Bayesian molecular clock dating of species divergences in the genomics era

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Abstract | It has been five decades since the proposal of the molecular clock hypothesis, which states that the rate of evolution at the molecular level is constant through time and among species. This hypothesis has become a powerful tool in evolutionary biology, making it possible to use molecular sequences to estimate the geological ages of species divergence events. With recent advances in Bayesian clock dating methodology and the explosive accumulation of genetic sequence data, molecular clock dating has found widespread applications, from tracking virus pandemics, to studying the macroevolutionary process of speciation and extinction, to estimating a timescale for Life on Earth.

Introduction

Five decades ago, Zuckerkandl and Pauling published two seminal papers proposing the concept of the molecular evolutionary clock[1, 2], that is, that the rate of evolution at the molecular level is approximately constant through time and among species. The idea arose when the pioneers of molecular evolution compared protein sequences (haemoglobins, cytochrome c, fibrinopeptides) from different species of mammals[1, 3, 4], and observed that the number of amino acid differences between species correlated with their divergence time based on the fossil record. The field of molecular evolution was revolutionized by this hypothesis (albeit not without controversy[5-8], Box 1) and biologists took on the task of using the molecular clock as

a technique to infer dates of major species divergence events in the Tree of Life[9].

From the outset, the molecular clock was not perceived as a perfect timepiece but, rather, as a stochastic clock, in which mutations accumulate at random intervals, albeit at roughly the same rate in different species, keeping time as a clock does. Initial statistical clock-dating methodology based on distance and maximum likelihood methods assumed a perfectly constant rate of evolution (the 'strict' clock), and used fossil-age calibrations that are point values even though the fossil record can never provide a precise date estimate for a clade. Subsequent tests of the molecular clock[10, 11] showed that it is often 'violated', that is, the molecular evolutionary rate is not constant, except in comparisons of closely related species, such as the apes. When the rates vary among species, multiple factors might influence the molecular evolutionary rate (such as generation time, population size, basal metabolic rate, etc.); however, the exact mechanisms of rate variation and the relative importance of these factors are still a matter of debate[7, 12, 13]. When the clock is violated, methods for dealing with the rate variation include removal of species exhibiting unusual rates[14], and the so-called local-clock models, which arbitrarily assign branches to rate classes[15, 16]. Sophisticated statistical models that take into account uncertainty in the fossil record as well as variation in evolutionary rate — and thus enable the strict clock assumption to be 'relaxed' — were not developed until the advent of Bayesian methods in the late 1990s to early 2000s. It is now generally acknowledged that the molecular clock cannot be applied globally, or for distantly related species. However, for closely related species, or in analysis of population data, the molecular clock is a good approximation of reality (Box 2).

Next-generation sequencing technologies and advances in Bayesian phylogenetics over the past decade have led to a dramatic increase in molecular clock dating studies. Examples of recent applications of the molecular clock include the rapid analysis of the 2014 Ebola virus outbreak[17], characterization of the origin and spread of HIV[18] and influenza[19, 20], ancient DNA studies to reconstruct a timeline for the origin and migration patterns of modern humans[21-23], use of timetrees to infer macroevolutionary patterns of speciation and extinction through time[24, 25], and the co-evolution of Life and the Planet[26, 27]. Knowledge of absolute times of species divergences has proven critically important for the interpretation of newly sequenced

genomes[23, 28]. Exciting new developments in Bayesian phylogenetics include relaxed clock models to accommodate the violation of the clock[29-31]; modelling of fossil preservation and discovery to generate prior probability distributions of divergence times to be used as calibrations in molecular clock dating[32]; and integration of morphological characters from modern and extinct species in a combined analysis with sequencing data[33, 34].

Here, we review the history, prospects and challenges of using molecular clock dating to estimate the timescale for the Tree of Life, particularly in the genomics era, and trace the rise of the Bayesian molecular clock dating method as a framework for integrating information from different sources, such as fossils and genomes. Non-Bayesian clock-dating methods, while still being proposed[35-38] typically do not accommodate the different sources of uncertainty in the dating analysis adequately, and are thus severely limited. They usually inolve less computation and may thus be useful for analysing very large datasets for which the Bayesian method is still computationally prohibitive. A detailed review of those methods can be found elsewhere[39].

Early attempts to estimate the time tree of life

Time trees, or phylogenies with absolute divergence times, provide incomparably richer information than a species phylogeny without temporal information, as they make it possible for species divergence events to be calibrated to geological time, from which correlations can be made to events in Earth History and, indeed, to other events in biotic evolution (i.e. by placing them in the correct palaeoclimate or geological environment), thus allowing for macroevolutionary hypotheses of species divergences and extinctions to be tested.

As the first protein and DNA sequences became available for a diversity of species, biologists started using the molecular clock as a simple, yet powerful, tool to estimate species divergence times. Underlying the notion that molecules can act as a clock is the theory that the genetic distance between two species, which is determined by the number of mutations accumulated in genes or proteins over time, is proportional to the time of species divergence (Box 1). If the time of divergence between two species is known — from fossil evidence, from a geological event, such

as continental break-up or island formation, or from sample dates for bacteria and viruses — the genetic distance between these species can be converted into an estimate of the rate of molecular evolution, which can be applied to all nodes on the species phylogeny to produce estimates of absolute geological times of divergence (Box 2). One of the first applications of this idea was by Sarich and Wilson[40], who used a molecular clock to infer the immunological distance of albumins. By assuming a divergence time of 30 Ma between the apes and New World monkeys, they calculated the age of the last common ancestor of humans and African apes (chimpanzee and gorilla) as 5 Ma. The work ignited one of the first 'fossils versus molecules' controversies as, at the time, the divergence between human and African apes had been thought to be over 14 Ma based on ages of the fossils *Ramapithecus* and *Sivapithecus*[41]. The controversy was settled once it was recognised that the fossils are more closely related to the orang-utan than to the African apes.

In response to expanding genetic sequence datasets resulting from the PCR revolution in the late 1990s, molecular clock dating was applied to a broad range of species. These studies generated considerable controversy because the clock estimates were much older than the dates suggested by the fossil record, sometimes twice as old[42], and many palaeontologists considered the discrepancy to be unacceptably large[43]. Examples include Mesoproterozoic estimates for the timing of origin and diversification of the animal phyla relative to their Phanerozoic fossil record[44], a Triassic origin of flowering plants relative to a fossil record beginning in the Cretaceous[45], and a Jurassic or Cretaceous origin of modern birds and placental mammals relative to fossil evidence confined largely to the period after the end-Cretaceous mass extinction[46, 47].

