INVITED REVIEW
Time-dependent rates of molecular evolution

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Abstract
For over half a century, it has been known that the rate of morphological evolution appears to vary with the time frame of measurement. Rates of microevolutionary change, measured between successive generations, were found to be far higher than rates of macroevolutionary change inferred from the fossil record. More recently, it has been suggested that rates of molecular evolution are also time dependent, with the estimated rate depending on the timescale of measurement. This followed surprising observations that estimates of mutation rates, obtained in studies of pedigrees and laboratory mutation-accumulation lines, exceeded long-term substitution rates by an order of magnitude or more. Although a range of studies have provided evidence for such a pattern, the hypothesis remains relatively contentious. Furthermore, there is ongoing discussion about the factors that can cause molecular rate estimates to be dependent on time. Here we present an overview of our current understanding of time-dependent rates. We provide a summary of the evidence for time-dependent rates in animals, bacteria and viruses. We review the various biological and methodological factors that can cause rates to be time dependent, including the effects of natural selection, calibration errors, model misspecification and other artefacts. We also describe the challenges in calibrating estimates of molecular rates, particularly on the intermediate timescales that are critical for an accurate characterization of time-dependent rates. This has important consequences for the use of molecular-clock methods to estimate timescales of recent evolutionary events.

Keywords: calibrations, divergence times, molecular clock, mutation rate, purifying selection, substitution rate

Introduction
More than half a century ago, Kurten (1959) found an inverse correlation between the rate of morphological evolution and the time interval over which the rate was measured. Specifically, the rate of morphological change between successive generations exceeded macroevolutionary rates by several orders of magnitude. This time-dependent rate pattern was confirmed in a number of subsequent studies, which consistently showed that morphological evolutionary rates appeared faster when measured over shorter timescales (Gingerich 1983, 2001; Roopnarine 2003). A similar phenomenon has recently emerged in rates of molecular evolution estimated from DNA sequence data. In particular, there is a striking disparity between spontaneous mutation rates, measured over a small number of generations in studies of pedigrees and laboratory mutation-accumulation lines, and the much lower substitution rates measured over geological time frames (e.g., Parsons et al. 1997; Howell et al. 2003; Santos et al. 2005; Gibbs et al. 2009).
The discrepancy between short- and long-term rates raises an obvious question: for how long does the short-term rate remain elevated? Following the initial studies of mutation rates in human pedigrees that were published in the late 1990s (e.g., Howell et al. 1996; Parsons et al. 1997), some authors suggested that high rates persist for only a few generations (Macaulay et al. 1997; Gibbons 1998). However, a growing number of empirical studies have found that rates remain elevated for prolonged periods, perhaps exceeding a hundred thousand generations in some taxa (e.g., Ho et al. 2005; Genner et al. 2007; Burridge et al. 2008; Papadopoulou et al. 2010). Some researchers have postulated that rates of evolution decline exponentially as a function of the age of the calibration used to estimate them (Fig. 1; Ho & Larson 2006; Ho et al. 2005; Penny 2005), with young calibrations yielding rate estimates reflecting (nonlethal) mutation rates and older calibrations giving rate estimates reflecting substitution rates.

A comprehensive characterization of time dependence remains elusive because it requires that molecular rates be quantified accurately across a range of timescales. In turn, this depends on the availability of reliable temporal information for calibrating rate estimates. While such calibrations can be found for analyses involving time frames that are either very short (e.g., documented pedigrees and laboratory mutation-accumulation lines) or very long (e.g., palaeontological evidence), there is a paucity of reliable calibrations for intermediate time frames (see Box 1). However, estimating rates on intermediate time frames is crucial for studying the decay of high short-term rates.

Validating and quantifying the time dependence of molecular rates has important consequences for studies in a number of fields. One of the key implications is that rates estimated over a given time frame are not transferable to analyses of other time frames, meaning that it is invalid to assume that a single molecular clock applies throughout all lineages over time (e.g., Ho & Larson 2006; Howell et al. 2008). The degree of this problem is greatest for studies that use molecular dating to understand recent evolutionary events, for example those concerning viral phylodynamics, phylogeographic processes, prehistoric human dispersal or the extinction of megafauna in the late Pleistocene. The impact of time-dependent rates extends beyond the inference of timescales, however, and can also affect the inference of demographic parameters that use estimates of the mutation rate, such as effective population size and migration rates.

To be able to address the problem of time dependence when estimating molecular rates, timescales and other parameters, it is necessary to have a thorough appreciation of the causes of elevated short-term rates. There are many factors that can lead to elevated short-term rates, but their relative importance will vary among taxa. Disentangling these factors has wider implications for understanding the molecular evolutionary process (e.g., Woodhams 2006; Peterson & Masel 2009; O’Fallon et al. 2010).

