall, C.R., 2008. A simple method for bracketing absolute divergence times on molecular phylogenies using multiple fossil calibration points. Am. Nat. 171, 726–742.
Meredith, R.W., Janečka, J.E., Gatesy, J., Ryder, O.A., Fisher, C.A., Teeling, E.C., Goodbla, A., Eizirik, E., Simão, T.L.L., Stadler, T., Rabosky, D.L., Honeycutt, R.L., Flynn, J.J., Ingram, C.M., Steiner, C., Williams, T.L., Robinson, T.J., Burk-Herrick, A., Westerman, M., Ayoub, N.A., Springer, M.S., Murphy, W.J., 2011. Impacts of the Cretaceous terrestrial revolution and KPg extinction on mammal diversification. Science 334, 521–524.
Miller, M.A., Pfeiffer, W., Schwartz, T., 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. Proceedings of the Gateway Computing Environments Workshop (GCE) 14 Nov. 20, 1–8.
Morlon, H., Parsons, T.L., Plotkin, J.B., 2011. Reconciling molecular phylogenies with the fossil record. Proc. Natl Acad. Sci. U. S. A. 108, 16327–21633.
Morrison, D.A., 2008. How to summarize estimates of ancestral divergence times. Evol. Bioinf. 4, 75–95.
Nagalingum, N.S., Marshall, C.R., Quental, T.B., Rai, H.S., Little, D.P., Mathews, S., 2011. Recent synchronous radiation of a living fossil. Science 334, 796–799.
O'Meara, B.C., 2012. Evolutionary inferences from phylogenies: a review of methods. Annu. Rev. Ecol. Evo. Syst. 43, 267–285.
Pagel, M., 1999. Inferring the historical patterns of biological evolution. Nature 401, 877–884.
Paradis, E., 2013. Molecular dating of phylogenies by likelihood methods: a comparison of models and a new information criterion. Mol. Phylogenet. Evol. 67, 436–444.
Parham, J.F., Donoghue, P.C.J., Bell, C.J., Calway, T.D., Head, J.J., Holroyd, P.A., Inoue, J.G., Irmis, R.B., Joyce, W.G., Ksepka, D.T., Patané, J.S.L., Smith, N.D., Tarver, J.E., Van Tuinen, M., Yang, Z., Angielczyk, K.D., Greenwood, J.M., Hipsley, C.A., Jacobs, L., Makovicky, P.J., Müller, J., Smith, K.T., Theodor, J.M., Warnock, R.C.M., Benton, M.J., 2012. Best practices for justifying fossil calibrations. Syst. Biol. 61, 346–359.
Peterson, K.J., Lyons, J.B., Nowak, K.S., Takacs, C.M., Wargo, M.J., McPeek, M.A., 2004. Estimating metazoan divergence times with a molecular clock. Proc. Natl Acad. Sci. U. S. A. 101, 6536–6541.
Phillips, M.J., 2009. Branch-length estimation bias misleads molecular dating for a vertebrate mitochondrial phylogeny. Gene 441, 132–140.
Pulquério, M.J.F., Nichols, R.A., 2007. Dates from the molecular clock: how wrong can we be? Trends Ecol. Evol. 22, 180–184.
Pyron, R.A., 2011. Divergence time estimation using fossils as terminal taxa and the origins of Lissamphibia. Syst. Biol. 60, 466–481.
Rambaut, A., Drummond, A.J., 2007. Tracer, version 1.5. University of Oxford, Oxford, UK (http://tree.bio.ed.ac.uk/software/tracer/).
Ramírez, S.R., Eltz, T., Fujiwara, M.K., Gerlach, G., Goldman-huertas, B., Tsutsui, N.D., Pierce, N.E., 2011. Asynchronous diversification in a specialized plant-pollinator mutualism. Science 333, 1742–1746.
Ree, R.H., Smith, S.A., 2008. Maximum likelihood inference of geographic range evolution by dispersal, local extinction, and cladogenesis. Syst. Biol. 57, 4–14.
Renner, S., 2005. Relaxed molecular clocks for dating historical plant dispersal events. Trends Plant Sci. 10, 550–558.
Ronquist, F., Klopfstein, S., Vilhelmsen, L., Schulmeister, S., Murray, D.L., Rasnitsyn, A.P., 2012a. A total-evidence approach to dating with fossils, applied to the early radiation of the Hymenoptera. Syst. Biol. 61, 973–999.
Ronquist, F., Teslenko, M., Van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012b. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61, 539–542.
Roure, B., Baurain, D., Philippe, H., 2013. Impact of missing data on phylogenies inferred from empirical phylogenomic data sets. Mol. Biol. Evol. 30, 197–214.
Ruane, S., Pyron, R.A., Burbrink, F.T., 2011. Phylogenetic relationships of the Cretaceous frog Beelzebufo from Madagascar and the placement of fossil constraints based on temporal and phylogenetic evidence. J. Evol. Biol. 24, 274–285.
