MOLECULAR ECOLOGY

Molecular Ecology (2011) **20**, 3087–3101 doi: 10.1111/j.1365-294X.2011.05178.x

INVITED REVIEW

Time-dependent rates of molecular evolution

SIMON Y. W. HO,*† ROBERT LANFEAR,* LINDELL BROMHAM,* MATTHEW J. PHILLIPS,*[1] JULIEN SOUBRIER,‡ ALLEN G. RODRIGO§¶ and ALAN COOPER‡

*Centre for Macroevolution and Macroecology, Evolution Ecology & Genetics, Research School of Biology, Australian National University, Canberra, ACT, Australia, †School of Biological Sciences, Edgeworth David Building A11, University of Sydney, Sydney, NSW, Australia, ‡Australian Centre for Ancient DNA, School of Earth & Environmental Sciences, University of Adelaide, Adelaide, SA, Australia, §Department of Biology, Duke University, Durham, NC, USA, ¶Bioinformatics Institute and School of Biological Sciences, University of Auckland, Auckland, New Zealand

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

Received 23 February 2011; revision received 24 May 2011; accepted 1 June 2011

Introduction

More than half a century ago, Kurtén (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).

Correspondence: Simon Y. W. Ho, Fax: +61 2 91140979; E-mail: simon.ho@sydney.edu.au

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.

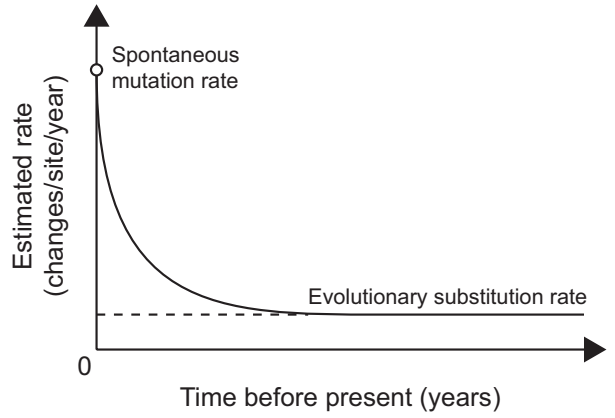

Fig. 1 Plot of time-dependent rates showing an exponentially declining rate estimate with increasing time depth. The spontaneous rate of non-lethal mutations is approached at a time depth of zero. As the time frame increases, the estimated rate tends towards the long-term substitution rate observed in 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.

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 delete-

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 divergences; 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

Box 1. *Continued*

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).

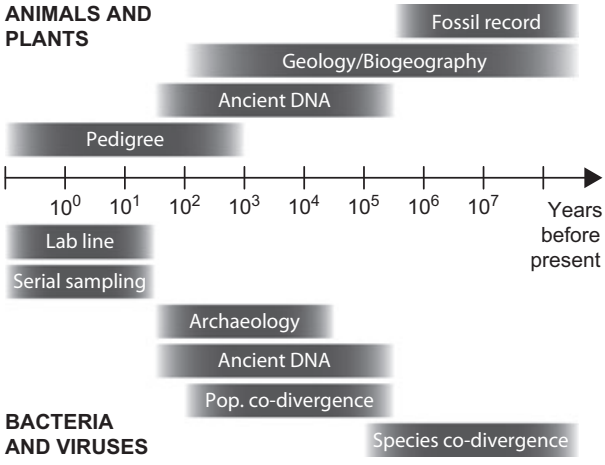

Fig. 2 Typical age ranges of different forms of calibrating information.

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; Loogväli *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

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).

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).

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

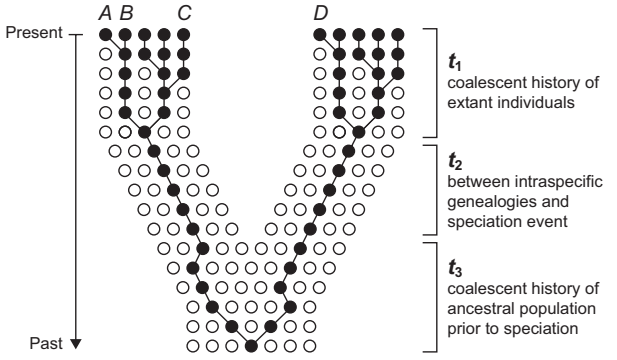

Fig. 3 A simplified representation of genealogical history at a single locus in a pair of species. Each circle represents a randomly mating individual and each row represents one generation. The ancestral species (spanning the time period t_3) splits into two descendent species (spanning the periods t_2 and t_1). Four contemporary individuals labelled *A*, *B*, *C* and *D* are referred to in the text.

are likely to be deleterious, but have not yet been removed by selection. If *A* and *B* are very closely related (e.g., siblings), then a larger proportion of the mutations can be detected and the molecular rate estimate will approach the per replication mutation rate (Howell *et al.* 2003; Santos *et al.* 2005).