Here we provide an overview of the known biological and methodological factors that can contribute to the time dependence of molecular evolutionary rates. We also list the various sources of information for calibrating rate estimates (Box 1) and survey the published evidence for time-dependent rates from studies of animals (Box 2) and bacteria and viruses (Box 3).

The basic biological framework

One of the most important biological reasons why we should expect molecular rate estimates to be time dependent is that rates measured on different timescales reflect different biological processes. Rates measured over very short timescales (e.g., between successive generations) can include genetic differences representing all but the most detrimental mutations. Therefore, these rate estimates approach the spontaneous mutation rate (discounting lethal mutations). On the other hand, rates measured over very long timescales (e.g., between distantly related species) will usually be dominated by substitutions (those mutations that were fixed in either diverging lineage) and will therefore approximate the substitution rate. Substitution rates are usually much lower than mutation rates because natural selection tends to remove deleterious mutations reflecting substitution rates.

Fig. 1 Plot of time-dependent rates showing an exponentially declining rate estimate with increasing time depth. The spontaneous rate of non-lethal mutations is approached at a time depth of zero. As the time frame increases, the estimated rate tends towards the long-term substitution rate observed in phylogenetic analyses calibrated using palaeontological or geological data. The exact form of the curve is likely to show considerable variation among taxa and among loci.
Box 1. Calibrating estimates of molecular evolutionary rates

The degree of divergence between two sequences is determined by two factors: the rate of change and the time since they last shared a common ancestor. Therefore, to estimate the rate of molecular change, it is necessary to include independent information about the evolutionary timescale. Age calibrations can come from a variety of sources, including the fossil record, dated geological events, archaeological evidence, heterochronous sampling, documented pedigrees and laboratory lines (Figure 2).

**Fossil record** – One of the most common methods of calibration, particularly in studies of vertebrates, is the use of palaeontological evidence. Fossil evidence provides the earliest appearance of identifiable members of a lineage, allowing a minimum age constraint to be specified for the divergence event that gave rise to the lineage. If the fossil record is sufficiently informative, it is possible to include maximum age constraints or to give calibrations in the form of parametric age distributions (Yang & Rannala 2006; Ho & Phillips 2009).

In most cases, calibrations based on the fossil record are at least several million years in age. On shorter timescales, there is usually an insufficient amount of inherited morphological variation for the fossil record to provide reliable diagnostic characters. Consequently, palaeontological calibrations find their primary employment in phylogenetic analyses conducted above the population level.

**Dated geological events** – In a biogeographic context, divergence of species or populations can sometimes be attributed to geophysical isolating mechanisms or the appearance of new habitats. These can result from the formation of islands, mountain ranges, seaways or other geological features. If the timing of such an event is known, for example through radiometric dating, this information can be used to calibrate phylogenetic estimates of rates. Geological calibrations span a wide range of ages and can include island formation events and ancient continental movements.

Biogeographic calibrations can come in several forms, depending on the nature of the geological event and its impact on lineage diversifications; for example, the appearance of the Panamanian isthmus might represent a barrier to gene flow between the Caribbean Sea and the Pacific Ocean (minimum age constraint), or a land bridge between the American continents (maximum age constraint). It is not always clear whether it is safe to assume a close correspondence between the age of the geological event and genetic divergence, because disparities can arise as a result of ancestral divergence or subsequent dispersal (Marko 2002; Heads 2005).

**Archaeological and anthropological evidence** – Molecular estimates of the timing of human migration have sometimes utilized calibrations informed by archaeological evidence. These calibrations include the arrival of humans in Australasia (about 45 000 years ago) and the Americas (about 14 000 years ago), as well as other migration events spanning a range of geographic and temporal scales (Endicott & Ho 2008; Henn et al. 2009; Subramanian 2009).

Diagnoses of human infection from archaeological sites or from historical documentation have been used to provide calibrations for analyses of viral and bacterial evolution; for example, Li et al. (2007) used descriptions of infections in ancient medical texts to infer the presence of smallpox in China in the 4th century AD; along with other archaeological evidence of smallpox infections, this allowed the authors to place a number of age calibrations at internal nodes in the phylogenetic tree of the variola virus.

