The molecular clock as a tool for understanding host-parasite evolution

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Abstract

The molecular clock in combination with evidence from the geological record can be applied to infer the timing and dynamics of evolutionary events. This has enormous potential to shed light on the complex and often evasive evolution of parasites. Here, we provide an overview of molecular clock methodology and recent advances that increase the potential for the study of host-parasite coevolutionary dynamics, with a focus on Bayesian approaches to divergence time estimation. We highlight the challenges in applying these methods to the study of parasites, including the nature of parasite genomes, the incompleteness of the rock and fossil records, and the complexity of host-parasite interactions. Developments in models of molecular evolution and approaches to deriving temporal constraints from geological evidence will help overcome some of these issues. However,

we also describe a case study in which the timescale of host-parasite coevolution cannot easily be inferred using existing methods – that of the alpha-proteobacteria *Wolbachia*. We conclude by providing a prospective on future methodological developments and data collection that will facilitate in understanding the role of parasitism in deep time.

1 Introduction

A timeline for events throughout geological history is required to address many questions in evolutionary biology, including establishing the sequence of key evolutionary transitions and assessing the role of environmental and evolutionary variables on the evolution of life (Rota-Stabelli et al., 2013; Betts et al., 2018; Morris et al., 2018; Silvestro et al., 2018). Due to the paucity of rocks from different time periods and environments, and the low fossilisation potential of many soft-bodied species, we cannot rely on the fossil record to establish this timeline very precisely (Benton et al., 2009; Parham et al., 2011; Holland, 2016). The genomes of living species provide an alternative source of evidence for inferring the sequence of events in deep time, although in themselves they provide no information about absolute time. Phylogenetic methods that enable us to leverage both sources of evidence simultaneously provide the most promising approach for generating a more precise timeline and a richer view of the past than either record can provide alone (Donoghue and Benton, 2007; Heath et al., 2014; Landis, 2017; Álvarez-Carretero et al., 2019).

It is estimated that more than half of all living species are parasites (Windsor, 1998; Bass et al., 2015), that is, species that rely on a host organism for survival, at the expense of the other species. The ubiquity of the parasitic lifestyle and the often strong selection pressure imposed by parasites on their hosts (Hughes et al., 2012) indicate that parasites have played an important role in shaping the evolution of life, but the challenge in detecting many parasite species, both now and in the past, suggest that this role is not well understood (Dobson et al., 2008; De Baets and Littlewood, 2015). Estimating the time of origin of parasitism across different groups enables us to answer key questions about the role of parasites throughout evolutionary history (De Baets and Littlewood, 2015; Cruaud and Rasplus, 2016). Time calibrated trees also enable us to infer cophylogenetic histories, to estimate phylodynamic parameters, including speciation and extinction rates, and to study the impact of different traits on diversification (Martínez-Aquino, 2016; Stadler, 2013; Harmon, 2018).

The evolutionary history of parasitism is especially challenging to infer from the geological record alone because the fossil record of parasites, and to a lesser degree of their hosts, is relatively sparse (De Baets and Littlewood, 2015; Leung, 2017). Molecular dating (or molecular clock analyses) provides a powerful approach to inferring species divergence times based on the genomes of living parasites and/or host species, in combination with evidence from the geological record (De Baets and Littlewood, 2015). However, the nature of parasites presents several challenges in applying this approach. Here, we review Bayesian approaches to molecular dating, with particular emphasis on the application of these methods to understanding the evolutionary history of parasitism. We explore the challenges encountered in applying conventional molecular clock and tree models to parasites, and highlight promising areas of methodological developments.

2 The molecular clock

The molecular clock hypothesis proposes that rates of molecular evolution are more or less constant over time (Zuckerkandl and Pauling, 1962, 1965). Following species divergence, substitutions accumulate along the genomes of discrete species units. Since the majority of molecular changes are neutral (i.e. confer little or no fitness advantage to the organism), changes occur at an approximately constant rate. This means that the expected number of substitutions observed between two species is a linear function of the time since they last shared a common ancestor. If evidence exists for the age of a given species pair, the rate of molecular evolution can be calibrated and used to infer the divergence times of other species pairs.

Two issues that make the application of the molecular clock challenging to apply in reality are that (1) rates of molecular evolution are often non-constant, and (2) calibrations are rarely known very precisely. In general, these issues also become increasingly problematic further back in time, and as we go into detail in the following sections may be particularly problematic in the case of many parasites, which are soft-bodied, microscopic and reside within their hosts (Littlewood and Donovan, 2003; De Baets and Littlewood, 2015; Leung, 2017).

3 Bayesian divergence time estimation

Mechanistic models enable us to describe evolutionary processes using explicit parameters that are meaningful in the context of evolutionary biology, including rates of molecular evolution or speciation. Bayesian statistical inference provides a natural framework in which to incorporate models that describe evolutionary processes along with multiple sources of uncertainty. Using Bayes' theorem, we aim to infer the joint posterior probability of our model parameters, θ, given our observed data, D,

$$P[\theta|D] = \frac{P[D|\theta]P[\theta]}{P[D]},$$

where $P[D|\theta]$ is the probability of the data given our model parameters, also known as the likelihood, and $P[\theta]$ is the prior, incorporating prior knowledge about our model parameters. The denominator $P[D]$ is the probability of our data and can be thought of as a normalising constant, which ensures the posterior density sums to one. To calculate the likelihood we require evolutionary models that allow us to calculate the probability of observing our data for a given set of parameter values. Estimates that best explain our observed data will produce the highest probability according to our model (known as the maximum likelihood estimates in a likelihood framework). The posterior reflects the likelihood in combination with our prior belief about the parameter values. In a conventional Bayesian problem, if there is sufficient information in the data then the parameter estimates will be insensitive to the priors. Ordinarily, we cannot calculate the posterior distribution analytically, so we use a numerical sampling approach, Markov chain Monte Carlo (MCMC), to sample the posterior distribution.

In any Bayesian divergence time analysis great care must be taken to consider the underlying model assumptions, and prior parameter choices. It is important to note that molecular sequence data are only informative about *relative* divergence times. Information about *absolute* time has to come from elsewhere (i.e. information from the geological record), meaning rate and time are typically only semi-identifiable (Yang and Rannala, 2006; Rannala and Yang, 2007; dos Reis and Yang, 2013). Strong prior information is therefore needed to constrain the substitution rate and species divergence times, and the data cannot fully overcome uncertainty associated with the priors. Unlike conventional Bayesian inference problems, this means the priors exert a large influence on the posteriors and there is a limit to the precision that can be achieved using Bayesian divergence

time estimation (Inoue et al., 2009; dos Reis and Yang, 2013; Zhu et al., 2015; Warnock et al., 2017). Note that what is referred to as the prior versus the likelihood can vary among authors. Here, we consider all model components that describe the evolutionary process to be part of the likelihood.

There are three essential model components required for Bayesian molecular clock analysis:

- **Substitution model**: also known as the site model, this describes the probability of observing a substitution from one character state to another (e.g. $A \rightarrow T$, $C \rightarrow G$, $Arg \rightarrow Leu$) and how this may vary across sites or partitions in a given alignment. Equivalent models are also available that describe the evolution of morphological characters.
- **Clock model**: this describes how evolutionary rates vary across lineages or branches in the phylogeny.
- **Branching process model**: also known as the tree model, this describes the shape of the phylogeny and branch durations. Temporal evidence used to calibrate the tree to absolute time can be incorporated using priors on the node ages or directly incorporated into the tree and considered in the calculation of the likelihood. These different approaches are sometimes referred to as prior- (or node-) versus process-based dating (Landis, 2017). This component ultimately describes the distribution of speciation times, in the absence of information from the sequence alignment.

The remainder of the chapter focuses on models that can be used to estimate divergence times in a Bayesian framework and discusses evidence useful for constraining the evolution of parasites. For recent technical reviews on Bayesian divergence time estimation we recommend dos Reis et al. (2016) and Bromham et al. (2018).

4 Substitution models

4.1 Models of molecular evolution

In Bayesian phylogenetic inference and divergence time estimation, the substitution model provides the basis for measuring genetic distances among individuals or species, expressed as the *expected number of substitutions per site* and used to represent the branch lengths in an uncalibrated tree. Both the clock model and calibration information, discussed in the subsequent sections, are required

to disentangle rate and time, i.e. to calculate the rate of molecular evolution in *expected substitutions per site per unit time* and to transform the branch lengths into units of time. If the substitution model fails to capture the complexity of the underlying process this can result in the inference of both spurious branch lengths and phylogenetic relationships (Williams et al., 2013), which has obvious consequences for timescaling and interpreting divergence events.

The role of the substitution model is to characterise the way in which homologous sites in a molecular (DNA or protein) sequence alignment evolve over time. For instance, how likely are we to observe a change from an adenine to a thymine, versus an adenine to cytosine? This is described using an instantaneous rate matrix, which will include the rate of change between a given character and any other. In general, the likelihood of change is determined by both the frequency with which each state is observed in the population or across species, and the relative rate of substitution between two character states. The simplest model of molecular evolution is the Jukes-Cantor (JC) model (Jukes and Cantor, 1969), which assumes the frequency of each state (i.e. A, T, C, G) is equal and that the rate of change between each combination of character states is the same. These assumptions are unrealistic for most molecular datasets. Perhaps the most widely implemented DNA substitution model is the general time reversible (GTR) model (Tavaré, 1986), which allows for each state to be associated with a different frequency and each combination of character states to be associated with a different rate.

Standard substitution models, including the GTR model, make the assumption that rates of change are independent of both alignment position and lineage. These assumptions are mainly applied for computational efficiency and tractability, however, we know there is enormous variation in rates of molecular evolution, even for sites within the same gene. For example, the third codon position can assume a variety of states and still encode for the same amino acid, in contrast to the first and second position, where a change in state is more likely to impact the encoded protein sequence. Thus, we tend to observe substantial rate variation between codon positions. The most common strategy for incorporating rate variation among sites is to use a discretized gamma distribution (Yang, 1994), where sites are assigned to different rate categorizes. The shape of the distribution can be estimated from the sequence data during inference and the approach is both computationally efficient and effective in modelling moderate rate variation. In reality, we often observe a greater degree of rate variation among sites than we can accommodate adequately using this approach (Yang et al., 1995; Shapiro et al., 2006), and typically we tend to partition the data, such that

a different rate matrix is used to model the molecular substitution process for different groups of sites (i.e. codon position 1 versus codons positions 2 and/or 3). One challenge is to select the most optimal set of partitions, while avoiding model overparameterization. For instance, should we partition by gene and by codon? Do some genes or regulatory regions evolve at similar rates? This choice is especially challenging for genome scale datasets, where a large number of partition strategies are possible. Efficient software and algorithms have been developed for selecting among partition strategies using model testing (Lanfear et al., 2012, 2016). Alternatively, if sequences vary in base or amino acid composition across sites, this can be accommodated using a mixture model, which allows the subdivision of sites into different categories to be estimated from the alignment during tree inference (Lartillot et al., 2007).

Evolutionary forces acting on parasites result in genomes that can be very distinct to their nearest free-living relatives (Hirt et al., 1999; Chang et al., 2015; Poulin and Randhawa, 2015; Schiffer et al., 2018). Parasite genomes are often short – intracellular parasites, for instance, have among some of the smallest known eurkaryote genomes (Corradi et al., 2010). Parasite evolution is generally associated with reduction and loss of complexity: as they evolve to take advantage of the metabolic pathways of their hosts, selective constrains on parts of the genome are released, resulting in loss of function (Frank et al., 2002). However, the perception of parasites as functionally degenerate is misleading. Instead, their genomes can be viewed as extremely genetically efficient and often encode complex mechanisms for host manipulation (Poulin and Randhawa, 2015). Further, gene families associated with parasitism have been found to have undergone massive expansion, in parallel to loss of function in other areas (Brayton et al., 2001; Hunt et al., 2016).