A and *C* represent distant relatives within the same population. The number of genetic differences between them is largely determined by the mutation rate but, in the time since their common ancestor, selection has had more opportunity to remove mildly deleterious mutations. For this reason, the estimate of the molecular rate in the *A*–*C* comparison will be considerably lower than that derived from the *A*–*B* comparison.

A and *D* represent individuals from different species. Many of the genetic differences between them are substitutions (mutations that are fixed during t_2; Fig. 3). However, some of the differences might also be polymorphisms that arose during t_1. Additionally, some differences might be due to mutations that occurred before the timing of the speciation event (i.e., during t_3). The relative proportions of each of these periods (t_1, t_2, and t_3) determine the relative influence of the mutation and substitution rates on the molecular rate estimate from the *A*–*D* comparison. If t_2 is long relative to both t_1 and t_3 (e.g., if *A* and *D* are individuals from distantly related species), then the rate estimate will approximate the long-term substitution rate.

The reduction in molecular rate estimates calculated from the *A*–*C* and *A*–*D* comparisons, relative to the *A*–*B* comparison, depends on the effective population size and on the distribution of fitness effects of new mutations in the two lineages. If the population size is large and the majority of mutations are deleterious, then the rate estimated from the *A*–*C* comparison will be considerably lower than from the *A*–*B* comparison. However, if both the population size and the fitness effects of mutations are very small, then the rates estimated from the *A*–*D* and *A*–*C* comparisons may be close to that estimated from the *A*–*B* comparison. Indeed, when all mutations are strictly neutral, the long-term substitution rate is equal to the per generation, per individual mutation rate, regardless of population size (Kimura 1968).

Looking beyond this basic framework, a range of other biological and methodological factors can amplify the disparity between estimates of short- and long-term rates. The identity, importance and relevance of these factors continue to be debated. Below, we broadly class these factors into those that relate to natural selection, calibration error, model misspecification and other artefacts. This classification is by no means definitive; most of the factors involve inadequate modelling of the biological process when estimating molecular evolutionary rates. We describe the factors and discuss their relative significance.

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; Loogväli *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 (Loogväli *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_3 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 descendent 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, homoplasmy and a lack of recombination. However, occurrences of biparental inheritance, paternal leakage, heteroplasmy 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; Debruyne & 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).

Ascertainment bias

For some types of markers, comparison of rates estimated over different timescales might be confounded if the rates have been calculated using different loci. This can be caused by ascertainment bias, whereby rapidly mutating markers are selected for pedigree studies while slowly evolving markers are chosen for evolutionary studies (Zhivotovsky *et al.* 2004; Bandelt 2008; Ballantyne *et al.* 2010). This can also be the case for single-nucleotide polymorphisms (SNPs), whereby highly variable sites are selected to provide resolution within species but might not provide resolution in interspecific comparisons; for example, this is a major problem with SNP-chips that have been designed to analyse a small subset of species (Decker *et al.* 2009). In some studies, fast-evolving and poorly aligned sites are discarded to improve the ratio of phylogenetic signal to noise and to increase confidence in homology (Castresana 2000), leading to a biased representation of the sequence data. Although it is unclear whether these ascertainment biases extend to any of the direct comparisons that have been made between pedigree and phylogenetic rates (Howell *et al.* 2008), the focus on rapidly evolving markers does lead to the additional problem of mutational saturation over long time frames.

Concluding remarks

Numerous factors have the potential to produce a time-dependent pattern in molecular rates, particularly via elevation of short-term rates. Short-term estimates of rates are likely to include mutations that will not be observed on longer timescales, owing to loss of mutations by selection. Therefore, short-term rates are closer to the nonlethal mutation rate than long-term rates, with the latter reflecting slower substitution rates.

In addition to the transience of deleterious mutations, various estimation biases can lead to an inflation of short-term measures of rates. In general, estimation biases and methodological artefacts tend to cause overestimation of short-term rates and underestimation of long-term rates. Overestimation bias tends to be proportionally greater on short timescales, which leads to a time-dependent pattern of rates. Although it is likely that their contributions will vary considerably among data sets, particularly among taxonomic groups, it seems plausible that negative selection, coalescent calibration error and perhaps model misspecification are the most important factors leading to elevated short-term rates. Some of these issues can be corrected by rigorous testing of model assumptions and careful selection of calibrating information.