**Host codivergence** – In some cases, the evolutionary timescale of pathogenic organisms can be inferred by assuming correspondence with the evolution of host organisms; for example, the estimated timescale of human movements has been used to provide indirect calibrations for associated pathogens (e.g., Lemey et al. 2005). At higher taxonomic levels, the codivergence of endosymbiotic bacteria with their host species has been used for calibrating estimates of long-term rates (Kuo & Ochman 2009). This usually represents the only available source of temporal information for analyses of pathogen evolution across geological scales. Conversely, stably inherited pathogens can...
be used to infer the recent evolutionary history of their hosts (Kitchen et al. 2008). Given that various studies have found evidence of host switching in pathogens (e.g., Ricklefs & Fallon 2002; Harkins et al. 2009), the general reliability of assuming host codivergence for age calibration is uncertain.

Heterochronous sampling – In some data sets, such as those containing ancient DNA or serially sampled viruses and bacteria, the sequences have distinct ages. Sometimes these dates are known exactly, for example through museum records or as part of the sampling strategy. Otherwise, they can be estimated radiometrically, stratigraphically or phylogenetically (Ho, Phillips 2009; Shapiro et al. 2011). If the age range of the sequences is large in relation to the evolutionary rate, the sampling times can provide sufficient calibrating information for the analysis (Rambaut 2000; Drummond et al. 2003). Because they usually apply to the tips of the phylogenetic tree rather than to the internal nodes, calibrations associated with heterochronous sequences differ markedly from those based on palaeontological, geological or archaeological data.

Ancient DNA sequences can be hundreds of thousands of years old (Willerslev et al. 2007). In the majority of cases, however, precise dating is bounded at around 50 000 years by the limits of radiocarbon methods. Sample ages can be indirectly obtained by association with dated strata (e.g., Lambert et al. 2002), but there is a risk of dating errors because of vertical migration of nucleic acids (Haile et al. 2007). Sample intrusions can also produce dating errors, particularly in archaeological contexts.

Heterochronous data can be obtained from viruses or bacteria by serially sampling from a single patient, from different patients over a period of months or years, or from preserved historical samples (e.g., Buonagurio et al. 1986; Li et al. 1988). The limits on the inclusion of ancient viral and bacterial data are governed by nucleic acid survival, sequence verifiability, access to reliable dating information and the extent of mutational saturation. Virus sequences have been obtained from samples dating to the early twentieth century (e.g., Taubenberger et al. 1997), although most data sets comprise samples from the past two or three decades.

Documented pedigrees and laboratory lines – In some studies, such as those of documented pedigrees and laboratory lines, the number of generations separating the individuals is known exactly. If the age of the common ancestor has not been recorded, it can be inferred using an estimate of the mean generation time. Pedigrees can span tens to hundreds of generations, depending on the organism. Meta-analyses of published human pedigree studies can include thousands of genetic transmission events (e.g., Howell et al. 2003; Goedbloed et al. 2009).

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**Box 1. Continued**

**Fig. 2** Typical age ranges of different forms of calibrating information.
Box 2. Evidence for time-dependent molecular rates in animals

Mitochondrial genomes – Perhaps the first observation of time-dependent molecular rates was made in a study of carnivores and primates using gel electrophoresis (Wayne et al. 1991), in which the authors noted that the molecular rate showed a linear decline with increasing time depth. This trend was most pronounced for time depths <3 Ma before present. More than a decade later, García-Moreno (2004) collected a number of published estimates of substitution rates for avian mtDNA, finding a distinctive decline in the estimated rate as the calibration point increased in age. Subsequent Bayesian phylogenetic analyses of many of the same avian data sets, along with mitochondrial sequences of primates, reproduced this pattern and clarified the curvilinear relationship (Ho et al. 2005, 2007c). Several authors have challenged these findings (Emerson 2007; Bandelt 2008), but a similar rate trend has emerged in a recent study of insect mtDNA (Papadopoulou et al. 2010).

The recent work on time-dependent rates followed a series of surprising observations made in pedigree-based studies of humans (Bendall et al. 1996; Howell et al. 1996, 2003; Mumm et al. 1997; Parsons et al. 1997; Sigurdardóttir et al. 2000; Santos et al. 2005). Analyses of mtDNA from individuals with documented relationships yielded remarkably high estimates of mutation rates, with some being more than an order of magnitude higher than estimates of substitution rates based on phylogenetic analyses of mammalian and avian mtDNA. Collectively, these studies pointed towards a very high short-term rate caused by the presence of transient polymorphisms. This raised the question of how long elevated rates might persist, and led to concerns about the accuracy of date estimates for recent evolutionary events (Howell & Mackey 1997; Macaulay et al. 1997; Gibbons 1998). High estimates of mtDNA rates have since been obtained in pedigree studies of other organisms, including Caenorhabditis (Denver et al. 2000), Drosophila (Haag-Liautard et al. 2008) and Adélie penguins (Millar et al. 2008).