The early dating studies suffer from a number of limitations[48, 49]. For example, many studies assumed a strict clock even for distantly related species, and most used point fossil calibrations without regard for their uncertainty[25, 47]. Sometimes secondary calibrations, that is, node ages estimated in previous molecular clock dating studies, were used[48]. Despite their limitations, these studies encouraged much discussion about the nature of the fossil record and the molecular clock[49], and inspired the development of more sophisticated methods. These early studies proposed a timescale for Life on Earth that has now been revised in the newer

genome-scale analyses[24, 50, 51].

The Bayesian method of clock dating

The Bayesian method was introduced into molecular clock dating around 2000 in a series of seminal papers by Jeff Thorne and colleagues[29, 52, 53]. The method has been developed greatly since then[30, 31, 54, 55], emerging as the dominant approach to divergence time estimation due to its ability to integrate different sources of information (in particular, fossils and molecules) while acommodating the uncertainties involved.

The Bayesian method is a general statistical methodology for estimating parameters in a model. Its main feature is the use of statistical distributions to characterize uncertainties in all unknowns. One assigns a prior probability distribution on the parameters, which is combined with the information in the data (in the form of the likelihood function) to produce the posterior probability distribution. In molecular clock dating, the parameters are the species divergence times ($\boldsymbol{t}$) and the evolutionary rates ($\boldsymbol{r}$). Given the sequence data (D), the posterior of times and rates is given by the Bayes theorem as

$$f(\boldsymbol{t}, \boldsymbol{r}|D) = \frac{1}{Z} f(\boldsymbol{t})\, f(\boldsymbol{r}|\boldsymbol{t})\, L(D|\boldsymbol{t}, \boldsymbol{r}). \quad (1)$$

Here $f(\boldsymbol{t})$ is the prior on divergence times, which is often specified using a model of cladogenesis (of speciation and extinction[54, 56], etc.) and incorporates the fossil calibration information[52, 54], $f(\boldsymbol{r}|\boldsymbol{t})$ is the prior on the rates for branches on the tree, which is specified by using a model of evolutionary rate drift[29-31], and $L(D|\boldsymbol{t}, \boldsymbol{r})$ is the likelihood or the probability of the sequence data, which is calculated using standard algorithms[11]. Figure 1 illustrates the Bayesian clock dating of equation (1) in a two-species case.

Direct calculation of the proportionality constant Z in equation (1) is not feasible. In practice, one uses a simulation algorithm called Markov Chain Monte Carlo (MCMC) to generate a sample from the posterior distribution. The MCMC algorithm is computationally expensive, and a typical MCMC clock-dating analysis may take from a few minutes to several months for large genome-scale datasets. Methods that approximate the likelihood, can speed up the analysis substantially[29, 57, 58]. Technical reviews on Bayesian and MCMC molecular clock-dating can be found in [59, 60].

Now nearly a dozen computer software packages exist for Bayesian dating analysis (Table 1), all of them incorporating models of rate variation among lineages (the episodic- or relaxed-clock models envisioned by Gillespie[61]). All of these programs can also analyze multiple gene loci, and accommodate multiple fossil calibrations in one analysis.

Limits of Bayesian divergence time estimation

Estimating species divergence times based on uncertain calibrations is challenging. The main difficulty is that the molecular sequence data provide information about the molecular distances (the product of times and rates) but not about times and rates separately. In other words, the time and rate parameters are unidentifiable. Thus, in Bayesian clock dating, the sequence distances are resolved into absolute times and rates through the use of priors. In a conventional Bayesian estimation problem, the prior becomes unimportant and the Bayesian estimates converge to the true parameter values as more and more data are analyzed. However, convergence on truth does not happen in divergence time estimation. The use of priors to resolve times and rates has two consequences. First, as more loci or longer and longer sequences are included in the analysis (but the calibration information does not change), the posterior time estimates will not converge to point values and will instead involve uncertainties[31, 53, 54]. Second, the priors on times and on rates will have an important impact on the posterior time estimates even if a huge amount of sequence data is used[62, 63]. Errors in the time prior and in the rate prior can lead to very precise but grossly inaccurate time estimates[62, 64]. Great care must always be taken in the construction of fossil calibrations and in the specification of priors on times and on rates in a dating analysis[65, 66].

As the amount of sequence data approximates genome scale, the molecular distances or branch lengths on the phylogeny are essentially determined without any uncertainty, as are the relative ages of the nodes. However, the absolute ages and absolute rates are cannot be known without additional information (in the form of priors). The joint posterior of times and rates is then one-dimensional. This reasoning has been used to determine the limiting posterior distribution when the amount of sequence data (i.e. the number of loci or the length of the sequences) increases

without bound[31, 54]. An infinite-sites plot can be used to determine whether the amount of sequence data is saturated or whether including more sequence data is likely to improve the time estimates (Fig. 2). The theory has been extended to the analysis of large but finite datasets, to partition the uncertainties in the posterior time estimates according to different sources: uncertain fossil calibrations and finite amount of sequence data [62, 63]. Application of the theory to analysis of a few real data sets (including genome-scale data) has indicated that most of the uncertainty in the posterior time estimates is due to uncertain calibrations rather than limited sequence data[24, 66].

Relaxed clock models — the prior on rates

Unsurprisingly, divergence time estimation under the strict molecular clock is highly unreliable when the clock is seriously violated. In early studies it was common to remove genes and/or lineages that violated the clock from the analysis[14], but this method does not make efficient use of the data, and is impractical when the clock is violated by too many genes or species. Relaxed clock models have been developed to allow the molecular rate to vary among species. The first methods were developed under the penalized-likelihood and maximum-likelihood frameworks[67, 68]. In Bayesian clock dating, such models are integrated in the analysis as the prior on rates.

Several types of relaxed-clock models have been implemented, using either continuous or discrete rates. In the geometric Brownian motion model[29, 31, 52], also called autocorrelated-rates model, the logarithm of the rate drifts over time as a Brownian motion process (Fig. 3a). Let $y_0 = \log(r_0)$ and $y_t = \log(r_t)$, where r_0 is the ancestral rate at time 0 while r_t is the rate time t later. Then $y_t \mid y_0 \sim N(y_0, t\nu)$; that is, given y_0 (or the ancestral rate r_0), y_t has a normal distribution with mean y_0 and variance $t\nu$ (or r_t has a log-normal distribution). Thus, rates on descendent branches are similar to the rate of the ancestral branch, especially if the branches cover short timescales, and furthermore, the variance of the rate increases with the passage of time. An unappealing property of Brownian motion is that it does not have a stationary distribution. Over a very long timescale, the log-rate can drift to very negative or very positive values with the rate becoming near 0 or very large, and the variance of the rate tends to infinity with time. This does not appear to be realistic. A model that does not have this property is the (geometric) Ornstein-Uhlenbeck model

(Fig. 3b). The logarithm of the rate follows Brownian motion with a dampening force, leading to a stationary distribution. This model (and the related Cox-Ingersoll-Ross model[55]) looks promising and merits further research. Note that an early implementation of the Ornstein-Uhlenbeck model[69] to clock inadvertedly assumed that evolutionary rates drift to zero with time[70]. Another type of relaxed-clock model assumes a small number of distinct rates on the tree, and assigns branches to the rate classes through a random process[71-73]. It is also possible to assume that the rates for branches on the tree are uncorrelated and are random draws from the same common distribution such as the log-normal[30, 31] (Fig. 3c).