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.

Acknowledgements

We thank the Genetics Society of Australasia for sponsoring the 2008 symposium in Adelaide that stimulated this review. We thank Jack da Silva for helpful discussions and Chris Burridge and two anonymous reviewers for constructive feedback. SYWH, RL, LB, MJP, and AC were funded by the Australian Research Council. SYWH was also supported by a start-up grant from the University of Sydney. JS was supported by the University of Adelaide.

References

Ballantyne KN, Goedbloed M, Fang R *et al.* (2010) Mutability of Y-chromosomal microsatellites: rates, characteristics, molecular bases, and forensic implications. *American Journal of Human Genetics*, **87**, 341–353.

Ballard JWO, Whitlock MC (2004) The incomplete natural history of mitochondria. *Molecular Ecology*, **13**, 729–744.

Bandelt HJ (2008) Time dependency of molecular rate estimates: tempest in a teacup. *Heredity*, **100**, 1–2.

Bandelt H-J, Kong QP, Richards M, Macaulay V (2006) Estimation of mutation rates and coalescence times: some caveats. In:*Human Mitochondrial DNA and the Evolution of* Homo sapiens (eds Bandelt H-J, Macaulay V, Richards M), pp. 47–90. Springer, Berlin.

Barr CM, Neiman M, Taylor DR (2005) Inheritance and recombination of mitochondrial genomes in plants, fungi and animals. *New Phytologist*, **168**, 39–50.

Bendall KE, Macaulay VA, Baker JR, Sykes BC (1996) Heteroplasmic point mutations in the human mtDNA control region. *American Journal of Human Genetics*, **59**, 1276–1287.

Bromham L, Eyre-Walker A, Smith NH, Maynard Smith J (2003) Mitochondrial Steve: paternal inheritance of mitochondria in humans. *Trends in Ecology and Evolution*, **18**, 2–4.

Buonagurio DA, Nakada S, Parvin JD *et al.* (1986) Evolution of human influenza A viruses over 50 years: rapid, uniform rate of change in NS gene. *Science*, **232**, 980–982.

Burridge CP, Craw D, Fletcher D, Waters JM (2008) Geological dates and molecular rates: fish DNA sheds light on time dependency. *Molecular Biology and Evolution*, **25**, 624–633.

Buschiazzo E, Gemmell NJ (2006) The rise, fall and renaissance of microsatellites in eukaryotic genomes. *Bioessays*, **28**, 1040–1050.

Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution*, **17**, 540–552.

Clark AG, Whittam TS (1992) Sequencing errors and molecular evolutionary analysis. *Molecular Biology & Evolution*, **9**, 744–752.

Davison J, Ho SYW, Bray SC *et al.* (2011) Late-quaternary biogeography scenarios for the brown bear (*Ursus arctos*), a wild mammal model species. *Quaternary Science Reviews*, **30**, 418–430.

Debruyne R, Poinar HN (2009) Time dependency of molecular rates in ancient DNA data sets, a sampling artifact? *Systematic Biology*, **58**, 348–360.

Decker JE, Pires JC, Conant GC *et al.* (2009) Resolving the evolution of extant and extinct ruminants with high-throughput phylogenomics. *Proceedings of the National Academy of Sciences, USA*, **106**, 18644–18649.

Denver DR, Morris K, Lynch M, Vassilieva LL, Thomas WK (2000) High direct estimate of the mutation rate in the mitochondrial genome of *Caenorhabditis elegans*. *Science*, **289**, 2342–2344.

Denver DR, Morris K, Lynch M, Thomas WK (2004) High mutation rate and predominance of insertions in the *Caenorhabditis elegans* nuclear genome. *Nature*, **430**, 679–682.

Drummond AJ, Suchard MA (2010) Bayesian random local clocks, or one rate to rule them all. *BMC Biology*, **8**, 114.

Drummond AJ, Pybus OG, Rambaut A, Forsberg R, Rodrigo AG (2003) Measurably evolving populations. *Trends in Ecology and Evolution*, **18**, 481–488.

Drummond AJ, Rambaut A, Shapiro B, Pybus OG (2005) Bayesian coalescent inference of past population dynamics from molecular sequences. *Molecular Biology and Evolution*, **22**, 1185–1192.