In addition to the disparity between pedigree-based and phylogenetic rates, various studies have found that molecular rate estimates are higher when calibrated within species than when calibrated with reference to sister species (e.g., Ho et al. 2008; Rajabi-Maham et al. 2008; Korsten et al. 2009; Davison et al. 2011). In some cases, calibration within species was made possible by the inclusion of ancient DNA sequences, which can be up to hundreds of thousands of years old (see Box 1). Most intraspecific ancient mtDNA data sets have produced relatively high estimates of rates, sometimes exceeding long-term phylogenetic rates by an order of magnitude (Lambert et al. 2002; Ho et al. 2007b, 2011; Kemp et al. 2007; Hay et al. 2008). In addition, some analyses of ancient mtDNA have found a relationship between estimated rate and calibration age (Ho et al. 2007c; Subramanian et al. 2009a).

Using calibrations based on dated geological events, such as river capture and lake separation, analyses of mtDNA from cichlids (Genner et al. 2007) and galaxiid fishes (Burridge et al. 2008) have yielded time-dependent patterns of rates. In both studies, the estimated rate declined over a few hundred thousand years.

There have been several detailed studies of time-dependent rates using human mtDNA. These have been made possible by the availability of complete mtDNA sequences from a large number of modern humans, as well as ancient mtDNA sequences from humans and Neanderthals. Some studies have noted substantial disparities between pedigree-based rates, genealogical rates and phylogenetic rates (Howell et al. 2003; Santos et al. 2005, 2008; Kemp et al. 2007; Endicott & Ho 2008; Endicott et al. 2009), while others have provided a more detailed characterization of the dependence of rate on time (Henn et al. 2009; Loogváli et al. 2009; Soares et al. 2009).

Nuclear genomes – Estimates of mutation rates from pedigree studies have indicated a short-term elevation in nuclear DNA which was similar to that seen in mitochondrial DNA. Such estimates have been made for Caenorhabditis (Denver et al. 2004), Drosophila (Houle & Nuzhdin 2004; Haag-Liautard et al. 2007; Keightley et al. 2009) and human (Nachman & Crowell 2000). These estimates are far higher than substitution rates estimated in phylogenetic analyses.

At the population level, most available nuclear data are in the form of microsatellites or SNPs. Mutation rates vary considerably among microsatellite loci, depending on characteristics such as genomic context, motif size and the existing number of repeats (Ellegren 2000; Buschiazzo & Gemmell 2006; Ballantyne et al. 2010). Despite this natural variation, studies of short tandem repeats in the human Y-chromosome have found an approximately threefold...

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rious mutations, which are thought to constitute a large proportion of spontaneous mutations, even in noncoding regions of the genome (Eyre-Walker & Keightley 2007).

To illustrate this point, it is helpful to consider a simple scenario in which we trace the history of a single locus in a pair of sister species in a coalescent framework (Fig. 3). In each species, mutations appear at a certain rate per individual, per generation and per nucleotide. These mutations are eventually lost or fixed (a substitution event) at a rate that depends on their fitness effects and on the population size (Ohta 1973). The fate of neutral mutations in a population is determined solely by genetic drift and, unless there is strong linkage to sites under selection, the fixation rate depends only on the rate at which the mutations are generated (Kimura 1968). At a given population size, the fixation rates of advantageous and deleterious mutations are higher and lower, respectively, than the neutral fixation rate.

In this setting, consider the hierarchy of genetic comparisons that can be made between four contemporary individuals, A, B, C and D in Fig. 3. A and B represent closely related individuals from the same population, so the observed genetic differences between them are dominated by mutations, the number of which is determined by the mutation rate. Many of these mutations

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**Box 2. Continued**

difference between the mean rates estimated in pedigree-based and population-level haplogroup-founder analyses (Forster et al. 2000; Kayser et al. 2000; Zhivotovsky et al. 2004, 2006; Vermeulen et al. 2009). A smaller disparity was found between a rate estimated from a 13-generation human pedigree and a phylogenetic rate calibrated using the human–chimpanzee divergence (Xue et al. 2009).

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**Box 3. Time-dependent molecular rates in bacteria and viruses**

*Bacteria* – Some degree of time dependence has been observed in rate estimates from bacteria, although there have been few studies of bacterial rates across significant evolutionary timescales. In a study of *Vibrio cholerae*, Feng et al. (2008) found that their estimate of the substitution rate, calibrated using heterochronous sequences, was 100-fold higher than the standard rate that is usually assumed. In contrast, slow rates for endosymbiotic bacteria were estimated using calibrations based on the assumption of host codivergence (Kuo & Ochman 2009). The ratio of nonsynonymous to synonymous mutations has been shown to be strongly time-dependent across several closely related bacterial taxa (Rocha et al. 2006).