Fossil calibrations — the prior on times

Molecular clock analyses are most commonly calibrated using evidence from the fossil record[74, 75]. Geological events such as the closure of the Isthmus of Panama or continental break-ups can also be used as calibrations, although such calibrations may involve a lot of uncertainties as well due to assumptions about vicariance, species dispersal potential, etc[76]. In Bayesian clock dating, calibration information is incorporated in analysis through the prior on times.

It has long been recognized that the fossil record is incomplete – temporally, spatially and taxonomically – and long time gaps may exist between the oldest known fossils and the last common ancestor of a group. The first known appearance of a fossil member of a group cannot be interpreted as the time and place of origination of the taxonomic group[77]. For example, during the 1980s the oldest known members of the human lineage were the Australopithecines, dating to around 4 Ma[41], providing a minimum age for the divergence time between human and chimpanzee. However, since 2000, several fossils belonging to the human lineage were discovered in quick succession: *Ardipithecus* (4.4 Ma), *Orrorin* (6 Ma), and *Sahelanthropus* (7 Ma), pushing the age of the human-chimpanzee ancestor to over 7 Ma[78]. Some groups have no known fossil record, such as the Malagasy lemurs (only a few hundred year old sub-fossils are known[79]). The oldest fossil in their sister lineage (the galagos and lorises) dates to 38 Ma, indicating a minimum 38 My gap in the fossil record of lemurs[80]. Clearly, fossil ages provide good minimum-age bounds on clade ages, but assuming that clade ages are the same as that of their oldest fossil is unwarranted and wrong[81, 82].

However, minimum-age bounds alone are insufficient for calibrating a molecular tree. Recent developments in Bayesian dating methodology have enabled 'soft bounds' and arbitrary probability curves to be used as calibrations[30, 54, 83]. Soft bounds assign small probabilities (such as 5% or 10%) for the violation of the bounds[54]. Those developments have motivated palaeontologists to formulate probabilistic densities for the *true clade age*s, rather than focusing on the minimum age. A program has been launched in palaeontology to reinterpret the fossil record to provide both sharp minimum bounds and soft maximum bounds on clade ages[84, 85].

We envisage several strategies for generating fossil calibrations, each of which may be appropriate depending on the available data. First, one may use the absence of evidence (the lack of occurrence of fossil species in the rock record) as weak evidence of absence and construct soft maximum age bounds[81, 82]. Together with hard or sharp minimum-age bounds, they can be used as calibrations. This procedure may involve some subjectivity. Second, fossil occurrences in the rock layers can be analyzed using probabilistic models of fossil preservation and discovery to generate posterior distributions of node ages, which can be used in later molecular dating studies[32, 56, 86-88]. Third, if morphological characters are scored for both modern and fossil species, they can be analyzed using models of morphological character evolution to estimate node ages, which serve as calibrations in molecular clock dating. It is advisable to fix the phylogeny for modern species while letting the placement of the fossil species to be determined by the data. Fossil remains are typically incomplete and their phylogenetic placement most often involve uncertainties[89]. It is also possible to analyse the fossil/morphological data and the molecular data in one joint analysis as discussed below (so-called total evidence dating[34]).

Joint analysis of molecular and morphological data

Morphological characters from both fossil species (which have dates) and modern species may be analyzed jointly with molecular data under models of morphological character evolution to estimate divergence times[33, 34]. The analysis is statistically similar to the analysis of serially sampled sequences in molecular dating of viral or ancient DNA or proteins (Box 3). A perceived advantage of such a 'tip dating' or 'total

evidence dating' approach is that they make it unnecessary to use constraints on node ages (the so-called node dating). The approach also facilitates coestimation of time and topology. Recent applications of this strategy to insects[34], spiders[90, 91], fish[92, 93] and mammals[94-96] have produced surprisingly ancient divergence times[97].

While tip dating offers a coherent framework for integrating information from molecules and fossils in one combined analysis, its current implementations involve a number of limitations, which may underlie these old date estimates. First, current models of morphological character evolution are simplistic and may not accommodate important features of the data well[98]. For example, morphological characters tend to be strongly correlated, but almost all current models assume independence. Furthermore all recent tip-dating studies analysed discrete morphological characters, but morphologists usually score only variable characters or parsimony-informative characters. Such ascertainment bias, even if accommodated correctly in the model[98], greatly reduces information about branch lengths and divergence times in the data. Whereas removal of constant characters can be easily accommodated[98], removal of parsimony-uninformative characters would require too much computation and is not achieved by any current dating software. Second, a tip dating analysis does not place any constraints on the ages of internal nodes on the tree and may thus be very sensitive to the prior of divergence times or the branching process used to generate that prior than dating using node calibrations. In a sense, node-dating, while using node calibrations that may be subjective, allows the paleontologist's common sense to be injected into the Bayesian analysis. In contrast, tip-dating may be unduly influenced by arbitrary choices of priors implemented in the computer program. Third, it is generally the case that there is far more molecular data than morphological characters, and that the rate of morphological character evolution is much more variable among lineages than molecular rates[6]. Box 2 presents a case of the cranial evolution within the hominoids, in which the rate in the human is about eight times as high as in the chimpanzee. Such drastic changes in morphological evolutionary rate contrast sharply with the near perfect clock-like evolution of the mitochondrial genome from the same species. Characters with drastically variable evolutionary rates, even if the rate variation is adequately accommodated in the model, will not provide much useful time information for the dating analysis. The small amount of morphological

data and the low information content (due to variable rates) mean that the priors on times and rates will remain important to the dating analysis. Finally, we note that most tip-dating studies have not integrated any of the uncertainty associated with fossil dating[97].