Rutschmann, F., 2006. Molecular dating of phylogenetic trees: a brief review of current methods that estimate divergence times. Divers. Distribut. 12, 35–48.
Rutschmann, F., Eriksson, T., Salim, K.A., Conti, E., 2007. Assessing calibration uncertainty in molecular dating: the assignment of fossils to alternative calibration points. Syst. Biol. 56, 591–608.
Sanderson, M.J., 1997. A nonparametric approach to estimating divergence times in the absence of rate constancy. Mol. Biol. Evol. 14, 1218–1231.
Sanderson, M.J., 2002. Estimating absolute rates of molecular evolution and divergence times: a penalized likelihood approach. Mol. Biol. Evol. 19, 101–109.
Sanderson, M.J., 2003. r8s: inferring absolute rates of molecular evolution and divergence times in the absence of a molecular clock. Bioinformatics 19, 301–302.
Sanderson, M.J., Thorne, J.L., Wikstrom, N., Bremer, K., 2004. Molecular evidence on plant divergence times. Am. J. Bot. 91, 1656–1665.
Sauquet, H., Ho, S.Y.W., Gandolfo, M.A., Jordan, G.J., Wilf, P., Cantrill, D.J., Bayly, M.J., Bromham, L., Brown, G.K., Carpenter, R.J., Lee, D.M., Murphy, D.J., Sniderman, J.M.K., Udovicic, F., 2012. Testing the impact of calibration on molecular divergence times using a fossil-rich group: the case of *Nothofagus* (Fagales). Syst. Biol. 61, 289–313.
Sauquet, H., Weston, P.H., Anderson, C.L., Barker, N.P., Cantrill, D.J., Mast, A.R., Savolainen, V., 2009. Contrasted patterns of hyperdiversification in Mediterranean hotspots. Proc. Natl Acad. Sci. U. S. A. 106, 221–222.
Schenk, J.J., Hufford, L., 2010. Effects of substitution models on divergence time estimates: simulations and an empirical study of model uncertainty using Cornales. Syst. Bot. 35, 578–592.
Smith, S.A., O'Meara, B.C., 2012. treePL: divergence time estimation using penalized likelihood for large phylogenies. Bioinformatics 28, 2689–2690.
Smith, S.A., Beaulieu, J.M., Donoghue, M.J., 2010. An uncorrelated relaxed-clock analysis suggests an earlier origin for flowering plants. Proc. Natl Acad. Sci. U. S. A. 107, 5897–6590.
Stadler, T., 2011. Mammalian phylogeny reveals recent diversification rate shifts. Proc. Natl Acad. Sci. U. S. A. 108, 6187–6192.
Stamatakis, A., 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22, 2688–2690.
Swofford, D.L., 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.

Tavaré, S., Marshall, C.R., Will, O., Soligo, C., Martin, R.D., 2002. Using the fossil record to estimate the age of the last common ancestor of extant primates. Nature 416, 726–729.
Webb, C.O., Ackerly, D.D., Kembel, S.W., 2008. Phylocom: software for the analysis of phylogenetic community structure and trait evolution. Bioinformatics 24, 2098–2100.
Weir, J.T., Schluter, D., 2008. Calibrating the avian molecular clock. Mol. Ecol. 17, 2321–2328.
Welch, J.J., Bromham, L., 2005. Molecular dating when rates vary. Trends Ecol. Evol. 20, 320–327.
Wiens, J.J., Morrill, M.C., 2011. Missing data in phylogenetic analysis: reconciling results from simulations and empirical data. Syst. Biol. 60, 719–731.
Wikström, N., Savolainen, V., Chase, M.W., 2001. Evolution of the angiosperms: calibrating the family tree. Proc. R. Soc. Lond. B Biol. Sci. 268, 2211–2220.
Wood, H.M., Matzke, N.J., Gillespie, R.G., Griswold, C.E., 2013. Treating fossils as terminal taxa in divergence time estimation reveals ancient vicariance patterns in the palpimanoid spiders. Syst. Biol. 62, 264–284.
Xie, W., Lewis, P.O., Fan, Y., Kuo, L., Chen, M.-H., 2011. Improving marginal likelihood estimation for Bayesian phylogenetic model selection. Syst. Biol. 60, 150–160.
Yang, Z., 2007. PAML 4: phylogenetic analysis by maximum likelihood. Mol. Biol. Evol. 24, 1586–1591.
Yang, Z., Rannala, B., 2006. Bayesian estimation of species divergence times under a molecular clock using multiple fossil calibrations with soft bounds. Mol. Biol. Evol. 23, 212–226.
Zuckerkandl, E., Pauling, L., 1962. Molecular disease, evolution, and genetic heterogeneity. In: Kasha, M., Pullman, B. (Eds.), Horizons in Biochemistry. Academic Press, New York, pp. 189–225.