*Viruses* – Rates of molecular evolution in viruses span several orders of magnitude. This wide variation is a consequence of numerous factors but is primarily influenced by the fidelity of the polymerase that is used during replication (Duffy et al. 2008; Holmes 2009). Generally, RNA viruses mutate more rapidly than retroviruses which, in turn, mutate more rapidly than DNA viruses. Nevertheless, there is considerable rate variation within each of these classes, making it difficult to evaluate the dependence of rate on the timescale of observation. This is compounded by the problem of rapid saturation in viral genomes.

Analyses of heterochronous virus samples have typically yielded high estimates of short-term rates, and these differ markedly from rate estimates based on an assumption of host codivergence (Jenkins et al. 2002; Ramsden et al. 2008; Harkins et al. 2009). In a survey of 15 rate estimates from plant viruses, Gibbs et al. (2009) found that the highest rate estimates were obtained from heterochronous data. Lower rates were estimated in analyses calibrated at internal nodes, with the very lowest estimates resulting from calibrations based on ancient codivergence events. There also appears to be a discrepancy between intrahost and interhost rates of molecular evolution (e.g., Lemey et al. 2006; Gray et al. 2011), but this can perhaps be explained by aspects of viral transmission dynamics (Pybus & Rambaut 2009).

There have also been studies of factors leading to the disparity between short- and long-term rates in viruses. There is extensive evidence of strong purifying selection in various viruses (Holmes 2003), with a compelling time-dependent pattern in the ratio of nonsynonymous to synonymous mutations in HIV (Sharp et al. 2001). Recent studies have discounted the impact of several potential biases in the high rate estimates obtained from heterochronous viral data (Duffy & Holmes 2009; Firth et al. 2010).
A simplified representation of genealogical history at a single locus in a pair of species. Each circle represents a randomly mating individual and each row represents one generation. The ancestral species (spanning the time period \( t_3 \)) splits into two descendent species (spanning the periods \( t_2 \) and \( t_1 \)). Four contemporary individuals labelled \( A, B, C \) and \( D \) are referred to in the text.

The effects of natural selection

Natural selection tends to remove mutations with negative fitness effects from populations. The ability of negative selection to remove a given mutation depends on both the fitness effect of that mutation and on the effective population size – mutations are removed more efficiently by negative selection when they are highly deleterious and when the effective population size is large (Ohta 1992). Because negative selection removes mutations from populations, it results in long-term rate estimates being lower than short-term rate estimates. The extent to which selection influences the time dependence of rate estimates will depend on the distribution of fitness effects of new mutations. It is possible for positive selection to cause a transient increase in substitution rates, although this will typically apply to only a very small proportion of sites in the genome.

Negative selection

Analyses of molecular data from pedigrees, laboratory mutation-accumulation lines, and intrahost viruses indicate that many new mutations are rapidly removed by selection (Denver et al. 2000; Holmes 2003; Haag-Liautard et al. 2008; Santos et al. 2008; Stewart et al. 2008). A large proportion of mutations are likely to be slightly deleterious for the metazoan mitochondrial and viral genomes (Ballard & Whitlock 2004; Duffy et al. 2008; Stewart et al. 2008; Galtier et al. 2009), the primary sources of evidence for time-dependent rates (see Boxes 2 and 3). If this is the case, we should expect to see a rapid decline from spontaneous mutation rates as...
we look back in time. However, Hill–Robertson interference, whereby a low rate of recombination prevents multiple selected sites from evolving independently (Hill & Robertson 1966), can substantially reduce the efficacy of selection in some sequences (Williamson & Orive 2002), consequently tempering the effects of negative selection on the time dependence of molecular rate estimates.

The effect of negative selection on molecular rate estimates is also apparent in the time-dependent decline in the ratio of nonsynonymous to synonymous changes in protein-coding sequences (Sharp et al. 2001; Elson et al. 2004; Rocha et al. 2006; Peterson & Masel 2009; Subramanian 2009). Analyses of mitochondrial DNA (mtDNA) sequences have shown that rates at nonsynonymous sites display stronger time dependence than those at synonymous sites (Endicott & Ho 2008; Loogváli et al. 2009; Soares et al. 2009; Subramanian et al. 2009a). These results suggest an important role for negative selection in the time dependence of rates. However, theoretical investigations have indicated that the action of purifying selection alone is insufficient to explain the time-dependent patterns of rates obtained in published empirical studies of primates and birds (Woodhams 2006; Peterson & Masel 2009). Sequences in noncoding regions, such as the mitochondrial D-loop and microsatellites in the human Y-chromosome, have also been shown to exhibit time-dependent rates (Box 2). This pattern might be the result of negative selection on these sequences, but it is perhaps more likely that it is because of negative selection in closely linked coding regions.