For reasons that are not well understood, the genomes of parasites also appear to evolve faster than both their hosts and/or free-living relatives (Hafner et al., 1994; Ricklefs and Outlaw, 2010; Bromham et al., 2013) – shorter generation times, smaller effective population sizes, selection, genetic drift and high mutation rates are all potential contributors (Cruaud and Rasplus, 2016). Rapid evolutionary rates among subsets of taxa creates challenges for modeling the process of molecular evolution, estimating branch lengths and inferring phylogenetic relationships. In particular, fast evolving lineages can be erroneously grouped together – an artefact commonly known as long branch attraction (Felsenstein, 1978). Model based approaches to phylogenetics, including maximum likelihood and Bayesian inference, are better at capturing the possibility that many unobserved changes may have occurred over time and can lead to more robust results, in contrast to

more traditional tree inference methods, such as parsimony or neighbour joining. However, model based approaches can also suffer long-branch attraction and result in the wrong topology if the model is misspecified (Williams et al., 2013). Parasites are often associated with long branches, and consequently it has been challenging to establish the phylogenetic position of several groups (De Baets and Littlewood, 2015). A good example is the classification of Myxozoa, now widely accepted to be a diverse group of parasitic cnidarians, which have sometimes been recovered as sister to all other bilaterians (Evans et al., 2010; Chang et al., 2015; Okamura and Grühl, in press). Other examples include Microsporidia, a parasitic relative of fungi (Hirt et al., 1999), and parasitic annelids (Orthonectida and Dicyemida) (Schiffer et al., 2018). In all cases the use of overly simple substitution models tends to recover the wrong topology.

In addition to undergoing rapid evolution, parasite genomes often exhibit extreme composition biases, compared to their free-living relatives. For example, several *Plasmodium* species, including the human malaria parasite *P. falciparum*, have among the most AT rich genomes of any known eukaryotes (Nikbakht et al., 2014). However, this varies tremendously across the genus, creating challenges for inferring the phylogeny of the entire group (Galen et al., 2018). Similarly to long branch attraction, where fast evolving lineages are erroneously grouped together, genomes with similar composition biases can also be grouped together, resulting in the wrong topology (Foster, 2004). Composition heterogeneity means that the probability of transitioning from one character state to another will vary across the tree, and requires substitution models that can accommodate this possibility. Relaxing the assumption of site rate variation across both sites *and* lineages is more challenging than dealing with either source of rate variation alone, but recent technical advances mean that these models are increasingly useable (Heaps et al., 2019). Modelling composition biases turns out to be extremely important for reliably recovering relationships among distantly related species, especially in studies focused on understanding the origins of life, where we are dealing with divergence events that occurred billions of years ago (Williams et al., 2013). There is evidence for composition biases among several groups of parasites – not only unicellular parasites but also more complex parasite species (e.g. flatworms, lice and vampire bats (Botero-Castro et al., 2018; Le et al., 2002; Yoshizawa and Johnson, 2013)) – but it remains unclear to what extent this has the potential to create issues in reconstructing a timeline for parasite evolution.

Despite the fundamental role of the substitution model in phylogenetics, this aspect of divergence time estimation has rarely been investigated. The is potentially due to the technical challenges

applying models that are more complex than the GTR model. It could also partly reflect the perception that the impact of the clock model and the calibration information (discussed below) will be greater and/or that partition strategy (i.e. how we subdivide the alignment into sites evolving at similar rates) is ultimately more important than the choice of substitution model applied to each partition. The impact of partitioning was recently shown to play an important role in divergence time estimation (Angelis et al., 2018), but this study focused mainly on clock model rather than substitution model violation. The results of one study indicated that the use of alternative substitution models may have a relatively minor impact on divergence estimates, based on simulations and empirical analysis of Cornales, a group of flowering plants, originating around 125 Ma (Schenk and Hufford, 2010). However, this study focused on relatively simple substitution models and did not apply a Bayesian framework. We suggest that this aspect of molecular dating is worth exploring in more detail, and may be especially relevant for groups of taxa, such as parasites, that exhibit composition biases and cases where very ancient divergence events are of prime interest.

4.2 Models of morphological evolution

Morphological data play an important role in species divergence dating, as it provides the only evidence for determining the phylogenetic position of most fossil species (Lee and Palci, 2015). It is also possible to incorporate morphology associated with fossils and living species directly into the estimation of divergence times using appropriate tree models that are described later in the chapter. The morphological data typically used for phylogenetic inference differs from molecular data in several key ways, with important implications for modelling character evolution (Wright, 2019). The most notable distinction is that discrete states do not carry a consistent meaning across characters. For example, binary characters with states “1” and “0” are often used to indicate the presence and absence of a particular morphological structure, such as an appendage. However, “1” and “0” can also be used to represent two alternative structural forms of a given appendage, rather than presence versus absence. The precise definition will depend on the character. This is very different from molecular characters, where for example, an “A” consistently represents an “adenine”, irrespective of alignment position. Morphological characters can also be multistate, with any number of states greater than two, where each state can correspond to a distinct morphology. Discrete states can also be used to represent subdivisions of continuous data, which can include measurement data (e.g. height, width) or count data (e.g. number of appendages). Depending on

the character, it can be extremely difficult to characterize discrete states objectively and to assign species to each category (Tarasov, 2019).

A much smaller set of models exist to describe the process of discrete morphological character evolution. The most widely used model used for tree inference is the Mk model (Lewis, 2001), which is a generalisation of the JC model for characters with any number of states. Recall that this simple model assumes the equilibrium frequency of each state is equal and that the relative rate of change between each combination of states is the same. The lack of consistency between character definitions and states makes it difficult to develop a more complex model that would be applicable across datasets or even characters. However, we can allow subsets of different characters to assume different rates, using the same strategies described previously for molecular data, e.g. partitioning characters or using a discrete gamma distribution to define different rate categories (Wright, 2019). To get around the assumption of equal, and therefore symmetric, rates of change between states we can use a variant of the Mk model that allows for variation in the state frequencies (Wright et al., 2016). This model can accommodate the possibility that the change between some states is asymmetrical (e.g. $0 \rightarrow 1$ may be more commonly observed than $1 \rightarrow 0$ transitions).

Morphologists also tend not to collect characters that are the same across all species (known as *invariant* characters) or those that differ in one species only (known as *parsimony non-informative* characters). This is because non-model based phylogenetic methods do typically not use this information. However, this data collection practice is not accounted for by standard substitution models and an explicit correction must be applied (Lewis, 2001; dos Reis et al., 2016; Wright, 2019). Unlike molecular data, where all potential states are generally observable, morphological characters can have both non-observable and hidden states. It is possible that additional layers of hidden states, such as gene regulatory networks, actually determine the observed states. Models that can accommodate this possibility have recently been introduced but have not yet been applied in the context of divergence dating (Tarasov, 2019).

Another important distinction with molecular alignments is that morphological matrices tend to be very small, containing tens or hundreds rather than thousands of characters, and are likely to be highly incomplete if the data contains fossils (O'Reilly et al., 2015). Extinct species are most commonly represented by fragments of fossilized hard tissue (e.g. bone or shell). Soft-tissue preservation is relatively rare. This is important for parasites, since most are soft-bodied and have low fossilisation potential (Littlewood and Donovan, 2003; De Baets and Littlewood, 2015;

Leung, 2017). In addition, morphology tends to exhibit higher levels of homoplasy than molecular data, i.e. shared character states that are due to convergence rather than ancestry (Lee and Palci, 2015). Parasites in particular show convergence among traits used to manipulate their hosts and to assume a parasitic lifestyle. Further, many species have evolved to become, at least superficially, more simplified than their free-living relatives (Poulin and Randhawa, 2015). Consequently it can be challenging to identify the taxonomy and phylogenetic affinity of parasite species, especially fossils, on the basis of morphology alone (De Baets and Littlewood, 2015). In reality, the phylogenetic placement of most fossils is highly uncertain, though this uncertainty is often not reported clearly (O'Reilly and Donoghue, 2017).

In contrast to molecular substitution models, the impact of morphological model violations remains hugely unexplored and the extent to which existing models fit available datasets remains largely unknown. A recent simulation study found that divergence estimates appear to be surprisingly robust to discrete morphological model violations, provided asymmetry in character transitions is accounted for and that the overall rate of evolution is not extremely high (Klopfstein et al., 2019). Although in theory small datasets should lead to more uncertainty but not lower accuracy, in general, the reliability of morphology-based trees is less well characterized and may depend on the impact of other variables such as taxon or fossil sampling (Luo et al., 2019). This aspect of divergence dating is also deserving of further scrutiny and development.

A family of models also exists for the analysis of continuous character or trait evolution. These models are more commonly used for phylogenetic comparative analyses, which typically aim to test hypotheses about evolutionary processes (Harmon, 2018), rather than to infer the topology and divergence times. The most general and widely used models of trait evolution are Gaussian phylogenetic models, including the Brownian motion (BM) and the Ornstein-Uhlenbeck (OU) process models. Under the BM model traits evolve along branches according to a random walk, while the OU model incorporates an additional parameter that constrains traits towards some optimum (Harmon, 2018). Elegant extensions of these models can allow for traits in different parts of the the tree to evolve under distinct processes (Mitov et al., 2019). Continuous trait models have only recently been applied to infer phylogenetic relationships (Parins-Fukuchi, 2017, 2018) or estimate divergence times (Álvarez-Carretero et al., 2019). These developments may be particularly advantageous for the analysis of fossils, as continuous trait data is potentially more phylogenetically informative than discrete characters (Parins-Fukuchi, 2017). This approach to modelling morpho-

logical evolution also overcomes many of the issues encountered with discrete characters (Wright, 2019; Álvarez-Carretero et al., 2019). For a recent comprehensive review of models available for morphological evolution see Wright (2019).

5 Molecular clock models

The clock model allows us to describe how the substitution rate varies (or not) across branches in the tree. The strict or *global molecular clock* model assumes that the rate is constant over time and across species – the same rate will apply to all branches. This is typically only appropriate over short and recent timescales, and will not be appropriate for many groups of parasite and their relatives (Hafner et al., 1994; Ricklefs and Outlaw, 2010; Bromham et al., 2013). *Relaxed* molecular clock models allow rates to vary over time and across branches. The most widely implemented clock model is the *uncorrelated lognormal relaxed* (UCLN) clock model (Drummond et al., 2006). Under this model the rate of each branch is drawn independently from a lognormal distribution, and the variance of the distribution can be estimated from the sequence data. The mean of the distribution may be fixed or estimated from the data when calibration information is available. In theory, independent rates could be sampled from a wide variety of distributions for continuous positive values. The *autocorrelated rates* clock models assume that rates will be more similar among ancestors and descendants (Kishino et al., 2001). For a detailed overview of available clock models see Heath and Moore (2014) and Ho and Duchêne (2014). Although the use of different relaxed clock models can have a large impact on divergence estimates (dos Reis et al., 2015; Bromham, 2019), in practice researchers rarely explore their effects.

Since different subsets of sites in the genome will evolve at different rates, as discussed in the section *Molecular substitution models*, we can apply separate clock models to different partitions, in addition to separate substitution models. Note that although it may seem to make sense to use the same set of partitions for the clock and substitution models, these do not necessarily need to be equivalent. Similarly to substitution model selection, computational tools are available to guide the selection of the optimal partition strategy for clock models (Duchêne et al., 2013). Different partition strategies for clock models have been shown to have a large impact on estimated divergence times, especially in combination with clock model violation and/or conflicting calibrations (Angelis et al., 2018). Complex patterns of rate variation may be especially important to consider in the

context of parasites, many of which evolve rapidly relative to their free-living relatives or host species.

It is possible to estimate species divergence times in the absence of any independent temporal information, by applying a fixed mean substitution rate, rather than coestimating the rate along with divergence times. This essentially requires "knowing" the substitution rate, which is only possible for some intensely studied model organisms and viruses. Substitution rates also appear to exhibit time dependency. In particular, lower rates are often recovered for trees that span longer time periods (Ho et al., 2005). This is thought to be caused by the action of purifying selection over surprisingly long intervals (i.e. the elimination of slightly deleterious mutations over 0.1–1 Myr), such that over shorter intervals we recover the *mutation rate* and not the slower, long-term substitution rate, (i.e. the rate of fixation of new mutations in a population or species) (Ho et al., 2005, 2007). This contributes to substantial uncertainty in the average substitution rate that could apply to a given phylogeny, even for closely related non-parasitic lineages. The use of a fixed substitution rate to estimate divergence times should therefore be implemented with caution, as it can demonstrably lead to unreliable estimates of node ages (Papadopoulou et al., 2010).

It is also possible to apply the clock models described in this section to morphological data (Ronquist et al., 2012; Gavryushkina et al., 2017; Zhang et al., 2015). However, substantially less is known about the clock-like nature of morphological evolution.