Resolving the timeline of the Tree of Life

The molecular clock is now serving as a framework to integrate genomic and palaeontological data to estimate time trees. Advancements in Bayesian clock dating methodology, increased computational power, and the accumulation of genome-scale sequence data have provided us with an unprecedented opportunity to achieve this objective. However, considerable challenges remain. Although next-generation sequencing technologies[99] now enable the cheap and rapid accumulation of genome data for many species[100], much work still remains to be done to obtain a balanced sampling of biodiversity: some estimates place the fraction of living eukaryotic species that have been described at about 14%[101], and sequence data is available for a much smaller and skewed fraction. More seriously, fossils are unavailable for most branches of the Tree of Life, and other sources of information (such as geological events[76] or experimentally measured mutation rates[23]) are available only rarely[102]. The amount of information in fossil morphological characters may never match the information about sequence distances in the genomic data, placing limits on the precision achievable in estimation of ancient divergence times, because fossil information is used to resolve sequence distances into absolute times and rates using that information. The problem seems particularly severe in dating ancient divergences, such as the origins of animal phyla[103], because at deeper divergences, the quality of fossil data tends to be poor, and the evolutionary rates for both morphological characters and sequence data are highly variable among distantly related species.

Challenges also remain in the development of the statistical machinery necessary for molecular clock dating. Current models of morphological evolution are simplistic and should be improved to accommodate different types of data and to account for the correlation between characters. In analysis of genomic-scale datasets under relaxed-clock models, data partitioning is an important but poorly studied area. The rationale for partitioning the sequence data is that sites in the same partition are expected to

share the same trajectory of evolutionary rate drift while those in different partitions do not, so that the different partitions constitute independent realizations of the rate-drift process (e.g., geometric Brownian motion). Theoretical analysis suggests that the precision of posterior time estimates is largely determined by the number of partitions rather than the number of sites in each partition[63]. However, the different strategies for partitioning large datasets for molecular clock dating analysis are poorly explored. Furthermore, the prior model of rate drift for data of multiple paritions appears to be very important to Bayesian divergence time estimation[53], but currently implemented rate models are highly unrealistic. All current dating programs assume independent rates among partitions, failing to accommodate the lineage effect, the fact that some evolutionary lineages or species tend to be associated with high (or low) rates for almost all genes in the genome[13]. Developing more realistic relaxed-clock models for multi-partition data and evaluating their effects on posterior time estimation will be a major research topic for the next few years. Another issue that has been underapreciated in clock dating studies is the fact that speciation events are more recent than gene divergences[104] (a result of the coalescent process of gene copies in ancestral populations), and ignoring this may cause important errors when estimating divergence times[105].

Despite the multitude of challenges, the prospect for a broadly reliable timescale for Life on Earth is looking more likely than ever before. Genome-scale sequence data are now being applied to resolve iconic controversies between fossils and molecules. For example, Bayesian clock dating using genome-scale data has demonstrated that modern mammals and birds diversified after the K-Pg boundary[24, 50], in contrast to non-Bayesian estimates based on limited sequence data that had suggested pre-K-Pg diversification[25, 47]. Similarly, Bayesian clock dating analysis of insect genomes has been used to elucidate the time of insect origination in the Early Ordovician[51]. We predict that the explosive increase in completely sequenced genomes, together with the development of efficient Bayesian strategies to analyse morphological and molecular data from both modern and fossil species, will eventually allow biologists to resolve the timescale for the Tree of Life. It seems that in reaching its half-century, the molecular clock has finally come of age.

Box 1 | The clock and the neutral theory of molecular evolution

Zuckerkandl and Pauling provided a justification for the clock by suggesting that amino acid changes that accumulate between species are mostly those with little or no effect on the structure and function of the protein, thus reflecting the background mutational process at the DNA level[1]. This hypothesis was formalised by Kimura[106] and King and Jukes[107] in the neutral theory of molecular evolution, which claims that most of the genetic variation we observe (either polymorphism within species or divergence between species) is due to chance fixation of selectively neutral mutations, rather than fixation of advantageous mutations driven by natural selection[6]. Thus, the molecular clock was soon entwined in the controversy surrounding the neutral theory, which was proposed initially to explain the *s*urprising finding of high levels of polymorphisms in natural populations[108, 109]. If molecular evolution is dominated by neutral mutations, which have little influence on the survival or reproduction of the individual, then an approximately constant rate of evolution is plausible. Indeed under the theory*,* the rate of molecular evolution is equal to the neutral mutation rate, which can be assumed to be similar among species with similar life histories.

Most mutations that arise in a generation in a large population get lost by chance within a small number of generations. This is true not only for deleterious and neutral mutations, but also for advantageous mutations unless the advantage is extremely large. For example, if a mutation offers a 1% selective advantage (which is a very large advantage), the chance is only about 2% that it will eventually spread through the whole population[110]. The minority of mutations that get fixed eventually in the population are called substitutions. Viewed over a very long time scale, this process of new mutations going to fixation, replacing previous wildtype alleles, is the process of molecular evolution. Suppose the total mutation rate is μ per generation, and a fraction f_0 of the mutations are neutral. The rest of mutations are deleterious and are removed by natural selection and do not contribute to the evolutionary process. There are $2N \times \mu f_0$ neutral mutations per generation for a diploid population of size N. The chance that a neutral mutation will eventually reach fixation is $1/(2N)$, because there are $2N$ alleles in the population and each has the same chance of reaching fixation. The molecular substitution rate per generation, r, (that is, the number of mutations per generation that reach fixation in the population) is thus equal to the number of new neutral mutations produced in each generation times the

probability that they will eventually reach fixation, that is,

$r = 2N\mu f_0 \times 1/(2N) = \mu f_0$.

In other words, the substitution rate is equal to the neutral mutation rate (μf_0) [111]. According to this neutral mutation-random drift theory (or the neutral theory), the rate of molecular evolution reflects the neutral mutation rate, independent of the population size. Thus the molecular clock holds if μ and f_0 are approximately constant through time and similar among closely related species.