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

Duffy S, Holmes EC (2009) Validation of high rates of nucleotide substitution in geminiviruses: phylogenetic evidence from East African cassava mosaic viruses. *Journal of General Virology*, **90**, 1539–1547.

Duffy S, Shackelton LA, Holmes EC (2008) Rates of evolutionary change in viruses: patterns and determinants. *Nature Reviews Genetics*, **9**, 267–276.

Edwards SV, Beerli P (2000) Gene divergence, population divergence, and the variance in coalescence time in phylogeographic studies. *Evolution*, **54**, 1839–1854.

Ellegren H (2000) Heterogeneous mutation processes in human microsatellite DNA sequences. *Nature Genetics*, **24**, 400–402.

Elson JL, Turnbull DM, Howell N (2004) Comparative genomics and the evolution of human mitochondrial DNA: assessing the effects of selection. *American Journal of Human Genetics*, **74**, 229–238.

Emerson BC (2007) Alarm bells for the molecular clock? No support for Ho et al.'s model of time-dependent molecular rate estimates. *Systematic Biology*, **56**, 337–345.

Endicott P, Ho SYW (2008) A Bayesian evaluation of human mitochondrial substitution rates. *American Journal of Human Genetics*, **82**, 895–902.

Endicott P, Ho SYW, Metspalu M, Stringer C (2009) Evaluating the mitochondrial timescale of human evolution. *Trends in Ecology and Evolution*, **24**, 515–521.

Eyre-Walker A, Keightley PD (2007) The distribution of fitness effects of new mutations. *Nature Reviews Genetics*, **8**, 610–618.

Feng L, Reeves PR, Lan R *et al.* (2008) A recalibrated molecular clock and independent origins for the cholera pandemic clones. *PLoS ONE*, **3**, e4053.

Firth C, Kitchen A, Shapiro B *et al.* (2010) Using time-structured data to estimate evolutionary rates of double-stranded DNA viruses. *Molecular Biology and Evolution*, **27**, 2038–2051.

Fleischer RC, McIntosh CE, Tarr CL (1998) Evolution on a volcanic conveyor belt: using phylogeographic reconstructions and K-Ar-based ages of the Hawaiian Islands to estimate molecular evolutionary rates. *Molecular Ecology*, **7**, 533–545.

Forster P (2003) To err is human. *Annals of Human Genetics*, **67**, 2–4.

Forster P, Röhl A, Lünnemann P *et al.* (2000) A short tandem repeat–based phylogeny for the human Y chromosome. *American Journal of Human Genetics*, **67**, 182–196.

Galtier N, Nabholz B, Glemin S, Hurst GD (2009) Mitochondrial DNA as a marker of molecular diversity: a reappraisal. *Molecular Ecology*, **18**, 4541–4550.

García-Moreno J (2004) Is there a universal mtDNA clock for birds? *Journal of Avian Biology*, **35**, 465–468.

Gatesy J (2007) A tenth crucial question regarding model use in phylogenetics. *Trends in Ecology and Evolution*, **22**, 509–510.

Genner MJ, Seehausen O, Lunt DH *et al.* (2007) Age of cichlids: new dates for ancient lake fish radiations. *Molecular Biology and Evolution*, **24**, 1269–1282.

Gibbons A (1998) Calibrating the mitochondrial clock. *Science*, **279**, 28–29.

Gibbs AJ, Fargette D, García-Arenal F, Gibbs MJ (2009) Time – the emerging dimension of plant virus studies. *Journal of General Virology*, **91**, 13–22.

Gingerich PD (1983) Rates of evolution: effects of time and temporal scaling. *Science*, **222**, 159–161.

Gingerich PD (2001) Rates of evolution on the time scale of the evolutionary process. *Genetica*, **112–113**, 127–144.

Goedbloed M, Vermeulen M, Fang RN *et al.* (2009) Comprehensive mutation analysis of 17 Y-chromosomal short tandem repeat polymorphisms included in the AmpFlSTR Yfiler PCR amplification kit. *International Journal of Legal Medicine*, **123**, 471–482.

Gray RR, Parker J, Lemey P *et al.* (2011) The mode and tempo of hepatitis C virus evolution within and among hosts. *BMC Evolutionary Biology*, **11**, 131.

Haag-Liautard C, Dorris M, Maside X *et al.* (2007) Direct estimation of per nucleotide and genomic deleterious mutation rates in *Drosophila*. *Nature*, **445**, 82–85.