6 Molecular clock calibrations

6.1 Minimum and maximum constraints on divergence times

Calibrating species trees to time requires temporal evidence. Here, we make the distinction between the fossil and the subfossil records. The latter refers to samples that are relatively young, often associated with archaeological finds and for which ancient DNA (aDNA) may be available. The upper limit for aDNA is ~ 1 Ma, though most aDNA samples are much youngest than this (Shapiro and Hofreiter, 2014; Krause and Pääbo, 2016) and aDNA associated with parasite is typically $< 50,000$ yrs (Wood, 2018). The inclusion of this type of data in divergence dating is discussed in the next section *Incorporating extinct samples into the tree*.

The most direct source of evidence for a speciation event in deep time is the first appearance of the

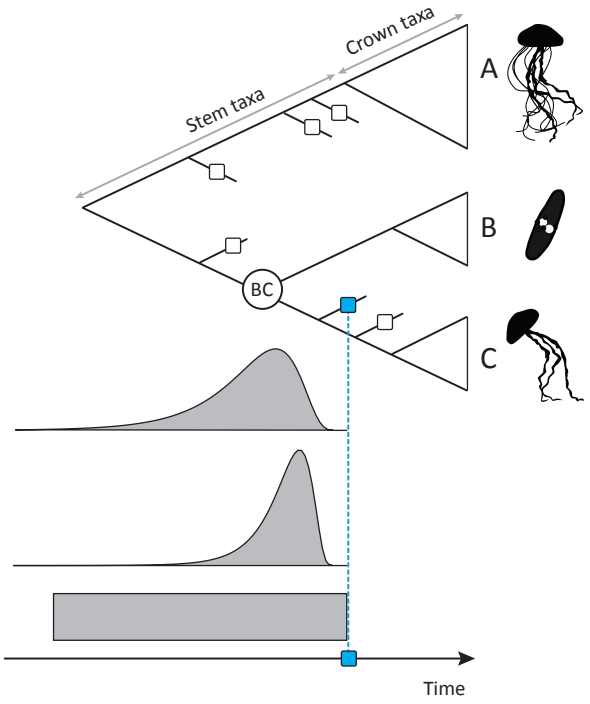

Figure 1: Evidence used to constrain speciation times using a node dating approach to calibration. The tree shows the relationships between three hypothetical groups of Cnidarians (A, B and C). Squares indicate sampled fossils. Triangles represent living representatives of each group. The distinction between the stem and crown group is indicated for group A. Three hypothetical probability densities (two lognormal and one uniform distribution) used to calibrate the age of the node representing the crown group BC are shown below the tree. The minimum of the distribution is based on the oldest fossil representative of BC (shown in blue). The maximum or 97.5% limit of the distribution is often chosen arbitrarily or may extend back in time to a point at which no taxa that appear similar to the stem or crown group members of ABC have been sampled, despite suitable environmental, taphonomic and sampling conditions (Parham et al., 2011).

descendants of that event in the fossil record. A critical aspect of this evidence is that the age of first appearances will not be coincident with the age of speciation events, but rather will provide an estimate for the minimum age of the divergence time before the present (Holland and Patzkowsky, 2002). Following molecular divergence, there will be some interval before independently evolving lineages acquire distinguishing morphological characteristics, and some subsequent interval before these changes are captured in the rock record (for a nice illustration of this see (Steiper and Young, 2008, figure 2) or (Brown et al., 2008, figure 1)). If preservation potential or fossil recovery is low, the interval between speciation and first appearances may be extremely large (e.g. > 10 Myr).

In a Bayesian framework the relationship between fossil evidence and divergence times can be modelled using a wide range of probability distribution (or density) functions (e.g. lognormal or uniform) (Drummond et al., 2006; Ho and Phillips, 2009). This approach to calibration is known as *node dating*, illustrated in Fig. 1. In theory, the parameters of the distribution can be chosen to reflect the degree to which first appearances approximate the true divergence time. For example, if we have reason to believe that fossil recovery is very high, we can select a distribution and parameters that place a high prior probability on the divergence event having occurred close to the age of the first appearance. Conversely, if fossil recovery is low, we can assign a diffuse distribution that places a lower prior probability on the divergence event having occurred close to the age of the first appearance, reflecting the possibility that speciation may be much more ancient than the earliest fossil evidence. In practice it is extremely challenging to select distributions and parameters objectively, and justification is rarely provided for either. This is problematic since calibration information exerts a large influence on posterior divergence times and seemingly minor changes in distribution parameters can shift estimates by millions to tens of millions of years (Warnock et al., 2011).

The fossil record can also be used to establish maximum constraints on divergence times using taphonomic, environmental and/or biogeographic controls. The geological record can be traced back to an interval during which we have strong evidence to believe that if a given lineage had been present, it would have been preserved (Benton et al., 2009; Parham et al., 2011). Since we anticipate speciation cannot postdate first appearances, minimum constraints are often implemented using *hard bounds* – that is, there is zero probability that speciation is younger than the minimum. Given the uncertainty inherent in maximum constraints, we may want to apply a *soft bound* – this means allowing for a small probability that speciation may be older than the maximum (e.g. the

maximum is used to specify the 97.5% quantile of the calibration density) (Yang and Rannala, 2006).

Specimen choice, including the evidence used to establish stratigraphic age and phylogenetic placement, is very important given the role of calibration in molecular dating. Few fossils have been recovered from rocks that have been directly dated so that usually, age must be established through a chain of evidence linking the litho-, bio- and chronostratigraphic records (Reisz and Müller, 2004; Erwin, 2006; Benton et al., 2009). Positioning fossils in the tree of life is challenging for several key reasons already noted – the overall number of morphological characters is low, specimens are typically very incomplete, and phenotypic traits are prone to homoplasy (Lee and Palci, 2015; O'Reilly et al., 2015). The placement of fossil species will be highly uncertain for many parasites (De Baets and Littlewood, 2015), as discussed below.

A set of best-practices was introduced to guide the selection of transparent and explicit evidence-based constraints (Parham et al., 2011). To maintain accuracy any ambiguous or putative group members are typically excluded. In the case of minimum constraints, insofar as possible, it should be established that specimens are members of the crown rather than stem group (i.e. are descendants of the last common ancestor of living group representatives and not members of the ancestral lineage leading to the crown, see Fig. 1). Similarly, maximum constraints should extend beyond the records of all putative stem group members. Constraints established using best practices can therefore be considered conservative and in many cases will span a large interval of time, especially for groups with poor preservation potential. Imprecise constraints tend to lead to imprecise posterior divergence estimates, even in combination with large sequence alignments (dos Reis and Yang, 2013; Warnock et al., 2017). However, it is preferable to have more accurate and less precise estimates of divergence times than overly precise, inaccurate results (De Baets et al., 2015). There are alternative approaches that utilize more paleontological data in deriving non-arbitrary node densities, by fitting a model of fossil recovery and/or the diversification process to fossil occurrence times (Nowak et al., 2013; Matschiner et al., 2017). These generally require a very large number of occurrences to generate precise constraints, which is unavailable for most lineages of parasites.

The age of non-calibration nodes is specified using the tree model. We typically use *birth-death process* models that describe the probability of observing a tree with extant tips only, where birth and death are equivalent to speciation and extinction in a macroevolutionary context (Yang and Rannala, 2006; Drummond et al., 2006). Different combinations of speciation and extinction rates

give rise to different tree shapes, and in particular, different distributions of expected node ages between extant species pairs. This means we assume that speciation and extinction processes gave rise to our tree, but we do not consider extinct species sampling as being part of this. Instead, the fossil evidence used to specify the node calibrations is considered separately. Alternative approaches to this are described in the next section.

6.2 Incorporating extinct samples into the tree

There are several important drawbacks associated with node dating. In particular, this approach limits the amount of information from the fossil record that can be used directly during inference, since typically only one fossil is assigned per calibration node. Best practice approaches also encourage the exclusive use of specimens that can be assigned to a specific node with a high degree of confidence. This results in minimum constraints that are more likely to be substantially younger than the speciation age, since the phylogenetic placement of many fossil taxa is very uncertain. Node calibrations can also interact with the tree prior and other calibrations in unintuitive ways, leading to a discrepancy between the user-specified calibration densities and the effective constraints on divergence times used during inference (Heled and Drummond, 2011; Warnock et al., 2015).

An alternative approach is to consider extinct samples as part of the tree. This approach is borrowed from epidemiology, where we have molecular sequence data associated with non-contemporaneous (i.e. extinct) samples. The sampling times of extinct tips are used to calibrate the substitution rate. aDNA can occasionally be recovered from the subfossil record, for relatively young specimens (i.e. typically <50,000 yrs) (Wood, 2018). This approach has been applied to date the spread of tuberculosis, using samples of *Mycobacterium tuberculosis* recovered from 1000 yr human skeletons (Bos et al., 2014). It has also been shown that extinct samples can only provide reliable calibration information if the extinct samples are well distributed throughout the tree between the root and the tips (i.e. low phylo-temporal clustering) (Tong et al., 2018). The use of aDNA to date parasite origins is therefore most useful for relatively shallow divergence events, e.g. those associated with archaeological finds. We are often interested in divergence events among hosts and parasites that are much older than the time span that can be captured by aDNA.

This principle can also be applied to extinct samples for which no molecular data is available. If morphological characters are available for both living and fossil species, the placement of fossils can be co-estimated, along with relationships and divergence times among extant species. The

uncertainty associated with the phylogenetic position of fossils will be reflected as part of the output. This approach is sometimes known as *total-evidence* or *tip-dating* (Ronquist et al., 2012). The same clock models used for molecular dating can also be applied to morphological data partitions.

Including non-contemporaneous tips requires using a different tree model. Several early applications of this approach within a Bayesian framework implemented a uniform tree model. This model places an equal probability on all possible tree topologies and draws internal node ages from a uniform distribution between the ages of the fossil samples and a user specified maximum applied at the root (Ronquist et al., 2012). This model does not incorporate an explicit diversification or fossil recovery process, and forces all fossil samples to be treated as tips, which may not be appropriate for some datasets.

The *fossilised birth-death* (FBD) *process* is a tree model that combines the diversification (speciation and extinction), fossil recovery and extant species sampling processes, and allows samples to occur along internal branches (Stadler, 2010; Heath et al., 2014; Gavryushkina et al., 2014). An example outcome of the processes described by this model is shown in Fig. 2. This model can be used in total evidence analysis (Gavryushkina et al., 2017), but an additional advantage is that fossil sampling times are informative about the FBD model parameters, regardless of whether their phylogenetic position can be resolved. This means all fossil samples (both with or without morphological character data) can be utilized during inference. The specimen level FBD model can be used for the analysis of fossil occurrence data (Stadler, 2010), while the FBD *range* process can be used for the analysis of stratigraphic ranges (i.e. when we only have information about the first and last occurrence times) (Stadler et al., 2018). A key distinction between this approach and alternatives to constraining divergence times based on fossil evidence is that the model actually describes the combined processes that generated our observed data. This allows us to estimate other parameters of interest, including diversification and fossil recovery rates. The FBD *skyline* model(s) also allow for non-uniform diversification, fossil recovery and extant species sampling (Gavryushkina et al., 2014; Zhang et al., 2015).