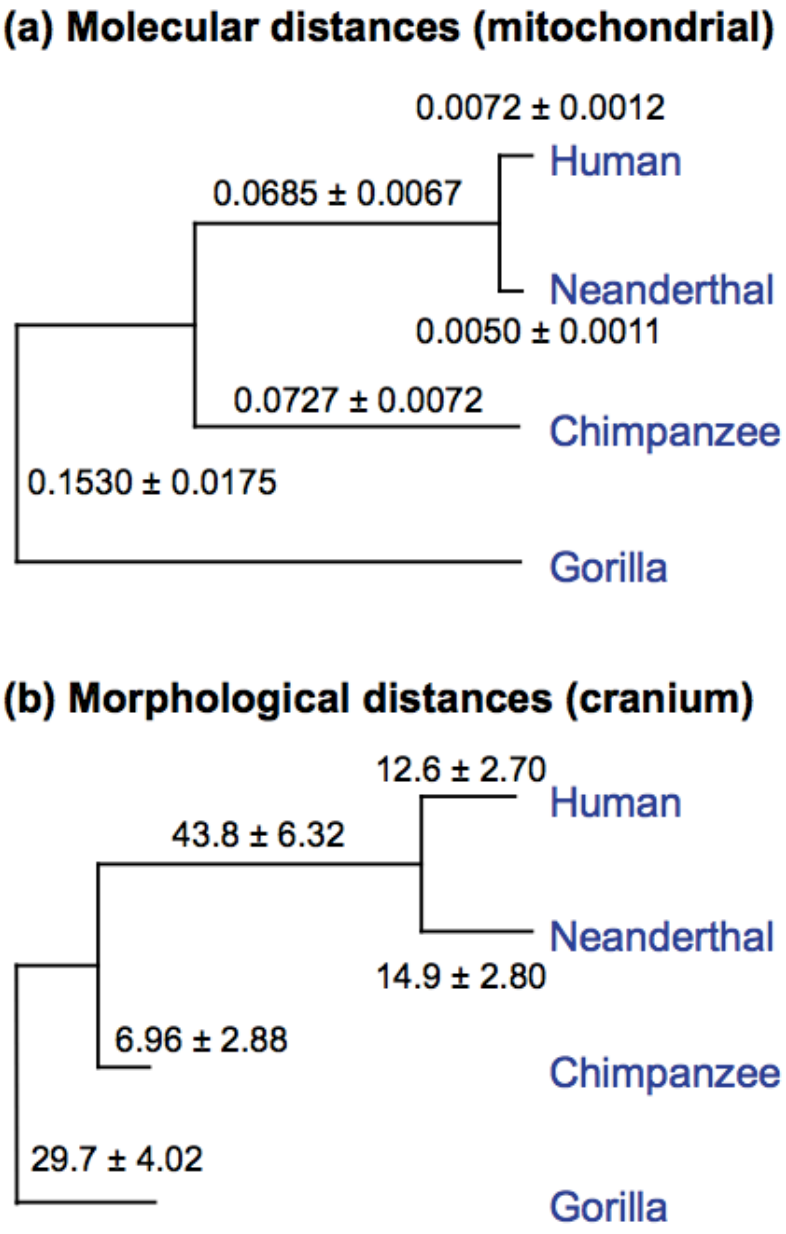
Hence, the neutral theory offers an explanation for the molecular clock and, for a time, the clock was considered the most important evidence supporting the neutral theory[6]. Proteins with different functional constraints may have different proportions of neutral mutations (f_0), so that they have different rates of neutral mutation, and their clocks tick at different rates. Extensive reviews of the clock-neutral theory controversy are given elsewhere[6, 7, 112].

Box 2 | Clocklike molecular evolution versus non-clocklike morphological evolution

Molecular sequences may evolve at a nearly constant rate among close species. An alignment of the mitochondrial genomes (15,889 bp) of human (H), Neanderthal (N), chimpanzee (C) and gorilla (G) was analyzed by maximum likelihood under the GTR+Γ_4 model[113, 114] to estimate the branch lengths without the assumption of a molecular clock. The molecular distance (± standard error) from the common ancestor of human-chimpanzee (HC) to the human is $d_{H\text{-}HC}$ = 0.0757 ± 0.00681 and that from HC to the chimpanzee is $d_{C\text{-}HC}$ = 0.0727 ± 0.00721. Those distances are nearly identical, as expected under the molecular clock hypothesis. Indeed, the strict clock hypothesis is not rejected by a likelihood-ratio test[11] (P = 0.60). The rate constancy of the mitochondrial genome allows us to date the age of the common ancestor of the human and Neanderthal (HN). Under the clock, the times are proportional to the distances, so that t_{HN}/t_{HC} = 0.0072/0.0757 = 0.0951. The fossil record suggests that the HC ancestor lived 10-6.5 Ma[115]. Thus, we obtain 0.95-0.62 Ma for the age of the HN ancestor.

By contrast, evolutionary rates of morphological characters may be much more variable. The 151 cranium landmark measurements from the same four species[116] were aligned and analyzed using maximum likelihood under Felsenstein's trait-

evolution model[117]. The morphological branch lengths (in units of expected accumulated variance) are shown on the tree. From the branch lengths $b_{H\text{-}HC}$ = 56.4 ± 6.87 and $b_{C\text{-}HC}$ = 6.96 ± 2.88, we see that the human cranium has changed 8.1 times as fast as the chimpanzee since the split of the two species. Driven by natural selection, the human cranium has rapidly become larger and rounder, with a smaller and more protracted face.



Box 3 | Dating divergences using serially sampled sequences

For viral sequences that evolve very fast, it is possible to observe mutations at the different times the viral sequences are sampled. The different sampling times in combination with the different amounts of evolution reflected in the genetic distances

can be used to date the divergence events[118-121]. For example, the genome of the 1918 pandemic influenza virus has been sequenced from samples obtained in individuals who died in 1918 and were buried in the Alaskan permafrost[122]. Analysis of the genomic sequences has allowed estimation of divergence times for the ancestors of the virus[19, 20] and propose scenarios for the origin of the pandemic, for example, a possible swine origin for the virus[123]. Similar approaches have also been used to study the origins of the HIV pandemic in humans, tracing its origins in the West Africa, its spread in African cities during the mid-20th century and its later spread to the Americas, Europe and the rest of the world[18, 124, 125].

The strategy of using sequences with sampling dates also applies to studies of ancient DNA (or proteins). Ancient sequence data are informative about times and rates separately, and divergence times can be estimated with high precision if the events to be dated are not much older than the sampling times covered by the data. Analysis of ancient DNA offers exciting prospects to elucidate evolutionary timelines. For example, analysis of several hundred ancient DNA samples from Bison, dating up to 60 Ka, allowed estimation of the timeline of evolution of bison populations, charting the rise and subsequent fall of bison populations in the northern hemisphere throught the late Pleistocene and Holocene epochs[126]. Other examples of ancient clock studies include dating the origins of horses[127], camels[128] and humans[129]. The approach is limited by our ability to sequence ancient, highly degraded material[130]. The oldest molecular material sequenced date to 0.78–0.56 Ma for DNA[127] and to 80 Ma (controversially) for proteins[131].