Haag-Liautard C, Coffey N, Houle D *et al.* (2008) Direct estimation of the mitochondrial DNA mutation rate in *Drosophila melanogaster*. *PLoS Biology*, **6**, e204.

Haile J, Holdaway R, Oliver K *et al.* (2007) Ancient DNA chronology within sediment deposits: are paleobiological reconstructions possible and is DNA leaching a factor? *Molecular Biology and Evolution*, **24**, 982–989.

Harkins GW, Delport W, Duffy S *et al.* (2009) Experimental evidence indicating that mastreviruses probably did not co-diverge with their hosts. *Virology Journal*, **6**, 104.

Hay JM, Subramanian S, Millar CD, Mohandesan E, Lambert DM (2008) Rapid molecular evolution in a living fossil. *Trends in Genetics*, **24**, 106–109.

Heads M (2005) Dating nodes on molecular phylogenies: a critique of molecular biogeography. *Cladistics*, **21**, 62–78.

Heled J, Drummond AJ (2008) Bayesian inference of population size history from multiple loci. *BMC Evolutionary Biology*, **8**, 289.

Heled J, Drummond AJ (2010) Bayesian inference of species trees from multilocus data. *Molecular Biology and Evolution*, **27**, 570–580.

Henn BM, Gignoux CR, Feldman MW, Mountain JL (2009) Characterizing the time dependency of human mitochondrial DNA mutation rate estimates. *Molecular Biology and Evolution*, **26**, 217–230.

Hill WG, Robertson A (1966) The effect of linkage on limits to artificial selection. *Genetical Research*, **8**, 269–294.

Ho SYW, Larson G (2006) Molecular clocks: when times are a-changin'. *Trends in Genetics*, **22**, 79–83.

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

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

Ho SYW, Heupink TH, Rambaut A, Shapiro B (2007a) Bayesian estimation of sequence damage in ancient DNA. *Molecular Biology and Evolution*, **24**, 1416–1422.

Ho SYW, Kolokotronis S-O, Allaby RG (2007b) Elevated substitution rates estimated from ancient DNA. *Biology Letters*, **3**, 702–705.

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

Ho SYW, Saarma U, Barnett R, Haile J, Shapiro B (2008) The effect of inappropriate calibration: three case studies in molecular ecology. *PLoS ONE*, **3**, e1615.

Ho SYW, Lanfear R, Phillips MJ *et al.* (2011) Bayesian estimation of substitution rates from ancient DNA sequences with low information content. *Systematic Biology*, **60**, 366–375.

Holmes EC (2003) Patterns of intra- and interhost nonsynonymous variation reveal strong purifying selection in dengue virus. *Journal of Virology*, **77**, 11296–11298.

Holmes EC (2009) *The Evolution and Emergence of RNA Viruses*. Oxford University Press, Oxford, UK.

Houle D, Nuzhdin SV (2004) Mutation accumulation and the effect of *copia* insertions in *Drosophila melanogaster*. *Genetics Research*, **83**, 7–18.

Howell N, Mackey D (1997) Reply to Macauley et al. *American Journal of Human Genetics*, **61**, 986–990.

Howell N, Kubacka I, Mackey DA (1996) How rapidly does the human mitochondrial genome evolve? *American Journal of Human Genetics*, **59**, 501–509.

Howell N, Smejkal CB, Mackey DA *et al.* (2003) The pedigree rate of sequence divergence in the human mitochondrial genome: there is a difference between phylogenetic and pedigree rates. *American Journal of Human Genetics*, **72**, 659–670.

Howell N, Howell C, Elson JL (2008) Time dependency of molecular rate estimates for mtDNA: this is not the time for wishful thinking. *Heredity*, **101**, 107–108.

Jenkins GM, Rambaut A, Pybus OG, Holmes EC (2002) Rates of molecular evolution in RNA viruses: a quantitative phylogenetic analysis. *Journal of Molecular Evolution*, **54**, 156–165.

Johnson PL, Slatkin M (2008) Accounting for bias from sequencing error in population genetic estimates. *Molecular Biology and Evolution*, **25**, 199–206.

Kayser M, Roewer L, Hedman M *et al.* (2000) Characteristics and frequency of germline mutations at microsatellite loci

from the human Y chromosome, as revealed by direct observation in father/son pairs. *American Journal of Human Genetics*, **66**, 1580–1588.