Positive selection

Positive selection can produce an increase in nonsynonymous rates, which is reflected in the overall substitution rate as well as the ratio of nonsynonymous to synonymous rates. Positive selection could explain some observations of time dependence if there has been recent adaptive evolution. It has been postulated that some of the nonsynonymous mutations towards the tips of the human mitochondrial genealogy represent adaptive changes in response to climatic variation (Mishmar et al. 2003; Ruiz-Pesini et al. 2004), although these claims have been contested (Elson et al. 2004; Sun et al. 2007). Moreover, it has been suggested that this form of positive selection does not lead to the persistent elevation of rates seen in empirical studies (Loogváli et al. 2009). Given the wide range of sequences and taxa in which time-dependent rates have been detected, it seems implausible that widespread recent increases in rates of adaptive molecular evolution could offer a common explanation.

The effects of calibration errors

To estimate absolute rates of molecular evolution, it is necessary to use independent temporal information to calibrate the age of a given node in the tree (Box 1). If calibrations are specified incorrectly, any resulting rate estimates might be biased. A number of errors can arise when choosing and implementing calibrations; most of these errors lead to overestimates of rates. This overestimation bias tends to be largest on short timescales, which can lead to a time-dependent pattern of rates (as seen in Fig. 1).

Coalescent calibration error

The genetic divergence of a given locus always precedes or coincides with population or species divergences \( t_2 \) in Fig. 3), unless there is subsequent gene flow. When calibrations are based on the presumed timing of a population- or species-divergence event, genetic divergence is typically assumed to coincide with population divergence. This leads to an underestimate of the time since genetic divergence, with a consequent overestimation of substitution rates. The magnitude of this bias will be greatest on short timescales, where the temporal difference between genetic and population divergences forms an appreciable proportion of the total time separating the two populations or species (Edwards & Beerli 2000). The severity of the effect also increases with the effective population size and generation time of the ancestral lineage. Some methods enable calibration of population divergences rather than genetic divergences (e.g., Heled & Drummond 2010), allowing the problem of coalescent calibration error to be avoided.

Recently, it was suggested that the rate overestimation caused by this coalescent calibration error provides a sufficient explanation for observed time-dependent trends in rates (Peterson & Masel 2009). In practice, however, there are some instances in which congruence between genetic and population splits is plausible. If there had been a bottleneck in the ancestral population prior to or during speciation, then there is a high probability that genetic divergence occurred close to the time of population divergence.

Coalescent calibration error almost certainly explains some portion of the observed time dependence of molecular rate estimates. However, the suggestion that it offers a sufficient explanation is challenged by the high rate estimates obtained in studies of data sets comprising sequences of varying ages (heterochronous data), including ancient mtDNA (e.g., Lambert et al. 2002; Ho et al. 2007b; Hay et al. 2008) and viruses (Box 3). In these studies, rate estimates are not cali-
brated at divergence events, but at the tips of the tree (see Box 1). Therefore, this approach does not entail any assumptions about the concurrence of genetic and population divergences. If the high rates estimated in analyses of heterochronous data are legitimate, then coalescent calibration error cannot be the exclusive cause of time-dependent rates. Therefore, it appears that heterochronous sequence data might play an important role in further explication of this phenomenon, although there has been some debate over the validity of rates estimated from such data sets (Ho et al. 2007b, 2011; Debruyne & Poinar 2009; Miller et al. 2009; Navascués & Emerson 2009; Subramanian et al. 2009b; Firth et al. 2010).

Palaeontological, biogeographic and archaeological calibration error

In phylogenetic analyses, estimates of molecular rates are usually calibrated using temporal information from the fossil record or from assumed biogeographic splits (Box 1). The timing of an evolutionary divergence event can be assumed to be older than the earliest appearance of either of its descendant lineages in the fossil record. Genetic divergence generally precedes the appearance of diagnostic morphological variation, meaning that there is a disparity between date estimates provided by molecular data and the fossil record. In addition, there is also a very low probability that a fossil taxon can be reliably placed close to a divergence event. Thus, genetic divergence times are underestimated by palaeontological evidence, which can lead to overestimates of molecular rates. The relative magnitude of this bias is most problematic for studies of short evolutionary timescales where the age underestimation can represent a substantial proportion of the true age of the calibration (Ho et al. 2005), but fossil calibrations are rarely used in such settings.