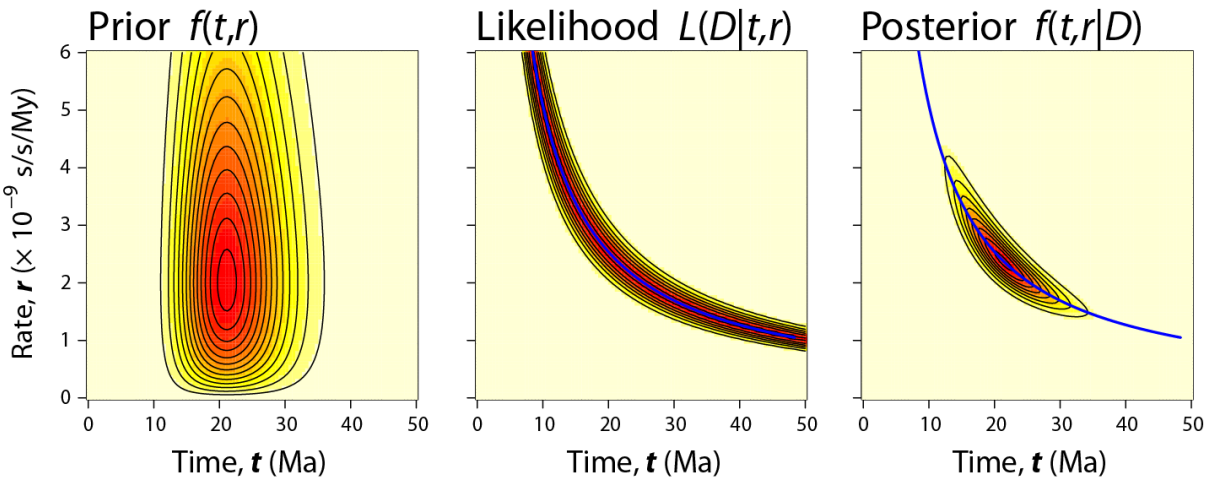


Figure 1. **Bayesian molecular clock dating.** We estimate the posterior distribuiton of divergence time (*t*) and rate (*r*) in a two-species to illustrate Bayesian molecular clock dating. The data is an alignment of the 12S RNA gene sequences from Human and Orangutan, with 90 differences at 948 nucleotides sites. The joint prior is composed of two gamma densities (reflecting our prior information on the molecular rate and on the geological divergence time of Human-Orangutan), and the likelihood is calculated under the Jukes-Cantor model. The posterior surface is the result of multiplying the prior and likelihood. The data are informative about the molecular distance, $d = tr$, but not about *t* and *r* separately. The posterior is thus very sensitive to the prior. The blue line indicates the maximum likelihood estimate of *t* and r, and the molecular distance *d*, with $\hat{t}\ \hat{r} = \hat{d}$. When the number of sites is infinite, the likelihood collapses onto the blue line, and the posterior becomes one-dimensional[62].

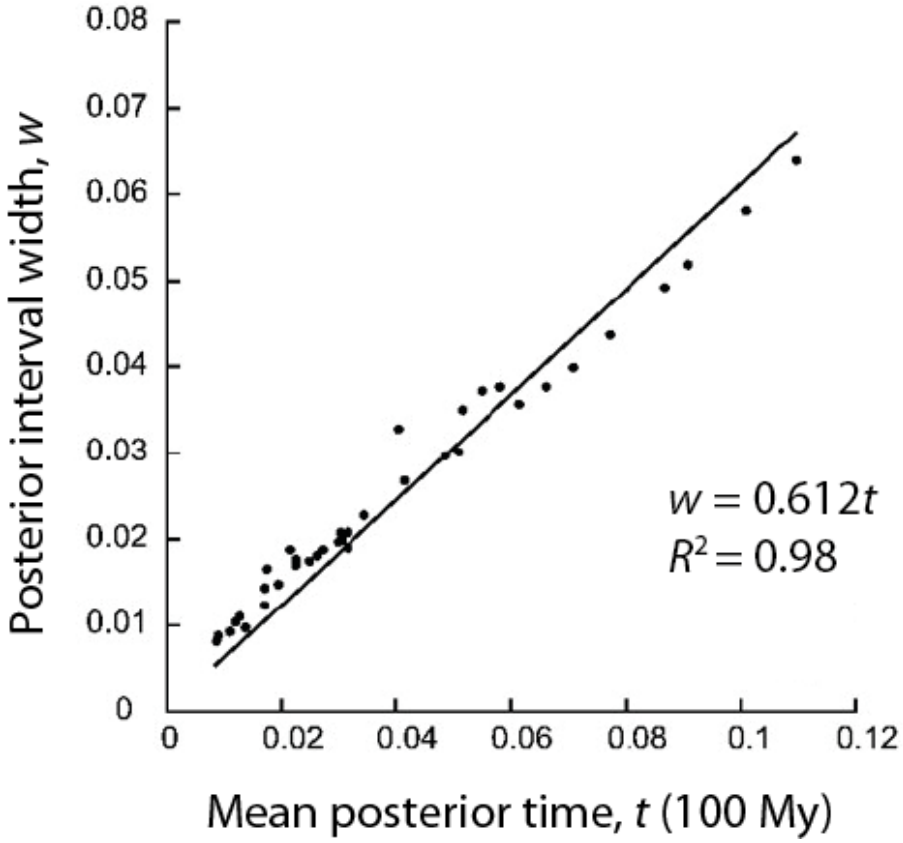


Figure 2. **Infinite-sites plot for Bayesian clock dating of divergences among 38 cat species.** There are 37 nodes on the tree and 37 points in the scatter plot. The *x*-axis is the posterior mean of the node ages, while the y-axis is the 95% posterior credibility interval (CI) width of the node ages. Here the slope (0.612) indicates that every million years of species divergence adds 0.612 million years of uncertainty in the posterior CI. When the amount of sequence data is infinite the points will fall onto a straight line. Here the high correlation (R^2 = 0.98) indicates that the amount of sequence data is very high and the large uncertainties in the posterior time estimates are mostly due to uncertainties in the fossil calibrations and including more sequence data will unlikely improve the posterior time estimates. Redrawn from figure 8c in[66].