Keightley PD, Trivedi U, Thomson M *et al.* (2009) Analysis of the genome sequences of three *Drosophila melanogaster* spontaneous mutation accumulation lines. *Genome Research*, **19**, 1195–1201.

Kemp BM, Malhi RS, McDonough J *et al.* (2007) Genetic analysis of early holocene skeletal remains from Alaska and its implications for the settlement of the Americas. *American Journal of Physical Anthropology*, **132**, 605–621.

Kimura M (1968) Evolutionary rate at the molecular level. *Nature*, **217**, 624–626.

Kitchen A, Miyamoto MM, Mulligan CJ (2008) Utility of DNA viruses for studying human host history: case study of JC virus. *Molecular Phylogenetics and Evolution*, **46**, 673–682.

Korsten M, Ho SYW, Davison J *et al.* (2009) Sudden expansion of a single brown bear maternal lineage across northern continental Eurasia after the last ice age: a general demographic model for mammals? *Molecular Ecology*, **18**, 1963–1979.

Kuo C-H, Ochman H (2009) Inferring clocks when lacking rocks: the variable rates of molecular evolution in bacteria. *Biology Direct*, **4**, 35.

Kurtén B (1959) Rates of evolution in fossil mammals. *Cold Spring Harbor Symposia on Quantitative Biology*, **24**, 205–215.

Lambert DM, Ritchie PA, Millar CD *et al.* (2002) Rates of evolution in ancient DNA from Adélie penguins. *Science*, **295**, 2270–2273.

Lemey P, Pybus OG, Van Dooren S, Vandamme AM (2005) A Bayesian statistical analysis of human T-cell lymphotropic virus evolutionary rates. *Infection, Genetics and Evolution*, **5**, 291–298.

Lemey P, Rambaut A, Pybus OG (2006) HIV evolutionary dynamics within and among hosts. *AIDS Reviews*, **8**, 125–140.

Lemmon AR, Moriarty EC (2004) The importance of proper model assumption in Bayesian phylogenetics. *Systematic Biology*, **53**, 265–277.

Li W, Tanimura M, Sharp P (1988) Rates and dates of divergence between AIDS virus nucleotide sequences. *Molecular Biology & Evolution*, **5**, 313–330.

Li Y, Carroll DS, Gardner SN *et al.* (2007) On the origin of smallpox: correlating variola phylogenics with historical smallpox records. *Proceedings of the National Academy of Sciences, USA*, **104**, 15787–15792.

Lindahl T (1993) Instability and decay of the primary structure of DNA. *Nature*, **362**, 709–715.

Loogväli E-L, Kivisild T, Margus T, Villems R (2009) Explaining the imperfection of the molecular clock of hominid mitochondria. *PLoS ONE*, **4**, e8260.

Macaulay VA, Richards MB, Forster P *et al.* (1997) mtDNA mutation rates – no need to panic. *American Journal of Human Genetics*, **61**, 983–985.

Marko PB (2002) Fossil calibration of molecular clocks and the divergence times of geminate species pairs separated by the Isthmus of Panama. *Molecular Biology and Evolution*, **19**, 2005–2021.

Mateiu LM, Rannala BH (2008) Bayesian inference of errors in ancient DNA caused by postmortem degradation. *Molecular Biology and Evolution*, **25**, 1503–1511.

Millar CD, Dodd A, Anderson J *et al.* (2008) Mutation and evolutionary rates in Adélie penguins from the Antarctic. *PLoS Genetics*, **4**, e1000209.

Miller HC, Moore JA, Allendorf FW, Daugherty CH (2009) The evolutionary rate of tuatara revisited. *Trends in Genetics*, **25**, 13–15.

Mishmar D, Ruiz-Pesini E, Golik P *et al.* (2003) Natural selection shaped regional mtDNA variation in humans. *Proceedings of the National Academy of Sciences, USA*, **100**, 171–176.

Mumm S, Whyte MP, Thakker RV, Buetow KH, Schlessinger D (1997) mtDNA analysis shows common ancestry in two kindreds with X-linked recessive hypoparathyroidism and reveals a heteroplasmic silent mutation. *American Journal of Human Genetics*, **60**, 153–159.

Nachman MW, Crowell SL (2000) Estimate of the mutation rate per nucleotide in humans. *Genetics*, **156**, 297–304.

Navascués M, Emerson BC (2009) Elevated substitution rate estimates from ancient DNA: model violation and bias of Bayesian methods. *Molecular Ecology*, **18**, 4390–4397.