In studies of recent evolutionary events, calibrations based on biogeography or archaeology are sometimes employed (Box 1); for example, the appearance of a geological feature can produce a barrier to gene flow, allowing the timing of genetic divergence to be estimated. In some cases, colonization events can be identified in phylogenetic trees and their timing estimated using geological or archaeological evidence (e.g., Fleischer et al. 1998; Henn et al. 2009). Biogeographic calibrations carry a number of risks because they usually involve strong assumptions, the most important of which is that there is a close correspondence between genetic and geological events. However, genetic divergence can substantially antedate the emergence of a barrier to gene flow, for example in the presence of undetected, extinct sister lineages (Emerson 2007). This can lead to a severe overestimation of molecular rates (e.g., Marko 2002). On the other hand, dispersal or other agents of gene flow can result in genetic divergence postdating geological events, resulting in an overestimation of the calibration age and subsequent underestimation of molecular rates.

The effect of model misspecification

In all estimates of rates, numerous simplifying assumptions are required. This is reflected in the use of relatively simple models of genetic inheritance, nucleotide substitution and demographic history. Computational and statistical tractability is the main purpose of these assumptions, but often this comes at the cost of biological realism. Biases in the estimates of various parameters, including rates, can arise if the model is misspecified (Swofford et al. 2001; Lemmon & Moriarty 2004). Although this problem can sometimes be minimized through a rigorous model-selection procedure, it can be the case that none of the available models is entirely adequate (Gatesy 2007).

Phylogenetic assumptions concerning inheritance

In most phylogenetic analyses, it is assumed that the sampled sequences are orthologous, which means that they diverged from each other via a speciation process and not by duplication events or horizontal gene transfer. In analyses of animal mtDNA, common assumptions include uniparental inheritance, homoplasy and a lack of recombination. However, occurrences of biparental inheritance, paternal leakage, heteroplasm and recombination have been reported for mtDNA from a variety of taxa (e.g., Schwartz & Vissing 2002; Bromham et al. 2003; Barr et al. 2005; Santos et al. 2005; Tsaousis et al. 2005). If these violations of the assumptions are not adequately dealt with, estimates of the rate of molecular evolution can be inaccurate (White et al. 2008). The presence of nontarget sequences (e.g., paralogous copies of genes in a single-gene analysis) will usually produce an artificial inflation of genetic diversity, leading to a rate overestimation that is greater on short timescales. However, it is difficult to envisage these problems occurring on a widespread scale in published analyses.

Substitution model misspecification and saturation

When the process of nucleotide substitution is modelled in a phylogenetic analysis, it is almost always assumed to be homogeneous throughout the tree. One of the functions of these models is to correct for unobserved substitutions; underestimation of these unseen changes
is the typical outcome of substitution model misspecification (Sullivan & Joyce 2005). Under-correction for mutational saturation can cause patterns of time-dependent rates, because saturation is likely to be less of a problem over very short time frames (such as those associated with analyses of recent events) but is an important factor over longer time frames (such as those associated with phylogenetic analyses). It has been suggested that this effect might be at least partly responsible for time-dependent rates (García-Moreno 2004; Emerson 2007; Ho et al. 2007c; Raquin et al. 2008).

Demographic factors
In Bayesian phylogenetic methods, a prior distribution needs to be specified for the tree (including topology and branch lengths). In some implementations, the prior distribution is generated using a stochastic branching or coalescent process (Drummond et al. 2006; Yang & Rannala 2006). For intraspecific data sets, the use of the coalescent also requires assumptions about the demographic history of the species being studied. Coalescent-based approaches typically involve simple parametric models of demographic change, built upon assumptions such as panmixia and random sampling. It has been demonstrated that violation of these assumptions, as well as incorrect modelling of demographic history, can lead to biases in rate estimates; for example, the presence of severe bottlenecks or marked population subdivision, combined with biased sampling, can artificially elevate the estimates of short-term rates made using heterochronous data (Miller et al. 2009; Navascués & Emerson 2009).

The problem of demographic model misspecification can be at least partly addressed using extensions of the coalescent that allow for population structure (Notohara 1990), while a model-selection procedure can be implemented to determine the most appropriate description of population history. Alternatively, some complexities in demographic history can be accommodated using flexible models of population change (e.g., Drummond et al. 2005; Heled & Drummond 2008). However, because population history can rarely be described with confidence, it remains possible that time-dependent rates will be a feature of coalescent analyses for some time.

Artefacts causing time dependence of molecular rates
Time dependence in rate estimates can also be influenced by a number of additional measurement errors. Some of these, such as sequence errors, can be difficult to detect, whereas certain statistical phenomena can be dealt with more readily.