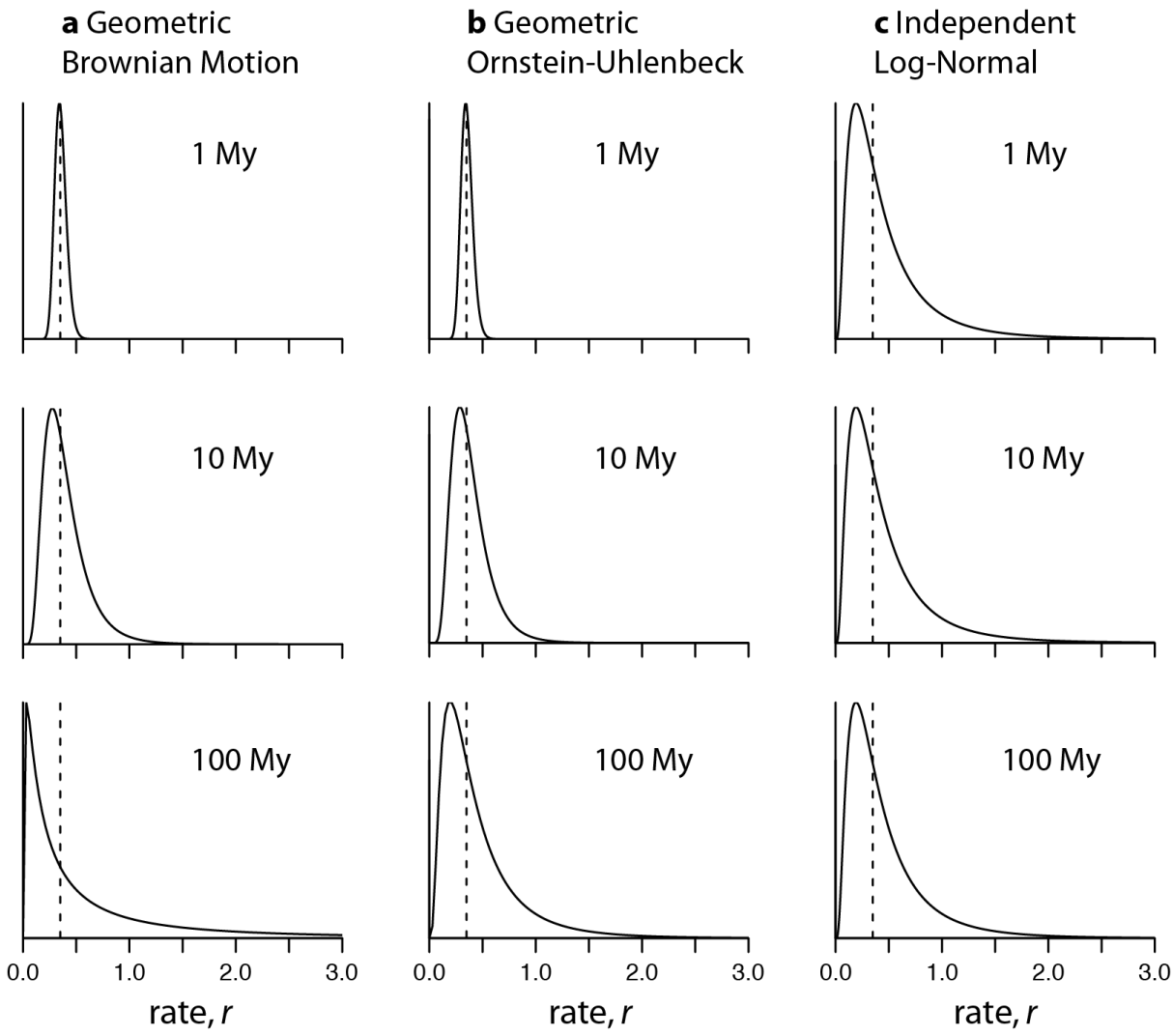


Figure 3. **Three relaxed-clock models of rate drift.** The rate of molecular evolution among lineages (species) is described by a time-dependent probability distribution (plotted here for three time points: 1 My, 10 My, 100 My) since the lineages diverged from a common ancestral rate (r_0 = 0.35 substitutions per site per 100 My, dashed line). **a |** The geometric Brownian process[29, 31, 52] (here with drift parameter v = 2.4 per 100 My). This model has the undesirable property that the variance increases with time and without bound, and that at large times, the mode of the distribution is pushed towards zero. **b** | The geometric Ornstein-Uhlenbeck process (here with v = 2.4 per 100 My and dampening force f = 2 per 100 My) converges to a stationary distribution with constant variance when time is large. **c** | The independent log-normal distribution[30, 31] is a stationary process, and the variance of rate among lineages remains constant through time (here with log-variance σ^2 = 0.6, the same as the long-term log-variance of the Ornstein-Ulhenbeck process above). Calculation

of the stochastic models in a and b is usually done approximately by Bayesian dating software[31, 52], however, progress has been made to find models that can be calculated exactly[55].

Table 1 | **Sample of Bayesian programs that use the molecular clock to estimate divergence times***

Program	Method	Brief description	Refs
BEAST	Bayesian	Comprehensive suite of models. Particularly strong for the analysis of serially sampled DNA sequences. Includes models of morphological traits.	[132]
DPPDIV	Bayesian	Dirichlet relaxed clock model[71]. Fossilised birth-death process prior to calibrate timetrees[56].	[133]
MCMCTREE	Bayesian	Comprehensive suite of models of rate variation. Fast approximate likelihood method that allows estimation of timetrees using genome alignments[57].	[134]
MRBAYES	Bayesian	Large suite of models for morphological and molecular evolutionary analysis. Comprehensive suite of models of rate variation.	[135]
MULTIDIVTIME	Bayesian	The first Bayesian clock-dating program. Introduced the geometric Brownian model and the approximate likelihood method.	[29, 53]
PHYLOBAYES	Bayesian	Broad suite of models. Uses data augmentation to speed up likelihood calculation and can be efficiently used in parallel computing environments (MPI enabled).	[136, 137]
R8S	Penalized likelihood	Very fast (uses Poisson densities on inferred mutations to approximate the likelihood). Suitable for analysis of large	[139]

		phylogenies. Suitable for estimating relative ages (by fixing the age of the root to 1). Does not deal with fossil and branch length uncertainty correctly[138].	
TREEPL	Penalized likelihood	Similar to R8S.	[140]

*Bayesian programs listed here were chosen for their ability to accommodate multiple calibrations with uncertainties (bounds or other probability densities), multiple loci of sequence data, and relaxed clock models. Penalized likelihood programs are listed as they are related to the Bayesian method[138].

Glossary definitions

ADVANTAGEOUS MUTATIONS. Advantageous mutations improves the fitness of the carrier and are favoured by natural selection.

BAYESIAN METHOD. A statistical inference methodology in which statistical distributions are used to represent uncertainties in model parameters. In Bayesian clock dating, priors on times and rates are combined with the likelihood (the probability of the sequence data) to produce the posterior of times and rates.

CLADE: A group of species descended from a common ancestor.

COALESCENT. The process of lineage joining when one traces the genealogical relationships of a sample backwards in time.