Notohara M (1990) The coalescent and the genealogical process in geographically structured population. *Journal of Mathematical Biology*, **29**, 59–75.

O'Fallon BD, Seger J, Adler FR (2010) A continuous-state coalescent and the impact of weak selection on the structure of gene genealogies. *Molecular Biology and Evolution*, **27**, 1162–1172.

Ohta T (1973) Slightly deleterious mutant substitutions in evolution. *Nature*, **246**, 96–98.

Ohta T (1992) The nearly neutral theory of molecular evolution. *Annual Review of Ecology and Systematics*, **23**, 263–286.

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

Parsons TJ, Muniec DS, Sullivan K *et al.* (1997) A high observed substitution rate in the human mitochondrial DNA control region. *Nature Genetics*, **15**, 363–368.

Penny D (2005) Relativity for molecular clocks. *Nature*, **426**, 183–184.

Peterson GI, Masel J (2009) Quantitative prediction of molecular clock and K_a/K_s at short timescales. *Molecular Biology and Evolution*, **26**, 2595–2603.

Phillips MJ (2009) Branch-length estimation bias misleads molecular dating for a vertebrate mitochondrial phylogeny. *Gene*, **441**, 132–140.

Pybus OG, Rambaut A (2009) Evolutionary analysis of the dynamics of viral infectious disease. *Nature Reviews Genetics*, **10**, 540–550.

Rajabi-Maham H, Orth A, Bonhomme F (2008) Phylogeography and postglacial expansion of *Mus musculus domesticus* inferred from mitochondrial DNA coalescent, from Iran to Europe. *Molecular Ecology*, **17**, 627–641.

Rambaut A (2000) Estimating the rate of molecular evolution: incorporating non-contemporaneous sequences into maximum likelihood phylogenies. *Bioinformatics*, **16**, 395–399.

Rambaut A, Ho SYW, Drummond AJ, Shapiro B (2009) Accommodating the effect of ancient DNA damage on inferences of demographic histories. *Molecular Biology and Evolution*, **26**, 245–248.

Ramsden C, Melo FL, Figueiredo LM *et al.* (2008) High rates of molecular evolution in hantaviruses. *Molecular Biology and Evolution*, **25**, 1488–1492.

Raquin AL, Depaulis F, Lambert A *et al.* (2008) Experimental estimation of mutation rates in a wheat population with a gene genealogy approach. *Genetics*, **179**, 2195–2211.

Ricklefs RE, Fallon SM (2002) Diversification and host switching in avian malaria parasites. *Proceedings of the Royal Society B*, **269**, 885–892.

Rocha EP, Smith JM, Hurst LD *et al.* (2006) Comparisons of dN/dS are time dependent for closely related bacterial genomes. *Journal of Theoretical Biology*, **239**, 226–235.

Rodrigo A, Bertels F, Heled J *et al.* (2008) The perils of plenty: what are we going to do with all these genes? *Philosophical Transactions of the Royal Society London Series B*, **363**, 3893–3902.

Roopnarine PD (2003) Analysis of rates of morphologic evolution. *Annual Review of Ecology, Evolution, and Systematics*, **34**, 605–632.

Ruiz-Pesini E, Mishmar D, Brandon M, Procaccio V, Wallace DC (2004) Effects of purifying and adaptive selection on regional variation in human mtDNA. *Science*, **303**, 223–226.

Santos C, Montiel R, Sierra B *et al.* (2005) Understanding differences between phylogenetic and pedigree-derived mtDNA mutation rate: a model using families from the Azores Islands (Portugal). *Molecular Biology & Evolution*, **22**, 1490–1505.

Santos C, Montiel R, Arruda A *et al.* (2008) Mutation patterns of mtDNA: empirical inferences for the coding region. *BMC Evolutionary Biology*, **8**, 167.

Schwartz M, Vissing J (2002) Paternal inheritance of mitochondrial DNA. *New England Journal of Medicine*, **347**, 576–580.

Shapiro B, Ho SYW, Drummond AJ *et al.* (2011) A Bayesian method to estimate unknown sequence ages in a phylogenetic context. *Molecular Biology and Evolution*, **28**, 879–887.

Sharp PM, Bailes E, Chaudhuri RR *et al.* (2001) The origins of acquired immune deficiency syndrome viruses: where and when? *Philosophical Transactions of the Royal Society London Series B*, **356**, 867–876.

Sigurdardóttir S, Helgason A, Gulcher JR, Stefánsson K, Donnelly P (2000) The mutation rate in the human mtDNA control region. *American Journal of Human Genetics*, **66**, 1599–1609.