This dating methodology has the advantage above node-dating that many more occurrences could be used to calibrate parasite divergences, though issues associated with deriving constraints for node dating remain relevant. In particular, this approach requires a large number of occurrences to produce reasonable or precise node age estimates. Similarly, better estimates are recovered if the phylogenetic placement of fossils can be resolved (Heath et al., 2014). In the context of

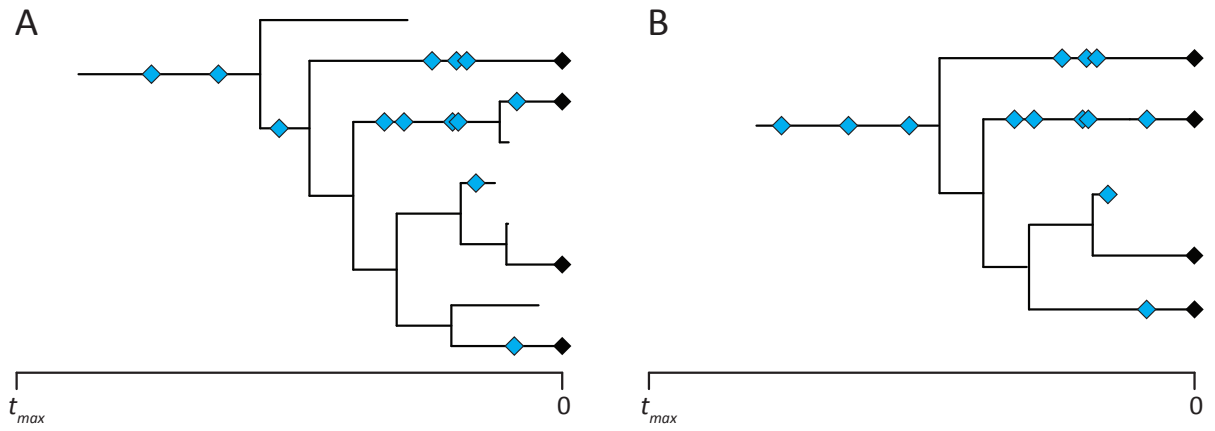

Figure 2: The fossilized birth-death (FBD) process as a tree model for coherent calibration. Panel (A) shows the complete outcome of the speciation, extinction, fossil recovery and extant species sampling processes incorporated into the model, which includes unsampled lineages. Blue squares represent fossils and black squares represent extant samples. Panel (B) shows the reconstructed or sampled tree. The FBD model is used to calculate the probability of observing the sampled tree, given that we assume the combined processes shown in (A) (Stadler, 2010; Heath et al., 2014). This modeling framework offers the potential to constrain parasite divergence times using the more abundant host fossil record, including evidence of parasites found associated with hosts. Trees and fossils were simulated and plotted using the R packages `TreeSim` (Stadler, 2011) and `FossilSim` (Barido-Sottani et al., 2019).

parasite evolution, this method may be best suited to some host groups that have abundant fossil occurrence data. In the subsequent sections we discuss temporal evidence available for the evolution of parasites.

6.3 The fossil record of parasites as a source of calibrations

The fossil record of most parasites is non-existent or includes extensive gaps. This is due to the intrinsic nature of parasites, many of which are small, soft-bodied and reside in other species. This is especially true for viruses, bacteria and unicellular eukaryotes but also applies to parasitic metazoans. For this reason the fossil record has often been overlooked in the study of parasite evolution, although evidence of parasitism in deep time is perhaps more extensive than many researchers have assumed (Conway Morris, 1981; De Baets and Littlewood, 2015; Leung, 2017).

Body fossils, complete or partial remains, of parasites are extremely rare. The best source for these finds are fossil Lagerstätten (sites of exceptional preservation) and in particular, amber deposits. Some spectacular finds show direct evidence of host-parasite associations and allow for straightfor-

ward assignment of both host and parasite to their living counterparts (Leung, 2017). However, several aspects of parasite evolution compound the challenges associated with phylogenetic inference using morphology. Species often exhibit high degrees of specialisation, reduction (simplification) or convergence, and can have complex life cycles featuring multiple ontogenetic stages (De Baets and Littlewood, 2015). Phylogenies of extant parasites based on morphology versus molecular characters often show considerable disagreement (e.g. among parasitic flatworms (Joffe and Kornakova, 2001)), highlighting the challenge of relying on morphology alone. More commonly, partial remains, such as attachment organs or eggs, are recovered. Some of these can be assigned with confidence to living lineages, but it can be hard to exclude the possibility that putative parasite remains actually belong to some now extinct parasite group (De Baets et al., 2015).

The trace fossil record of parasites is more extensive. Some parasites leave distinctive marks on their hosts and can be taxonomically identified by taking advantage of pathologies left by extant parasites. However, similar pathologies can also be caused by distantly related parasites (e.g. due to convergence), caused by parasites that have no extant counterparts or attributable to causes unrelated to parasitism (Poulin and Randhawa, 2015; Leung, 2017). In general it is extremely difficult to definitively assign traces to a specific lineage of parasites. Alternatively, the fossil record of free-living relatives may provide a more abundant source for constraints, depending on the phylogenetic scope of available molecular data. However, in some cases the fossil record of parasites is actually better or at least more ancient than the body fossil record of free-living relatives (e.g. platyhelminthes or nematodes) (De Baets et al., 2015; Poinar, 2015a).

Node calibrations require that taxonomic affinity is established *a priori*. The nature of the fossil record of parasites means that calibrations will be broad at best. For example, molecular clock analyses place the origin of ticks in the Carboniferous (359–299 Ma) or early Permian (299–273 Ma) (Jeyaprakash and Hoy, 2009; Mans et al., 2012), but the unambiguous first appearance of ticks in the fossil record is not observed until the Late Cretaceous (ca. 100 Ma) (Dunlop, in press). Although novel fossil finds and advanced non-destructive imaging techniques will increase the potential of the fossil record of parasites (De Baets and Littlewood, 2015), it can never provide a temporally comprehensive picture of host-parasite evolution. This creates good motivation to utilise molecular clock methodology but we still rely on temporal evidence to calibrate the clock. Most known groups of parasites leave no traces that would be readily detectable in the fossil record, even under exceptional circumstances, and so we require other sources of evidence for calibration.

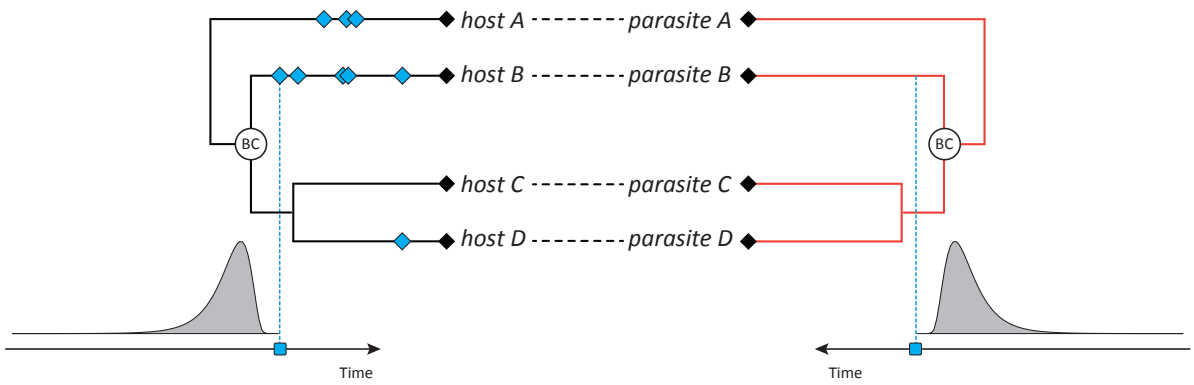

Figure 3: The host fossil record as a potential source of evidence for constraining divergence times among parasites. In the idealized scenario shown here, the host phylogeny (shown on the left) and the parasite phylogeny (shown in red on the right) are fully congruent. Sampled occurrences from the host fossil record are shown in blue along the host tree; no fossil samples are available for parasites. In this example node BC in the parasite tree is constrained using a probabilistic node calibration derived for the corresponding speciation event in the host phylogeny. Here we assume that the first appearance in the fossil record of host species B or C postdates the divergence event separating parasite species B and C.

6.4 The fossil record of hosts as a source of calibrations

Many hosts (e.g. shelled mollusks or vertebrates) will have a substantially better record that their attendant parasites. In some cases it may be possible to apply constraints derived from the host fossil record to corresponding nodes in the parasite phylogeny, as illustrated in Fig. 3. This approach has been widely used to infer divergence times among parasites, including flatworms, nematodes, tapeworms, myxozoans, haemosporidians (*Plasmodium*) and feline papillomaviruses (Verneau et al., 2002; Li et al., 2018; Olson et al., 2010; Holzer et al., 2018; Pacheco et al., 2017; Rector et al., 2007). A key assumption required is that the constraints derived for a given host speciation event are appropriate to use for the corresponding speciation event among parasites. In an idealized scenario, such as the one shown in Fig. 3, the two phylogenies would be identical and we could infer that cospeciation has occurred without intra-host speciation, host-switching or loss. However, as we later discuss, this scenario is rare, and biased host shifts can even lead to misleadingly congruent trees (de Vienne et al., 2007).

To maintain a best practice approach to calibration, we recommend statements regarding the phylogenetic congruence between the host and parasite phylogenies are stated explicitly, along with the evidence used to establish phylogenetic affinity and age of the host specimens. In addition, any

hypotheses being tested based on the time calibrated tree of parasites should be clear. For example, if the goal is to test whether the origin of parasites was coincident with the origin of their hosts, then applying calibrations derived from the host fossil record can introduce a degree of circularity (De Baets et al., 2015).

Another option, if molecular and calibration data are available for hosts, is to first estimate divergence times among the host species and then to apply the posterior node age estimates as priors, known as *secondary calibrations*, in a subsequent analysis of the parasite tree. This approach was used to date the origins of feline papillomaviruses, which have fully congruent host-parasite phylogenies, using posterior estimates of divergence times among felids (Rector et al., 2007). However, this approach is not strictly Bayesian, as the uncertainty associated with the primary calibration priors will not be reflected properly in the secondary posterior age estimates, and has been shown to produce overly precise and/or erroneous ages (Schenk, 2016).

How do we test whether the age of a given parasite group is coincident with the age of a host, if we are relying on the host fossil record? For example, we may want to estimate the origin of blood parasites versus blood feeding insects, or the origin of lice versus the evolution of certain integumentary traits, such as skin, fur or feathers (Leung, 2017). This is very challenging if we want to avoid circularity and we have to bear in mind that the time of trait acquisition may not be coincident with speciation. One possible solution is to utilize constraints for nodes within the host phylogeny that are younger than the most recent common ancestor (MRCA) of descendant lineages known to be associated with host specific parasites.

Phylogenetic models that describe the process of fossil recovery create the potential for more data to be included in molecular dating analyses. Of particular interest in the context of host-parasite evolution, is the potential application of the FBD model to the trace fossil record of parasites or evidence of parasitism associated with the host fossil record. For example, the appendages of parasitic platyhelminths found associated with several vertebrates species (De Baets et al., 2015) or the pathologies observed in bivalve hosts that are associated with trematodes (Huntley and De Baets, 2015) could be utilised in this modelling framework. Parasitic eggs found in fossil coprolites could also provide a particularly valuable source of evidence for nematodes or platyhelminths (De Baets and Littlewood, 2015). Here, the fossil recovery rate is tied to the sampling of host species, which displays direct evidence of host-parasite association.

6.5 Caveats to using hosts as calibrations

Using the host fossil record to derive constraints requires making assumptions that can be hard to verify. If extant host and parasite phylogenies are fully congruent and the parasites are known be be highly host specific, this is strong evidence to suggest that the host fossil record is a reliable source for calibrations. However, without further evidence it is hard to know if modern associations have been this way throughout history (Leung, 2017). For example, the origin of bed bugs associated with host bat species were found to have evolved ~ 30 Myr earlier than their hosts based on molecular clock estimates obtained using independent calibrations (Roth et al., 2019). Host ranges can clearly vary over time to become more specialised, host switching is common among many parasites, and evidence of extinct associations are observable from the fossil record, even for some very recent associations (Leung, 2017; Boast et al., 2018). In addition, many host-parasite relationships of interest are very ancient, originating 100s of Myr ago, and the long-term pattern of coevolution may be totally obscured from extant phylogenies. Evidence supporting the same associations could come from the fossil record, but direct evidence of parasitism in extremely rare and brings us back to the challenges outlined in the section *The fossil record of parasites as a source of calibrations.*

Cophylogenetic methods allow historical interactions between between two coevolving groups of species – including cospeciation, intra-host speciation (duplication), host-switching or loss – to be reconstructed based on their independent phylogenies (Charleston and Libeskind-Hadas, 2014; Martínez-Aquino, 2016) (discussed in more detail in the section *Prospects for constraining coevolutionary dynamics of hosts and parasites*). These inferences are typically based on branching patterns and estimated node ages of the host and parasite trees, which is problematic if our goal is determine whether the host fossil record can be used to time calibrate the parasite tree. In general, it is extremely difficult to gauge the level of congruence between two trees (Poisot, 2015; de Vienne, 2018).

Unfortunately, even if the host and parasite phylogenies appear topologically congruent, the branching times may still be very different. This situation can arise when host shifts are frequent but occur preferentially between closely related host species (Charleston and Robertson, 2002; Engelstädter and Hurst, 2006). Figure 4 illustrates this situation using a simulated cophylogeny. The parasites invade the host tree halfway through host diversification, at a time when the main host clades have already arisen (Figure 4A). They then undergo cospeciation, extinction and host switching events, with the latter usually restricted to crossing only short host phylogenetic distances. The outcome

of this process in terms of the host and parasite phylogenies and the associations between hosts and parasites (the 'tanglegram') appears to exhibits a high degree of congruence despite the fact that host and parasite diversification took place on different timescales (Figure 4B). This demonstrates that great caution needs to be exerted when inferring cospeciation from apparent congruence (de Vienne et al., 2007) and using host fossils to calibrate parasite trees. Both theoretical models and empirical evidence suggest that true cospeciation is extremely rare (de Vienne et al., 2013; Poisot, 2015). Unless there is good evidence (beyond host and parasite tree congruence) indicating that host shifts have been infrequent and the parasites codiversified with the hosts predominantly by cospeciation, using host speciation times may be a poor proxy for parasite speciation times.