DELETERIOUS MUTATIONS. Deleterious mutations reduce the fitness of the carrier and are removed from the population by negative selection.

FOSSIL-AGE CALIBRATION. Minimum and maximum time constraints, based on the fossil record, that are placed on the age of a node in a phylogeny in molecular clock dating.

JUKES & CANTOR (JC) MODEL. A model of nucleotide substitution in which the rate of substitution between any two nucleotides is the same.

K-PG BOUNDARY. The boundary between Cretaceous and Paleogene at 66 Ma. It coincides with a mass extinction that wiped-out the dinosaurs and many more species.

LIKELIHOOD. The probability of the observed data given the model parameters viewed as a function of the parameters with the data fixed. In Bayesian clock dating the likelihood is calculated using the sequence data (and possibly morphological data) under a model of character evolution.

LIKELIHOOD RATIO TEST. A general hypothesis-testing method that uses the likelihood to compare two nested hypotheses, often using the χ^2.

MARKOV CHAIN MONTE CARLO (MCMC) ALGORITHM is a Monte Carlo simulation algorithm that generates a sample from a target distribution (often a Bayesian posterior distribution).

MOLECULAR CLOCK. The hypothesis that the rate of molecular evolution is constant over time or among species. Thus mutations accumulate at a uniform rate after species divergence, keeping time like a timepiece.

MORPHOLOGICAL CHARACTER. Discrete features or continuous measurements of different species that are informative about phylogenetic relationships.

MUTATION. Mutations are changes in the genes or genomes of an organism.

NEUTRAL MUTATION. A mutation that does not affect the fitness (survival or reproduction) of the individual.

NEUTRAL THEORY. The neutral mutation-random drift theory claims that evolution at the molecular level is mainly random fixation of mutations that have little fitness effect.

PARSIMONY-INFORMATIVE CHARACTERS. A discrete character is informative to the parsimony method of phylogenetic reconstruction if at least two states are observed among species each at least once.

PHYLOGENY. A tree structure representing the evolutionary relationship of the species.

PRIOR PROBABILITY DISTRIBUTION. The distribution assigned to parameters before the analysis of the data. In Bayesian clock dating, the prior on divergence times is specified using a branching model, possibly incorporating fossil calibration information, while the prior on evolutionary rates is specified using a model of rate drift (a relaxed-clock model).

POSTERIOR PROBABILITY DISTRIBUTION. The distribution of the parameters (or models) depending on the observed data. It combines the information in the prior and in the data (likelihood).

RELAXED MOLECULAR CLOCK. Models of evolutionary rate drift over time or across lineages developed to relax the molecular clock hypothesis.

SELECTIONIST THEORY. The theory that maintains that molecular evolution is dominated by fixation of advantageous mutations driven by natural selection.

SOFT BOUNDS. Minimum or maximum constraints on a node age with small error probabilities (such as 1% or 5%) used as bounds in clock dating.

SUBSTITUTION. Substitutions are mutations that spread into the population and become fixed, driven either by chance or by natural selection.

TREE OF LIFE. The evolutionary tree depicting the relationships among all the living species of organisms, calibrated to the geological time.

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Selected papers:

- Zuckerkandl and Pauling (1962). The earliest clock dating paper. Used the idea of approximate rate constancy to calculate the age of the alpha and beta globin duplication event.
- Zuckerkandl and Pauling (1965). The seminal paper proposing the concept of a 'molecular evolutionary clock'. Provides a justification for the clock based on the idea that most amino acid changes may not change the structure and function of the protein.
- Felsenstein (1981). This seminal paper describes how to calculate the likelihood for a molecular sequence alignment and describes a likelihood-ratio test of the clock.
- Kimura (1983). Authoritative book outlining the neutral theory. Chapter 4 has an extensive discussion of morphological vs. molecular rates of evolution.
- Gillespie (1984). Proposes the idea of an episodic clock, modelling rate evolution through time and among lineages as an stochastic process.
- Thorne et al. (1998). Describes the first Bayesian molecular clock dating method. Introduces the geometric Brownian motion model of rate variation among species.
- Yang and Rannala (2006). Develops a method to integrate the birth-death process to construct the time prior jointly with fossil calibrations with soft bounds. Introduces the limiting theory of uncertainty in divergence time estimates.
- dos Reis et al. (2012). An example of using the molecular clock with genome-scale datasets to infer the timeline of diversification of modern mammals relative to the end-Cretaceous mass extinction.
- Ronquist et al. (2012). This paper develops a Bayesian 'total-evidence' dating method for the joint analysis of morphological and molecular data.
- Wilkinson et al. (2011). Develops a model of species origination, extinction and fossil preservation and discovery to construct time priors based on data of fossil

occurrences.

- Parham et al. (2012). Sets out the criteria required for the establishment of fossil calibrations.

Key points

- 2015 celebrated five decades of the proposal of the molecular clock hypothesis by Emile Zuckerkandl and Linus Pauling in 1965.
- The molecular clock has become an essential tool in evolutionary biology, from tracking virus pandemics to estimating the timeline of evolution of Life on Earth.
- Early molecular clock dating studies made simplistic assumptions about the evolutionary process and proposed scenarios of species diversification that contradicted the fossil record.
- Bayesian clock dating methodology has become the standard tool to integrate information from fossils and molecules to estimate the timeline of the Tree of Life.
- Exciting developments in Bayesian clock dating include relaxed clock models, sophisticated fossil calibration curves and joint analysis of morphology and sequence data.
- Bayesian clock dating analysis of genome-scale data has resolved many iconic controversies between fossils and molecules, such as the pattern of diversification of mammals and birds relative to the end-Cretaceous mass extinction.

Author biographies

Mario dos Reis received his PhD from Birkbeck College, University of London. He is a lecturer at Queen Mary University of London. His research focusses on understanding the causes of uncertainty in Bayesian models of the molecular clock and applications to estimate the time of diversification of animal groups.

Philip CJ Donoghue received his PhD from Leicester University. He is Professor of Palaeobiology at the School of Earth Sciences, University of Bristol. His research focusses on formative episodes in evolutionary history, such as the origin of plants,

animals and vertebrates, which he studies through the integration of palaentological, anatomical, and molecular evidence. Donoghue has a long-standing interest in divergence time estimation and he has been influential in establishing the role of palaeontological data in molecular clock studies.

Ziheng Yang received his PhD from Beijing Agricultural University, China. He currently holds the RA Fisher Chair of Statistical Genetics at University College London, UK, and is the Director of RA Fisher Centre for Computational Biology at UCL. His research focuses on the development of statistical methods and computer algorithms for phylogenetic and phylogeographic analyses of DNA sequence data.

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