Sequence error and postmortem damage
Errors in DNA sequences can be introduced at various stages of analysis, including base misincorporation during the polymerase chain reaction and misinterpretation of sequence traces. In studies of sequences acquired from ancient samples, the DNA molecule can be altered by postmortem damage, the level of which is usually higher than typical sequencing error by an order of magnitude (Lindahl 1993). Regardless of the source, sequence errors will manifest themselves as spurious recent mutations, leading to time-dependent rate estimates as well as biases in other parameters (Clark & Whittam 1992; Ho et al. 2005, 2007a; Johnson & Slatkin 2008). The manifold sequencing coverage achieved by next-generation sequencers might lead to a better characterization of sequence quality, but the degree of error in published sequences is unknown (e.g., Forster 2003; Bandelt et al. 2006).

The impact of sequence errors is most pronounced when there is low diversity among sequences, which is often the case for population-level data. Assuming a fixed frequency of sequence error, the estimation bias in rates declines with increasing sequence divergence (Ho et al. 2005). In phylogenetic analyses of ancient DNA sequences, this estimation bias can be alleviated using models of sequence damage (Ho et al. 2007a; Mateiu & Rannala 2008; Rambaut et al. 2009). However, because all sequencing errors will be incorrectly recorded as recent mutations, they are likely to explain some proportion of the time dependence of rates in a large number of studies.

Skewed rate distributions
Molecular rates are described by scale parameters, which have a natural lower bound of zero and lack a rigid upper bound. This asymmetric constraint can introduce a bias in Bayesian rate estimates, particularly if they are associated with wide credibility intervals, leading to a positively skewed posterior distribution in which the point probability of a very high rate is higher than normal. Consequently, the mean can give a biased reflection of the rate, especially if the rate is estimated from a data set with very low sequence variability (Heled & Drummond 2008; Debryune & Poinar 2009). Thus, the use of mean rate estimates in Bayesian studies of substitution rates might explain some cases of time-dependent rates. A recent simulation-based study demonstrated that this bias can be reduced or removed through the use of other measures of central tendency, such as the median or mode (Ho et al. 2011).
Given the number of potential factors causing time-dependent rates, as well as differences in their effects among loci, it might prove very difficult to correct for these factors when making estimates of evolutionary timescales. Until further studies have quantitatively addressed the importance of and interactions between each of these factors, building mechanistic models to account for time-dependent rates is unlikely to be straightforward. Perhaps the most pragmatic approach to obtaining accurate estimates of evolutionary timescales is to be aware of the factors causing time-dependent rates and to attempt to avoid them.

An important practical consequence of time-dependent rates is that a strict molecular clock is rarely appropriate for investigating phylogenetic trees that cross the barrier between populations and species. Even if a relaxed molecular clock is employed in an attempt to deal with this problem (e.g., Korsten et al. 2009), it is typically assumed that all of the branch-specific rates come from a single distribution (Drummond et al. 2006). A potential solution is to perform separate analyses at the intra- and interspecific levels, or perhaps to use local-clock models to distinguish between short- and long-term rates (Yoder & Yang 2000; Drummond & Suchard 2010). Alternatively, it may be appropriate to define substitution rate mathematically as a function of time, applied across all lineages of a phylogenetic tree (Rodrigo et al. 2008).

Further empirical studies, including detailed comparisons among the factors discussed in this review, will help to identify the scale and impact of the various factors. Evidence from nuclear data, including noncoding regions, should also prove to be illuminating. The growth of genomic sequence data provides an excellent opportunity to investigate time-dependent rates in detail, particularly when sequences are available from multiple individuals within a population as well as from closely related species. The identification of reliable age calibrations, particularly those relating to recent evolutionary timescales, is also an important avenue of research.

It should be noted that some of the factors described in this review can also affect estimates of rates over much longer time frames; for example, the problems of saturation and model misspecification will grow in severity as the timescale of measurement increases. On long timescales, however, it is likely that the effects of time-dependent factors will be small in comparison with other sources of error, including those relating to calibrations and models of rate variation among lineages. Additional factors such as changes in the substitution process and differences in equilibrium nucleotide frequencies will also become significant (e.g., Phillips 2009).

Understanding the causes of time-dependent rates is important for a number of reasons, not only for quanti-
fying rates of molecular change but also for constructing evolutionary timescales. This is likely to have an impact on estimates of the timing of events that have occurred in the past few hundred thousand years, and perhaps over longer timescales. Disentangling the factors influencing rate estimation will also lead to an improvement in our understanding of the molecular evolutionary process.

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