In many cases the phylogenies recovered for host and parasite will appear extremely incongruent, and the host fossil record cannot simply be applied to calibrate the rate of evolution for the parasite tree. Although it is worth emphasising that if the host and parasite phylogenies are incongruent, this does not mean that the evolution of each group has occurred independent of the other. Instead the pattern of coevolution is likely be more complex (Poisot, 2015). Sometimes, subsets of the phylogenies may be congruent. In this circumstance, constraints could potentially be applied to nodes where congruence can be established with confidence, preferentially with the support of other lines of evidence.

An additional major challenge for many groups of interest is that both the parasite and host group have a poor fossil record. For example, insects have many parasites that are of broad relevance from a biomedical and environmental, as well as evolutionary, perspective (Eigenbrode et al., 2018). However, the fossil record of insects (though also often overlooked) is sparse for many groups relative to their extant diversity (Labandeira and Sepkoski, 1993). In these cases it is necessary to investigate other sources of evidence to constrain the evolutionary history of the host and parasite group.

6.6 Biogeographic constraints on divergence times

In the absence of any fossil evidence, biogeography can provide an alternative source of evidence for deriving node constraints. For example, if speciation can be linked to a specific tectonic event, such as mountain or island formation, the opening and closing of seas, or the splitting and merging of continents, the age of that event can be used to inform node calibrations (Ho et al., 2015; De Baets et al., 2016). This requires making the assumption that the main driver of the lineage split represented by the calibration node was genetic isolation that resulted from the geological

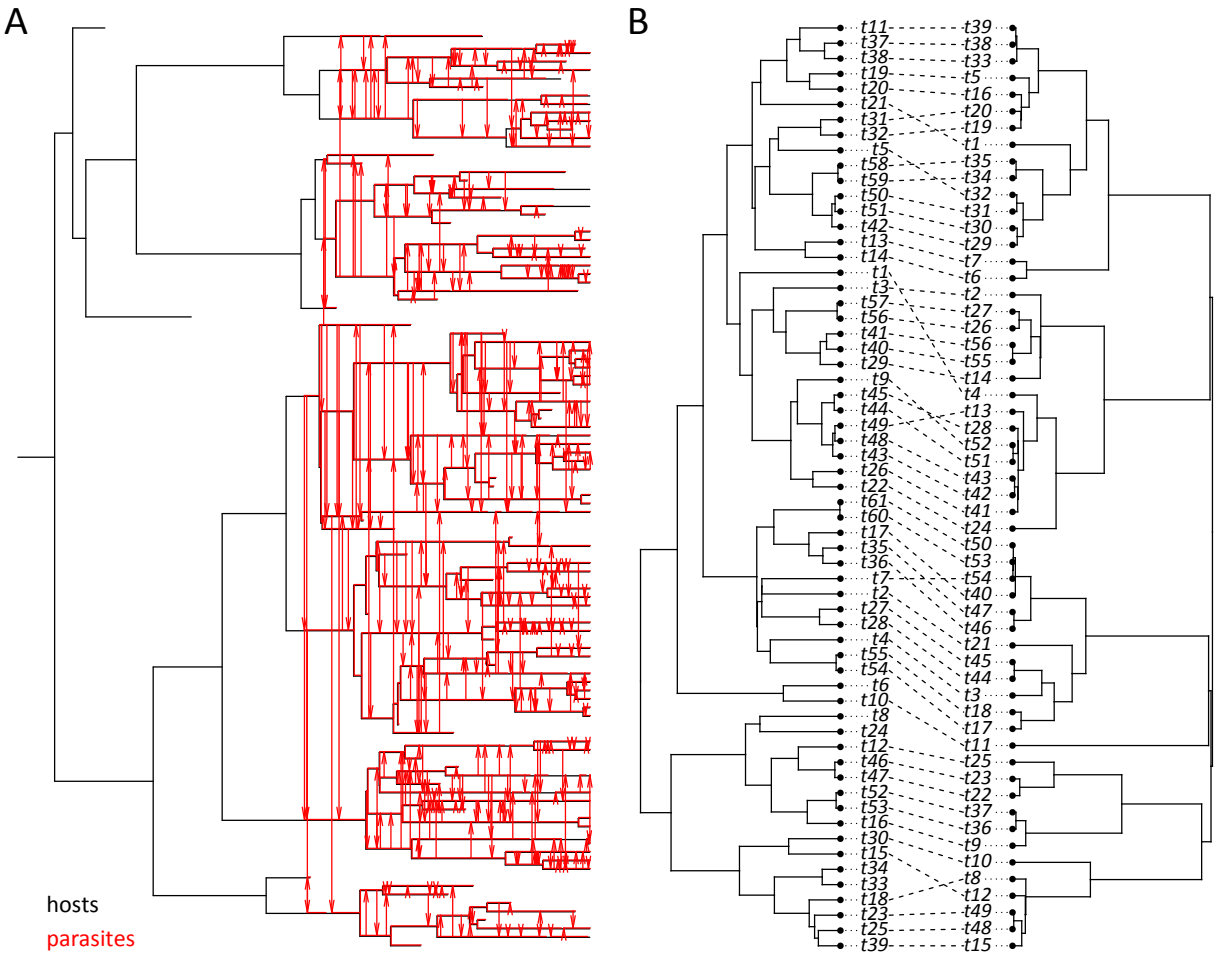

Figure 4: Host parasite codiversification with preferential host switching of parasite between closely related hosts. Panel (A) shows the complete cophylogenetic process, which was generated using a simulation routine implemented in the R package `cophy` (Engelstädter and Fortuna, 2019). The plot in B, a 'tanglegram' generated using the R package `phytools` (Revell, 2012), shows the two individual phylogenies of extant hosts (left) and parasites (right) resulting from the same process, with dashed lines indicating the associations between hosts and parasites. Note that the two trees are scaled differently as the age of the host tree is twice that of the parasite tree in this example.

event. Evidence for this typically comes from the biogeographic distribution of living species. Events that show evidence of having impacted the distribution of many species and have frequently been adopted as molecular clock calibrations include the rise of the Isthmus of Panama, which separated the Atlantic and Pacific oceans ($\sim$ 3.5 Ma), and the separation of New Zealand from the supercontinent Gondwana ($\sim$ 102 Ma). However, it can be challenging to date many tectonic events, and to determine whether these events are actually responsible for speciation. Tectonic events are protracted, rather than geologically instantaneous, events (e.g. the separation of New Zealand from Gondwana has a minimum age of 22 Ma), such that even if the age range of the event can be established, it will not be known at which point it became a barrier to dispersal and genetic isolation was fully established. Furthermore, it will depend on the environmental preferences and dispersal abilities of the group (e.g. aquatic versus terrestrial species). Assigning speciation events to biogeographic processes also becomes more challenging further back in time, as evidence is overprinted by younger events (De Baets et al., 2016).

To apply biogeographic events as age constraints, a best practice approach can also be adopted, by being explicit and transparent about the assumptions being made and available evidence. Statements should be provided in support of phylogenetic relationships, how they relate to biogeographic events, the source of evidence used to date the events and the range of uncertainty associated with the age of events. We should also be explicit about the hypotheses being tested and attempt to avoid circularity. For example, if we want to investigate the role of biogeography in shaping the distribution of host and/or parasites, we should be careful about using biogeographic events of interest as calibrations (Goswami and Upchurch, 2010; Kodandaramaiah, 2011).

One of the caveats associated with the node dating approach to utilising biogeographic evidence to calibration is that it requires committing to a given biogeographic/speciation scenario, and making strong assumptions about biogeographic history. In reality, there will be a lot of uncertainty in the role of tectonic processes in driving speciation, along with uncertainty in the topology. An alternative is to use process-based biogeographic models (Landis, 2017; Landis et al., 2019). This involves modelling the evolution of biogeographic ranges along the tree, using biogeographic evidence available for extant tips. This is conceptually similar to the FBD model, in that it describes the generation of our observed data (e.g. fossils or biogeographic ranges). One of the main advantages of this approach is that it can incorporate uncertainty in the tree topology, divergence times, and biogeographic histories. Because we also estimate the biogeographic history, we can

actually use this approach to test hypotheses about the role of biogeography in driving the evolution of the group. This approach has not yet been applied to date the origin of parasites but has great potential for estimating divergence times and testing biogeographic hypotheses of host-parasite coevolution.

7 *Wolbachia*: a case study

Wolbachia are alpha-proteobacteria that live as intracellular endosymbionts in arthropods and nematodes (Werren et al., 2008). A recent meta-analysis estimates that globally, around 50% of all terrestrial arthropods are infected with *Wolbachia* (Weinert et al., 2015), which translates to an enormous number of host species. The vast majority of described strains cluster in two supergroups A and B and in what follows we will focus exclusively on these two groups. *Wolbachia* belonging to these groups are often parasitic, manipulating their hosts reproduction to their own advantage, but they may also have beneficial effects on their hosts (Werren et al., 2008; Engelstädter and Hurst, 2009; Zug and Hammerstein, 2015).

Pinpointing the time when supergroups A and B *Wolbachia* emerged and diversified is difficult for two reasons. First, there is, to the best of our knowledge, no definitive fossil evidence of *Wolbachia*. *Wolbachia* live exclusively inside the cells of their hosts, and even if traces of endosymbionts are found in arthropod fossils it seems unlikely that they could ever be definitively identified as A/B *Wolbachia* and distinguished from other intracellular bacteria, let alone from other supergroups of *Wolbachia*. The best source of microscopic parasites found associated with arthropod hosts are amber deposits (Poinar, 2018). A single record of *Rickettsial*-like cells has been found in a tick preserved in Cretaceous amber, and while the finding is spectacular, the parasite's taxonomic placement within the order Rickettsiales is tentative and probably impossible to identify at a higher level of taxonomic resolution (Poinar, 2015b). Second, phylogenies of *Wolbachia* and their hosts are generally very discordant, indicating frequent host shifts of *Wolbachia*, often across large host phylogenetic distances (Werren et al., 1995), and over short evolutionary timescales (Siozios et al., 2018; Turelli et al., 2018).

Early attempts to date the arrival of *Wolbachia* in arthropods assumed a constant molecular clock, arriving at estimates of 100 Ma for the split between supergroup D (infecting nematodes) and supergroups A and B (Bandi et al., 1998), and around 60 Ma for the origin of the last common

ancestor of the A and B supergroups (Werren et al., 1995). However, these estimates relied on neutral substitutions in only a single gene (*ftsZ*) and an earlier estimate for the neutral substitution rate in unrelated bacteria (Ochman and Wilson, 1987). To improve upon these dates, a recent study took a different approach that is not based on estimated substitution rates in other bacteria but instead uses the host phylogeny for calibration (Gerth and Bleidorn, 2016). In this study, the authors analysed whole genome sequencing data of four closely related supergroup A *Wolbachia* strains infecting four bee species of the genus *Nomada*. Assuming an uncorrelated relaxed molecular clock model calibrated with a dated host tree, they estimated the age of the last common ancestor of A and B *Wolbachia* to have lived 216 Ma. This estimate puts the origin of *Wolbachia* diversification within arthropods (the *Wolbachia* 'pandemic') at a much earlier time than the previous estimates but coincides with the diversification of large host groups such as the Lepidoptera and Diptera. Unfortunately, this estimate comes with a very large credible interval (460 to 76 Ma based on the 95% highest posterior density interval), which is likely due to the use of only a single, very recent calibration point. Moreover, a caveat with this approach, as discussed above, is that strict cospeciation of the four *Wolbachia* strains with their bee host was assumed. Although *Wolbachia* and host phylogenies were found to be perfectly congruent, this could also have been the result of host shifts of the initial *Wolbachia* strain arriving in this clade of hosts, as discussed above. Further phylogenomic studies of different *Wolbachia* strains and the use of alternative calibration approaches are clearly needed to assess the robustness of this estimate. In particular, novel cophylogenetic models that can account for complex patterns of host-parasite coevolution (Braga et al., 2019, discussed below) may provide a promising direction for constraining the evolution of *Wolbachia*.