Soares P, Ermini L, Thomson N *et al.* (2009) Correcting for purifying selection: an improved human mitochondrial molecular clock. *American Journal of Human Genetics*, **84**, 1–20.

Stewart JB, Freyer C, Elson JL *et al.* (2008) Strong purifying selection in transmission of mammalian mitochondrial DNA. *PLoS Biology*, **6**, e10.

Subramanian S (2009) Temporal trails of natural selection in human mitogenomes. *Molecular Biology and Evolution*, **26**, 715–717.

Subramanian S, Denver DR, Millar CD *et al.* (2009a) High mitogenomic evolutionary rates and time dependency. *Trends in Genetics*, **25**, 482–486.

Subramanian S, Hay JM, Mohandesan E, Millar CD, Lambert DM (2009b) Molecular and morphological evolution in tuatara are decoupled. *Trends in Genetics*, **25**, 16–18.

Sullivan J, Joyce P (2005) Model selection in phylogenetics. *Annual Review of Ecology and Systematics*, **36**, 445–466.

Sun C, Kong QP, Zhang YP (2007) The role of climate in human mitochondrial DNA evolution: a reappraisal. *Genomics*, **89**, 338–342.

Swofford DL, Waddell PJ, Huelsenbeck JP *et al.* (2001) Bias in phylogenetic estimation and its relevance to the choice between parsimony and likelihood methods. *Systematic Biology*, **50**, 525–539.

Taubenberger JK, Reid AH, Krafft AE, Bijwaard KE, Fanning TG (1997) Initial genetic characterization of the 1918 ''Spanish'' influenza virus. *Science*, **275**, 1793–1796.

Tsaousis AD, Martin DP, Ladoukakis ED, Posada D, Zouros E (2005) Widespread recombination in published animal mtDNA sequences. *Molecular Biology and Evolution*, **22**, 925–933.

Vermeulen M, Wollstein A, van der Gaag K *et al.* (2009) Improving global and regional resolution of male lineage differentiation by simple single-copy Y-chromosomal short tandem repeat polymorphisms. *Forensic Science International: Genetics*, **3**, 205–213.

Wayne RK, van Valkenburgh B, O'Brien SJ (1991) Molecular distance and divergence time in carnivores and primates. *Molecular Biology & Evolution*, **8**, 297–319.

White DJ, Wolff JN, Pierson M, Gemmell NJ (2008) Revealing the hidden complexities of mtDNA inheritance. *Molecular Ecology*, **17**, 4925–4942.

Willerslev E, Cappellini E, Boomsma W *et al.* (2007) Ancient biomolecules from deep ice cores reveal a forested southern Greenland. *Science*, **317**, 111–114.

Williamson S, Orive ME (2002) The genealogy of a sequence subject to purifying selection at multiple sites. *Molecular Biology and Evolution*, **19**, 1376–1384.

Woodhams M (2006) Can deleterious mutations explain the time dependency of molecular rate estimates? *Molecular Biology and Evolution*, **23**, 2271–2273.

Xue Y, Wang Q, Long Q *et al.* (2009) Human Y chromosome base-substitution mutation rate measured by direct sequencing in a deep-rooting pedigree. *Current Biology*, **19**, 1453–1457.

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

Yoder AD, Yang ZH (2000) Estimation of primate speciation dates using local molecular clocks. *Molecular Biology & Evolution*, **17**, 1081–1090.

Zhivotovsky LA, Underhill PA, Cinnioglu C *et al.* (2004) The effective mutation rate at Y chromosome short tandem repeats, with application to human population-divergence time. *American Journal of Human Genetics*, **74**, 50–61.

Zhivotovsky LA, Underhill PA, Feldman MW (2006) Difference between evolutionarily effective and germ-line mutation rate due to stochastically varying haplogroup size. *Molecular Biology and Evolution*, **23**, 2268–2270.

S.Y.W.H. is interested in molecular clocks, evolutionary timescales, and ancient DNA. R.L. and L.B. study the causes and consequences of molecular evolution. M.J.P. uses phylogenetic inference to understand evolutionary and ecological processes. J.S. is a doctoral candidate investigating how evolutionary timescales could be refined in the light of ancient DNA and improved molecular clock methods. A.R. is the director of NESCent and develops computational evolutionary genetic methods to model the processes that shape the diversity of rapidly evolving pathogens. A.C. is the director of the Australian Centre for Ancient DNA at the University of Adelaide.