8 Prospects for understanding the coevolutionary dynamics of host and parasites

Host-parasite interactions have undoubtedly had a major impact on the long-term evolutionary dynamics of many groups of organisms. However, this topic is challenging to address, partly due to the different timescales involved, i.e. short term selection pressure at the population level versus long-term macroevolutionary trends at the species level (de Vienne et al., 2013). In addition, studying phylogenetic processes requires reconstructing events that are almost never directly observable (Bromham, 2019). This places enormous significance on the reliable estimation of evolutionary

timescales using molecular (or morphological) clocks and the fossil record (Martínez-Aquino, 2016; Cruaud and Rasplus, 2016).

The branching patterns and node ages of trees provide valuable information for inferring the history of interactions using cophylogenetic methods (Charleston and Libeskind-Hadas, 2014; Martínez-Aquino, 2016). The most widely implemented approaches are *event-based cophylogenetic methods*, which take as input a set of unconstrained or preferably time calibrated trees for the host and parasite. The cophylogenetic history, incorporating cospeciation, intra-host speciation, host-switching or loss events, that maximises the congruence between the two trees is considered the most optimal. Some methods can also take biogeography into account (Berry et al., 2018). However, there are several potential drawbacks with event-based methods. First, the user must assign a cost to each type of event, which is extremely difficult to do objectively (Charleston and Libeskind-Hadas, 2014). Second, methods assume that the most congruent set of trees provides the best explanation for the data, with cospeciation being the preferred driver of congruence (de Vienne et al., 2013). However, events such as host-switching appear to be extremely common, as has been discussed above (Fig. 4) and elsewhere (Poisot, 2015), and cophylogenetic methods therefore have a tendency to overestimate cospeciation events (de Vienne et al., 2013). Additional information is ultimately required to definitively test for cospeciation (de Vienne et al., 2013; Cruaud and Rasplus, 2016). Third, the reliability of event-based methods hinges on the input trees being correct, but as we emphasise throughout this chapter there is often a large degree of uncertainty associated with both the topology and divergence times. Finally, a related issue is that since these methods are non-model based (i.e. not probabilistic), it is not straightforward to determine the uncertainty associated with the inferred cophylogenetic history. Furthermore, if we want to utilize the host fossil record for calibration, at least broad congruence between the host and parasite phylogenies must be established *a priori*. What if we were able to estimate all these parameters in combination?

Bayesian phylogenetics provides a probabilistic framework for linking evolutionary processes, where we can define explicit models that incorporate biologically meaningful parameters and obtain intuitive estimates of uncertainty. Since our goal may be to infer both the dynamics and the timeline of host-parasite interactions, it may be desirable to estimate all our parameters of interest in combination. A Bayesian approach has already been proposed for cophylogenetic inference, which allows for the simultaneous estimation of the host and parasite trees, along with the cophylogenetic history (Huelsenbeck et al., 2000). This model includes a rate of shifting between hosts, rather than a cost

associated with each type of event, and although it still makes the assumption that cospeciation is the main driver of congruence, in principle this assumption can be relaxed. An alternative phylogenetic model for inferring ancestral host-parasite interactions was recently proposed that fully relaxes the assumption that congruence is the null model (Braga et al., 2019). The approach is based on models of biogeography and allows parasites to have a *host repertoire*, where parasites can interact with multiple host species. Parasites can evolve an affinity with new hosts – analogous to the colonisation of a new geographic area – taking into account phylogenetic distance among host and potential host species. Similarly, host-parasite associations can be lost, analogous to local extinctions. Initial applications of this approach have relied on fixed time calibrated input trees. Further extensions of this framework could enable the co-estimation of time and topology based on additional evidence, such fossil occurrences or biogeographic information. In addition to coupling the diversification of distinct clades, hierarchical Bayesian models can also be used to link phylogenetic and environmental processes and extended to incorporate other available information. For example, sampling proxy data, such as variation in the number of rock formations over time, could be used to constrain the fossil recovery rate. The recent development of Bayesian phylogenetic software for extendable and flexible model specification, including BEAST2.5 (Bouckaert et al., 2019) and RevBayes (Höhna et al., 2016), provide enormous potential for developing and implementing complex hierarchical models.

Another potentially valuable avenue for molecular dating is the use of horizontal gene transfer (HGT) events, the lateral exchange of genes between species, in combination with evidence from the fossil record. For a given set of donor and recipient species, where only the latter has a known fossil record, since the transfer of genetic material must post-date the divergence of the donor lineage, the earliest fossil evidence of the recipient also provides evidence for the minimum age of the donor (dos Reis, 2018). This approach has been used to date divergence times among microbial lineages, most of which lack a substantive fossil record (Davín et al., 2018; Wolfe and Fournier, 2018a; Magnabosco et al., 2018; Gruen et al., 2019). This approach has the potential for constraining parasite evolution, as HGTs have been documented between some hosts and their parasites, including several parasitic plant groups (Molina et al., 2014; Kado and Innan, 2018). The use of HGTs is likely to be most useful for groups that are more closely related and therefore have a higher chance of exchanging genetic material. That said, evidence of HGT has been discovered between some distantly related host-parasite species, for example, *Wolbachia* and multiple host species, including nematodes and arthropods (Hotopp, 2011; Koutsovoulos et al., 2014). However, patterns of host switching and

HGTs appears to be extremely difficult to disentangle for *Wolbachia* and their hosts (Lefoulon et al., 2016), potentially due to rapid host shifts (Siozios et al., 2018; Turelli et al., 2018). The same approach could also be applied using other horizontally transferred genetic material, such as retrotransposons, which show evidence of exchange between birds and nematodes (Suh et al., 2016). Like other approaches to calibration, the HGT events and constraints must be selected carefully (Roger and Susko, 2018; Wolfe and Fournier, 2018b; Gruen et al., 2019), and species tree/HGT tree conflict introduces another aspect of uncertainty (dos Reis, 2018). Nevertheless, the innovative use of genetic and palaeontological data represents an exciting step towards dating divergence times that were once considered impossible to date.

Outstanding challenges from a methodological perspective include the development of phylogenetic models that are biologically reasonable, and determining whether our parameters of interest are identifiable given our available data. From the empirical perspective, the challenge will be collating valuable datasets, and exploring the limits of our data. Comprehensive sequence data is increasingly available, however, curating this data and delineating operational taxonomic units remains challenging. In addition, we need to be able to identify species using morphological data, and interpret the environmental context in which extinct species existed, meaning the role of taxonomists, palaeontologists and geologists will remain critically relevant (Cruaud and Rasplus, 2016). Ideally, we want to utilise as much information as possible in our quest to understand the evolution of parasites (De Baets and Littlewood, 2015), and novel methodology increasingly makes this possible. A multidisciplinary approach will create the highest potential for unravelling the major, yet elusive, roles of parasites throughout evolutionary history.

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References

Álvarez-Carretero S, Goswami A, Yang Z, dos Reis M (2019) Bayesian estimation of species divergence times using correlated quantitative characters. Systematic Biology p. syz015

Angelis K, Álvarez-Carretero S, dos Reis M, Yang Z (2018) An evaluation of different partitioning strategies for Bayesian estimation of species divergence times. Systematic Biology 67:61–77

Bandi C, Anderson TJC, Genchi C, Blaxter ML (1998) Phylogeny of *Wolbachia* in filarial nematodes. Proceedings of the Royal Society of London. Series B: Biological Sciences 265:2407–2413

Barido-Sottani J, Pett W, O'Reilly JE, Warnock RCM (2019) FossilSim: an R package for simulating fossil occurrence data under mechanistic models of preservation and recovery. Methods in Ecology and Evolution

Bass D, Stentiford GD, Littlewood DTJ, Hartikainen H (2015) Diverse applications of environmental DNA methods in parasitology. Trends in Parasitology 31:499–513

Benton MJ, Donoghue PCJ, Asher RJ (2009) Calibrating and constraining molecular clocks. In: The Timetree of Life, Oxford University Press Oxford, pp. 35–86

Berry V, Chevenet F, Doyon JP, Jousselin E (2018) A geography-aware reconciliation method to investigate diversification patterns in host/parasite interactions. Molecular Ecology Resources 18:1173–1184

Betts HC, Puttick MN, Clark JW, Williams TA, Donoghue PCJ, Pisani D (2018) Integrated genomic and fossil evidence illuminates lifes early evolution and eukaryote origin. Nature Ecology and Evolution 2:1556

Boast AP, Weyrich LS, Wood JR, Metcalf JL, Knight R, Cooper A (2018) Coprolites reveal ecological interactions lost with the extinction of New Zealand birds. Proceedings of the National Academy of Sciences 115:1546–1551

Bos KI, Harkins KM, Herbig A, et al. (11 co-authors) (2014) Pre-columbian mycobacterial genomes reveal seals as a source of new world human tuberculosis. Nature 514:494

Botero-Castro F, Tilak MK, Justy F, Catzeflis F, Delsuc F, Douzery EJP (2018) In cold blood: compositional bias and positive selection drive the high evolutionary rate of vampire bats mitochondrial genomes. Genome Biology and Evolution 10:2218–2239

Bouckaert R, Vaughan TG, Barido-Sottani J, et al. (22 co-authors) (2019) BEAST 2.5: An advanced software platform for Bayesian evolutionary analysis. PLoS Computational Biology 15:e1006650

Braga MP, Landis MJ, Nylin S, Janz N, Ronquist F (2019) Bayesian inference of ancestral host-parasite interactions under a phylogenetic model of host repertoire evolution. bioRxiv p. 675017

Brayton KA, Knowles DP, McGuire TC, Palmer GH (2001) Efficient use of a small genome to generate antigenic diversity in tick-borne ehrlichial pathogens. Proceedings of the National Academy of Sciences 98:4130–4135

Bromham L (2019) Six impossible things before breakfast: Assumptions, models, and belief in molecular dating. Trends in Ecology and Evolution

Bromham L, Cowman PF, Lanfear R (2013) Parasitic plants have increased rates of molecular evolution across all three genomes. BMC Evolutionary Biology 13:126

Bromham L, Duchêne S, Hua X, Ritchie AM, Duchêne DA, Ho SYW (2018) Bayesian molecular dating: opening up the black box. Biological Reviews 93:1165–1191

Brown JW, Rest JS, García-Moreno J, Sorenson MD, Mindell DP (2008) Strong mitochondrial DNA support for a cretaceous origin of modern avian lineages. BMC Biology 6:6

Chang ES, Neuhof M, Rubinstein ND, Diamant A, Philippe H, Huchon D, Cartwright P (2015) Genomic insights into the evolutionary origin of myxozoa within cnidaria. Proceedings of the National Academy of Sciences 112:14912–14917

Charleston M, Libeskind-Hadas R (2014) Event-based cophylogenetic comparative analysis. In: Modern phylogenetic comparative methods and their application in evolutionary biology, Springer, pp. 465–480

Charleston MA, Robertson DL (2002) Preferential host switching by primate lentiviruses can account for phylogenetic similarity with the primate phylogeny. Systematic Biology 51:528–535

Conway Morris S (1981) Parasites and the fossil record. Parasitology 82:489–509

Corradi N, Pombert JF, Farinelli L, Didier ES, Keeling PJ (2010) The complete sequence of the smallest known nuclear genome from the microsporidian encephalitozoon intestinalis. Nature Communications 1:77

Cruaud A, Rasplus JY (2016) Testing cospeciation through large-scale cophylogenetic studies. Current Opinion in Insect Science 18:53–59

Davín AA, Tannier E, Williams TA, Boussau B, Daubin V, Szöllősi GJ (2018) Gene transfers can date the tree of life. Nature Ecology and Evolution 2:904

De Baets K, Antonelli A, Donoghue PCJ (2016) Tectonic blocks and molecular clocks. Philosophical Transactions of the Royal Society B: Biological Sciences 371:20160098

De Baets K, Dentzien-Dias P, Upeniece I, Verneau O, Donoghue PCJ (2015) Constraining the deep origin of parasitic flatworms and host-interactions with fossil evidence. In: Advances in Parasitology, Elsevier, volume 90, pp. 93–135

De Baets K, Littlewood DTJ (2015) The importance of fossils in understanding the evolution of parasites and their vectors. In: Advances in Parasitology, Elsevier, volume 90, pp. 1–51

de Vienne D, Refrégier G, López-Villavicencio M, Tellier A, Hood ME, Giraud T (2013) Cospeciation vs host-shift speciation: methods for testing, evidence from natural associations and relation to coevolution. New Phytologist 198:347–385

de Vienne DM (2018) Tanglegrams are misleading for visual evaluation of tree congruence. Molecular Biology and Evolution 36:174–176

de Vienne DM, Giraud T, Shykoff JA (2007) When can host shifts produce congruent host and parasite phylogenies? A simulation approach. Journal of Evolutionary Biology 20:1428–1438

Dobson A, Lafferty KD, Kuris AM, Hechinger RF, Jetz W (2008) Homage to linnaeus: how many parasites? How many hosts? Proceedings of the National Academy of Sciences 105:11482–11489

Donoghue PCJ, Benton MJ (2007) Rocks and clocks: calibrating the tree of life using fossils and molecules. Trends in Ecology and Evolution 22:424–431

dos Reis M (2018) Fossil-free dating. Nature Ecology and Evolution 2:771

dos Reis M, Donoghue PCJ, Yang Z (2016) Bayesian molecular clock dating of species divergences in the genomics era. Nature Reviews Genetics 17:71

dos Reis M, Thawornwattana Y, Angelis K, Telford MJ, Donoghue PCJ, Yang Z (2015) Uncertainty in the timing of origin of animals and the limits of precision in molecular timescales. Current Biology 25:2939–2950

dos Reis M, Yang Z (2013) The unbearable uncertainty of Bayesian divergence time estimation. Journal of Systematics and Evolution 51:30–43

Drummond AJ, Ho SYW, Phillips MJ, Rambaut A (2006) Relaxed phylogenetics and dating with confidence. PLoS Biology 4:e88

Duchêne S, Molak M, Ho SYW (2013) Clockstar: choosing the number of relaxed-clock models in molecular phylogenetic analysis. Bioinformatics 30:1017–1019

Dunlop JA (in press) Chelicerates as parasites

Eigenbrode SD, Bosque-Pérez NA, Davis TS (2018) Insect-borne plant pathogens and their vectors: ecology, evolution, and complex interactions. Annual Review of Entomology 63:169–191

Engelstädter J, Fortuna NZ (2019) The dynamics of preferential host shifting: host phylogeny as a key predictor of parasite distribution. Evolution in press

Engelstädter J, Hurst GDD (2006) The dynamics of parasite incidence across host species. Evolutionary Ecology 20:603–616

Engelstädter J, Hurst GDD (2009) The ecology and evolution of microbes that manipulate host reproduction. Annual Review of Ecology Evolution and Systematics 40:127–149

Erwin DH (2006) Dates and rates: temporal resolution in the deep time stratigraphic record. Annual Review of Earth and Planetary Sciences 34:569–590

Evans NM, Holder MT, Barbeitos MS, Okamura B, Cartwright P (2010) The phylogenetic position of myxozoa: exploring conflicting signals in phylogenomic and ribosomal data sets. Molecular Biology and Evolution 27:2733–2746

Felsenstein J (1978) Cases in which parsimony or compatibility methods will be positively misleading. Systematic Zoology 27:401–410

Foster PG (2004) Modeling compositional heterogeneity. Systematic Biology 53:485–495

Frank CA, Amiri H, Andersson SGE (2002) Genome deterioration: loss of repeated sequences and accumulation of junk DNA. Genetica 115:1–12

Galen SC, Borner J, Martinsen ES, Schaer J, Austin CC, West CJ, Perkins SL (2018) The polyphyly of plasmodium: comprehensive phylogenetic analyses of the malaria parasites (order haemosporida) reveal widespread taxonomic conflict. Royal Society Open Science 5:171780

Gavryushkina A, Heath TA, Ksepka DT, Stadler T, Welch D, Drummond AJ (2017) Bayesian total-evidence dating reveals the recent crown radiation of penguins. Systematic Biology 66:57–73

Gavryushkina A, Welch D, Stadler T, Drummond AJ (2014) Bayesian inference of sampled ancestor trees for epidemiology and fossil calibration. PLoS Computational Biology 10:e1003919

Gerth M, Bleidorn C (2016) Comparative genomics provides a timeframe for *Wolbachia* evolution and exposes a recent biotin synthesis operon transfer. Nature Microbiology 2:16241

Goswami A, Upchurch P (2010) The dating game: a reply to Heads (2010). Zoologica Scripta 39:406–409

Gruen DS, Wolfe JM, Fournier GP (2019) Paleozoic diversification of terrestrial chitin-degrading bacterial lineages. BMC Evolutionary Biology 19:34

Hafner MS, Sudman PD, Villablanca FX, Spradling TA, Demastes JW, Nadler SA (1994) Disparate rates of molecular evolution in cospeciating hosts and parasites. Science 265:1087–1090

Harmon L (2018) Phylogenetic comparative methods: learning from trees. EcoEvoRxiv

Heaps SE, Nye TM, Boys RJ, Williams TA, Cherlin S, Embley TM (2019) Generalizing rate heterogeneity across sites in statistical phylogenetics. Statistical Modelling p. 1471082X18829937

Heath TA, Huelsenbeck JP, Stadler T (2014) The fossilized birth–death process for coherent calibration of divergence-time estimates. Proceedings of the National Academy of Sciences 111:E2957–E2966

Heath TA, Moore BR (2014) Bayesian inference of species divergence times. In: Bayesian phylogenetics: methods, algorithms, and applications, Sinauer Associates, Sunderland, MA, pp. 277–318

Heled J, Drummond AJ (2011) Calibrated tree priors for relaxed phylogenetics and divergence time estimation. Systematic Biology 61:138–149

Hirt RP, Logsdon JM, Healy B, Dorey MW, Doolittle WF, Embley TM (1999) Microsporidia are related to fungi: evidence from the largest subunit of RNA polymerase ii and other proteins. Proceedings of the National Academy of Sciences 96:580–585

Ho SYW, Duchêne S (2014) Molecular-clock methods for estimating evolutionary rates and timescales. Molecular Ecology 23:5947–5965

Ho SYW, Phillips MJ (2009) Accounting for calibration uncertainty in phylogenetic estimation of evolutionary divergence times. Systematic Biology 58:367–380

Ho SYW, Phillips MJ, Cooper A, Drummond AJ (2005) Time dependency of molecular rate estimates and systematic overestimation of recent divergence times. Molecular Biology and Evolution 22:1561–1568

Ho SYW, Shapiro B, Phillips MJ, Cooper A, Drummond AJ (2007) Evidence for time dependency of molecular rate estimates. Systematic Biology 56:515–522

Ho SYW, Tong KJ, Foster CS, Ritchie AM, Lo N, Crisp MD (2015) Biogeographic calibrations for the molecular clock. Biology Letters 11:20150194

Höhna S, Landis MJ, Heath TA, Boussau B, Lartillot N, Moore BR, Huelsenbeck JP, Ronquist F (2016) RevBayes: Bayesian phylogenetic inference using graphical models and an interactive model-specification language. Systematic Biology 65:726–736

Holland SM (2016) The non-uniformity of fossil preservation. Philosophical Transactions of the Royal Society B: Biological Sciences 371:20150130

Holland SM, Patzkowsky ME (2002) Stratigraphic variation in the timing of first and last occurrences. Palaios 17:134–146

Holzer AS, Bartošová-Sojková P, Born-Torrijos A, Lövy A, Hartigan A, Fiala I (2018) The joint evolution of the Myxozoa and their alternate hosts: a cnidarian recipe for success and vast biodiversity. Molecular Ecology 27:1651–1666

Hotopp JCD (2011) Horizontal gene transfer between bacteria and animals. Trends in Genetics 27:157–163

Huelsenbeck JP, Rannala B, Larget B (2000) A Bayesian framework for the analysis of cospeciation. Evolution 54:352–364

Hughes DP, Brodeur J, Thomas F (2012) Host manipulation by parasites. Oxford University Press

Hunt VL, Tsai IJ, Coghlan A, et al. (11 co-authors) (2016) The genomic basis of parasitism in the Strongyloides clade of nematodes. Nature Genetics 48:299

Huntley JW, De Baets K (2015) Trace fossil evidence of trematodebivalve parasitehost interactions in deep time. In: Advances in parasitology, Elsevier, volume 90, pp. 201–231

Inoue J, Donoghue PCJ, Yang Z (2009) The impact of the representation of fossil calibrations on Bayesian estimation of species divergence times. Systematic Biology 59:74–89

Jeyaprakash A, Hoy MA (2009) First divergence time estimate of spiders, scorpions, mites and ticks (subphylum: Chelicerata) inferred from mitochondrial phylogeny. Experimental and Applied Acarology 47:1–18

Joffe BI, Kornakova EE (2001) Flatworm phylogeneticist: between molecular hammer and morphological anvil. In: Interrelationships of the Platyhelminthes, Taylor and Francis London, pp. 279–291

Jukes TH, Cantor CR (1969) Evolution of protein molecules. Mammalian Protein Metabolism 3:132

Kado T, Innan H (2018) Horizontal gene transfer in five parasite plant species in Orobanchaceae. Genome Biology and Evolution 10:3196–3210

Kishino H, Thorne JL, Bruno WJ (2001) Performance of a divergence time estimation method under a probabilistic model of rate evolution. Molecular Biology and Evolution 18:352–361

Klopfstein S, Ryser R, Corio M, Spasejovic T (2019) Mismatch of the morphology model is mostly unproblematic in total-evidence dating: insights from an extensive simulation study. bioRxiv p. 679084

Kodandaramaiah U (2011) Tectonic calibrations in molecular dating. Current Zoology 57:116–124

Koutsovoulos G, Makepeace B, Tanya VN, Blaxter M (2014) Palaeosymbiosis revealed by genomic fossils of *Wolbachia* in a strongyloidean nematode. PLoS Genetics 10:e1004397

Krause J, Pääbo S (2016) Genetic time travel. Genetics 203:9–12

Labandeira CC, Sepkoski JJ (1993) Insect diversity in the fossil record. Science 261:310–315

Landis MJ (2017) Biogeographic dating of speciation times using paleogeographically informed processes. Systematic Biology 66:128–144

Landis MJ, Freyman WA, Baldwin BG (2019) Retracing the hawaiian silversword radiation despite phylogenetic, biogeographic, and paleogeographic uncertainty. Evolution 72:2343–2359

Lanfear R, Calcott B, Ho SYW, Guindon S (2012) PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. Molecular Biology and Evolution 29:1695–1701

Lanfear R, Frandsen PB, Wright AM, Senfeld T, Calcott B (2016) PartitionFinder 2: new methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. Molecular Biology and Evolution 34:772–773

Lartillot N, Brinkmann H, Philippe H (2007) Suppression of long-branch attraction artefacts in the animal phylogeny using a site-heterogeneous model. BMC Evolutionary Biology 7:S4

Le TH, Blair D, McManus DP (2002) Mitochondrial genomes of parasitic flatworms. Trends in Parasitology 18:206–213

Lee MSY, Palci A (2015) Morphological phylogenetics in the genomic age. Current Biology 25:R922–R929

Lefoulon E, Bain O, Makepeace BL, dHaese C, Uni S, Martin C, Gavotte L (2016) Breakdown of coevolution between symbiotic bacteria *Wolbachia* and their filarial hosts. PeerJ 4:e1840

Leung TLF (2017) Fossils of parasites: what can the fossil record tell us about the evolution of parasitism? Biological Reviews 92:410–430

Lewis PO (2001) A likelihood approach to estimating phylogeny from discrete morphological character data. Systematic Biology 50:913–925

Li L, Lü L, Nadler SA, Gibson DI, Zhang LP, Chen HX, Zhao WT, Guo YN (2018) Molecular phylogeny and dating reveal a terrestrial origin in the early carboniferous for ascaridoid nematodes. Systematic Biology p. syy018

Littlewood DTJ, Donovan SK (2003) Fossil parasites: a case of identity. Geology Today 19:136–142

Luo A, Duchene D, Zhang C, Zhu CD, Ho S (2019) A simulation-based evaluation of total-evidence dating under the fossilized birth-death process. Systematic Biology p. syz038

Magnabosco C, Moore KR, Wolfe JM, Fournier GP (2018) Dating phototrophic microbial lineages with reticulate gene histories. Geobiology 16:179–189

Mans BJ, de Klerk D, Pienaar R, de Castro MH, Latif AA (2012) The mitochondrial genomes of *Nuttalliella namaqua* (Ixodoidea: Nuttalliellidae) and *Argas africolumbae* (Ixodoidae: Argasidae): estimation of divergence dates for the major tick lineages and reconstruction of ancestral blood-feeding characters. PLoS One 7:e49461

Martínez-Aquino A (2016) Phylogenetic framework for coevolutionary studies: a compass for exploring jungles of tangled trees. Current Zoology 62:393–403

Matschiner M, Musilová Z, Barth JM, Starostová Z, Salzburger W, Steel M, Bouckaert R (2017) Bayesian phylogenetic estimation of clade ages supports trans-atlantic dispersal of cichlid fishes. Systematic Biology 66:3–22

Mitov V, Bartoszek K, Stadler T (2019) Automatic generation of evolutionary hypotheses using mixed Gaussian phylogenetic models. Proceedings of the National Academy of Sciences

Molina J, Hazzouri KM, Nickrent D, et al. (11 co-authors) (2014) Possible loss of the chloroplast genome in the parasitic flowering plant *Rafflesia lagascae* (Rafflesiaceae). Molecular Biology and Evolution 31:793–803

Morris JL, Puttick MN, Clark JW, Edwards D, Kenrick P, Pressel S, Wellman CH, Yang Z, Schneider H, Donoghue PCJ (2018) The timescale of early land plant evolution. Proceedings of the National Academy of Sciences 115:E2274–E2283

Nikbakht H, Xia X, Hickey DA (2014) The evolution of genomic GC content undergoes a rapid reversal within the genus Plasmodium. Genome 57:507–511

Nowak MD, Smith AB, Simpson C, Zwickl DJ (2013) A simple method for estimating informative node age priors for the fossil calibration of molecular divergence time analyses. PLoS One 8:e66245

Ochman H, Wilson AC (1987) Evolution in bacteria: evidence for a universal substitution rate in cellular genomes. Journal of Molecular Evolution 26:74–86

Okamura B, Grühl A (in press) Evolution, origins and diversification of parasitic cnidarians

Olson PD, Caira JN, Jensen K, Overstreet RM, Palm HW, Beveridge I (2010) Evolution of the trypanorhynch tapeworms: parasite phylogeny supports independent lineages of sharks and rays. International Journal for Parasitology 40:223–242

O'Reilly JE, Donoghue PCJ (2017) The efficacy of consensus tree methods for summarizing phylogenetic relationships from a posterior sample of trees estimated from morphological data. Systematic Biology 67:354–362

O'Reilly JE, dos Reis M, Donoghue PCJ (2015) Dating tips for divergence-time estimation. Trends in Genetics 31:637–650

Pacheco MA, Matta NE, Valkiūnas G, et al. (11 co-authors) (2017) Mode and rate of evolution of haemosporidian mitochondrial genomes: timing the radiation of avian parasites. Molecular Biology and Evolution 35:383–403

Papadopoulou A, Anastasiou I, Vogler AP (2010) Revisiting the insect mitochondrial molecular clock: the mid-aegean trench calibration. Molecular Biology and Evolution 27:1659–1672

Parham JF, Donoghue PCJ, Bell CJ, et al. (11 co-authors) (2011) Best practices for justifying fossil calibrations. Systematic Biology 61:346–359

Parins-Fukuchi C (2017) Use of continuous traits can improve morphological phylogenetics. Systematic Biology 67:328–339

Parins-Fukuchi C (2018) Bayesian placement of fossils on phylogenies using quantitative morphometric data. Evolution 72:1801–1814

Poinar GO (2015a) The geological record of parasitic nematode evolution. Advances in Parasitology 90:53–92

Poinar GO (2015b) Rickettsial-like cells in the Cretaceous tick, *Cornupalpatum burmanicum* (Ixodida: Ixodidae). Cretaceous Research 52:623–627

Poinar GO (2018) Vertebrate pathogens vectored by ancient hematophagous arthropods. Historical Biology pp. 1–14

Poisot T (2015) When is co-phylogeny evidence of coevolution? Parasite diversity and diversification: Evolutionary ecology meets phylogenetics p. 420

Poulin R, Randhawa HS (2015) Evolution of parasitism along convergent lines: from ecology to genomics. Parasitology 142:S6–S15

Rannala B, Yang Z (2007) Inferring speciation times under an episodic molecular clock. Systematic Biology 56:453–466

Rector A, Lemey P, Tachezy R, et al. (11 co-authors) (2007) Ancient papillomavirus-host co-speciation in felidae. Genome Biology 8:R57

Reisz RR, Müller J (2004) Molecular timescales and the fossil record: a paleontological perspective. Trends in Genetics 20:237–241

Revell LJ (2012) phytools: An R package for phylogenetic comparative biology (and other things). Methods in Ecology and Evolution 3:217–223

Ricklefs RE, Outlaw DC (2010) A molecular clock for malaria parasites. Science 329:226–229

Roger AJ, Susko E (2018) Molecular clocks provide little information to date methanogenic Archaea. Nature Ecology and Evolution 2:1676

Ronquist F, Klopfstein S, Vilhelmsen L, Schulmeister S, Murray DL, Rasnitsyn AP (2012) A total-evidence approach to dating with fossils, applied to the early radiation of the Hymenoptera. Systematic Biology 61:973–999

Rota-Stabelli O, Daley AC, Pisani D (2013) Molecular timetrees reveal a Cambrian colonization of land and a new scenario for ecdysozoan evolution. Current Biology 23:392–398

Roth S, Balvín O, Siva-Jothy MT, et al. (11 co-authors) (2019) Bedbugs evolved before their bat hosts and did not co-speciate with ancient humans. Current Biology 29:1847–1853

Schenk JJ (2016) Consequences of secondary calibrations on divergence time estimates. PLoS One 11:e0148228

Schenk JJ, Hufford L (2010) Effects of substitution models on divergence time estimates: Simulations and an empirical study of model uncertainty using Cornales. Systematic Botany 35:578–592

Schiffer PH, Robertson HE, Telford MJ (2018) Orthonectids are highly degenerate annelid worms. Current Biology 28:1970–1974

Shapiro B, Hofreiter M (2014) A paleogenomic perspective on evolution and gene function: new insights from ancient DNA. Science 343:1236573

Shapiro B, Rambaut A, Drummond AJ (2006) Choosing appropriate substitution models for the phylogenetic analysis of protein-coding sequences. Molecular Biology and Evolution 23:7–9

Silvestro D, Tejedor MF, Serrano-Serrano ML, Loiseau O, Rossier V, Rolland J, Zizka A, Höhna S, Antonelli A, Salamin N (2018) Early arrival and climatically-linked geographic expansion of New World monkeys from tiny African ancestors. Systematic Biology 68:78–92

Siozios S, Gerth M, Griffin JS, Hurst GDD (2018) Symbiosis: *Wolbachia* host shifts in the fast lane. Current Biology 28:R269–R271

Stadler T (2010) Sampling-through-time in birth–death trees. Journal of Theoretical Biology 267:396–404

Stadler T (2011) Simulating trees with a fixed number of extant species. Systematic Biology 60:675–684

Stadler T (2013) Recovering speciation and extinction dynamics based on phylogenies. Journal of Evolutionary Biology 26:1203–1219

Stadler T, Gavryushkina A, Warnock RCM, Drummond AJ, Heath TA (2018) The fossilized birth-death model for the analysis of stratigraphic range data under different speciation modes. Journal of Theoretical Biology 447:41–55

Steiper ME, Young NM (2008) Timing primate evolution: lessons from the discordance between molecular and paleontological estimates. Evolutionary Anthropology: Issues, News, and Reviews: Issues, News, and Reviews 17:179–188

Suh A, Witt CC, Menger J, et al. (11 co-authors) (2016) Ancient horizontal transfers of retrotransposons between birds and ancestors of human pathogenic nematodes. Nature Communications 7:11396

Tarasov S (2019) Integration of anatomy ontologies and evo-devo using structured markov models suggests a new framework for modeling discrete phenotypic traits. Systematic Biology p. Published online ahead of print

Tavaré S (1986) Some probabilistic and statistical problems in the analysis of DNA sequences. Lectures on Mathematics in the Life Sciences 17:57–86

Tong KJ, Duchêne DA, Duchêne S, Geoghegan JL, Ho SYW (2018) A comparison of methods for estimating substitution rates from ancient DNA sequence data. BMC Evolutionary Biology 18:70

Turelli M, Cooper BS, Richardson KM, et al. (11 co-authors) (2018) Rapid global spread of wri-like *Wolbachia* across multiple Drosophila. Current Biology 28:963–971

Verneau O, Bentz S, Sinnappah ND, Preez Ld, Whittington I, Combes C (2002) A view of early vertebrate evolution inferred from the phylogeny of polystome parasites (Monogenea: Polystomatidae). Proceedings of the Royal Society of London Series B: Biological Sciences 269:535–543

Warnock RCM, Parham JF, Joyce WG, Lyson TR, Donoghue PCJ (2015) Calibration uncertainty in molecular dating analyses: there is no substitute for the prior evaluation of time priors. Proceedings of the Royal Society of London. Series B: Biological Sciences 282:20141013

Warnock RCM, Yang Z, Donoghue PCJ (2011) Exploring uncertainty in the calibration of the molecular clock. Biology Letters p. rsbl20110710

Warnock RCM, Yang Z, Donoghue PCJ (2017) Testing the molecular clock using mechanistic models of fossil preservation and molecular evolution. Proceedings of the Royal Society of London. Series B: Biological Sciences 284:20170227

Weinert LA, Araujo-Jnr EV, Ahmed MZ, Welch JJ (2015) The incidence of bacterial endosymbionts in terrestrial arthropods. Proceedings of the Royal Society of London. Series B: Biological Sciences 282:20150249

Werren JH, Baldo L, Clark ME (2008) *Wolbachia*: master manipulators of invertebrate biology. Nature Reviews Microbiology 6:741–751

Werren JH, Zhang W, Guo LR (1995) Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. Proceedings of the Royal Society of London. Series B: Biological Sciences 261:55–63

Williams TA, Foster PG, Cox CJ, Embley TM (2013) An archaeal origin of eukaryotes supports only two primary domains of life. Nature 504:231

Windsor DA (1998) Controversies in parasitology, most of the species on earth are parasites. International Journal for Parasitology 28:1939–1941

Wolfe JM, Fournier GP (2018a) Horizontal gene transfer constrains the timing of methanogen evolution. Nature Ecology and Evolution 2:897

Wolfe JM, Fournier GP (2018b) Reply to molecular clocks provide little information to date methanogenic archaea. Nature Ecology and Evolution 2:1678

Wood JR (2018) DNA barcoding of ancient parasites. Parasitology pp. 1–10

Wright AM (2019) A systematists guide to estimating Bayesian phylogenies from morphological data. Insect Systematics and Diversity 3:2

Wright AM, Lloyd GT, Hillis DM (2016) Modeling character change heterogeneity in phylogenetic analyses of morphology through the use of priors. Systematic Biology 65:602–611

Yang Z (1994) Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: approximate methods. Journal of Molecular Evolution 39:306–314

Yang Z, Lauder IJ, Lin HJ (1995) Molecular evolution of the hepatitis B virus genome. Journal of Molecular Evolution 41:587–596

Yang Z, Rannala B (2006) Bayesian estimation of species divergence times under a molecular clock using multiple fossil calibrations with soft bounds. Molecular Biology and Evolution 23:212–226

Yoshizawa K, Johnson KP (2013) Changes in base composition bias of nuclear and mitochondrial genes in lice (Insecta: Psocodea). Genetica 141:491–499

Zhang C, Stadler T, Klopfstein S, Heath TA, Ronquist F (2015) Total-evidence dating under the fossilized birth–death process. Systematic Biology 65:228–249

Zhu T, dos Reis M, Yang Z (2015) Characterization of the uncertainty of divergence time estimation under relaxed molecular clock models using multiple loci. Systematic Biology 64:267–280

Zuckerkandl E, Pauling L (1962) Molecular disease, evolution and genetic heterogeneity. In: Horizons in Biochemistry, Academic Press, pp. 189–225

Zuckerkandl E, Pauling L (1965) Evolutionary divergence and convergence in proteins. In: Evolving Genes and Proteins, Elsevier, pp. 97–166

Zug R, Hammerstein P (2015) Bad guys turned nice? A critical assessment of *Wolbachia* mutualisms in arthropod hosts. Biological Reviews of the Cambridge Philosophical Society 90:89–111