

Points of View

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Evidence for Time Dependency of Molecular Rate Estimates

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Long-term changes in the genetic composition of a population occur by the fixation of new mutations, a process known as substitution. The rate at which mutations arise in a population and the rate at which they are fixed are expected to be equal under neutral conditions (Kimura, 1968). Between the appearance of a new mutation and its eventual fate of fixation or loss, there will be a period in which it exists as a transient polymorphism in the population (Kimura and Ohta, 1971). If the majority of mutations are deleterious (and nonlethal), the fixation probabilities of these transient polymorphisms are reduced and the mutation rate will exceed the substitution rate (Kimura, 1983). Consequently, different apparent rates may be observed on different timescales of the molecular evolutionary process (Penny, 2005; Penny and Holmes, 2001). The substitution rate of the mitochondrial protein-coding genes of birds and mammals has been traditionally recognized to be about 0.01 substitutions/site/million years (Myr; Brown et al., 1979; Ho, 2007; Irwin et al., 1991; Shields and Wilson, 1987), with the noncoding D-loop evolving several times more quickly (e.g., Pesole et al., 1992; Quinn, 1992). Over the past decade, there has been mounting evidence that instantaneous mutation rates substantially exceed substitution rates, in a range of organisms (e.g., Denver et al., 2000; Howell et al., 2003; Lambert et al., 2002; Mao et al., 2006; Mumm et al., 1997; Parsons et al., 1997; Santos et al., 2005). The immediate reaction to the first of these findings was that the polymorphisms generated by the elevated mutation rate are short-lived, perhaps extending back only a few hundred years (Gibbons, 1998; Macaulay et al., 1997). That is, purifying selection was thought to remove these polymorphisms very rapidly.

Recently, we suggested that these polymorphisms persist for much longer and, if not accounted for, can lead to biased estimates of short-term substitution rates (Ho et al., 2005). Furthermore, we posited that the influence of this bias declines predictably with increasing age of the

calibration, with the result that it can be accommodated while estimating divergence times. This hypothesis was based on analyses of mitochondrial sequences from the protein-coding genes of birds and primates, as well as D-loop sequences from primates. The results of these analyses indicated that the bias in rate estimates may persist for a period of up to 1 million years, whereupon estimated rates eventually converge on the values estimated in phylogenetic studies. The range of data sets we analyzed, however, was far too limited to provide accurate estimates of the exact time frame over which this transition occurs, apart from indicating that it appears to occur over a much longer period than previously thought. The noticeable consequence of this relationship is that rates estimated over the short-term are timescale dependent, which has enormous implications for studies of recent evolutionary events (Ho and Larson, 2006; Penny, 2005).

The time dependency of molecular rate estimates, far from being a new hypothesis, had been present in the literature for well over a decade. Wayne et al. (1991) first described the relationship explicitly in a study of carnivores and primates, finding that substitution rate estimates decreased linearly with increasing time depth. García-Moreno (2004) discovered a similar pattern in birds, but the decline was exponential rather than linear; this was mirrored in our study, in which we analyzed similar data sets (Ho et al., 2005). Macaulay et al. (1997) recognized the implications of such a relationship: "Given that the pedigree rate may be measurably higher than the phylogenetic rate, one would expect a monotonic decline from one to the other as the time depth increases. The problem would lie in deciding on the rate that is appropriate to any particular data set." This "timescale problem" was one of the issues discussed at the Fifth Annual New Zealand Phylogenetics Meeting in 2001, where it was noted that "different time scales lead to different 'rates' of evolution" (Penny and Holmes, 2001).

Based on a reanalysis of our data, Emerson (ibid.) argues that the observed time dependency is an artefact of poor model selection and, consequently, that there is little cause for concern among evolutionary biologists. Here we discuss the results of his analyses, demonstrate that our original estimates are still largely valid when a conservative approach is used, and collate some of the widespread evidence supporting the time dependency of molecular rate estimates. We also present a novel analysis of a large bison data set, in which we find a time-dependent pattern in a series of rate estimates calibrated using radiocarbon-dated sequences. All of the mutation and substitution rate estimates given in this paper refer to per lineage rates (cf. “divergence” rates, which are twice as large).

RESPONSE AND REANALYSIS

In his critique, Emerson raises a number of concerns about the analyses performed in our paper (Ho et al., 2005). His primary criticism is that model selection was not conducted in our study, so that analyses of certain data sets were overparameterized. Generally, this leads to an increase in the variance of estimates without the benefit of additional explanatory power, but in more serious cases it can lead to parameter unidentifiability (Rannala, 2002). Emerson reanalyzes our data after implementing a model selection procedure, but his rate estimates appear to be broadly similar to ours, suggesting that the parameter of interest is largely unaffected and that the elevated short-term rate estimates are not an artefact of overparameterization. Nevertheless, on account of various methodological differences, it is unclear whether or not the results of Emerson’s reanalysis can be directly compared to our original results. For example, we analyzed the data using a relaxed clock with an autocorrelated exponential model of rate change, whereas he used an uncorrelated lognormal model (see Drummond et al., 2006). Additionally, Emerson does not describe his selection of the prior on the tree,

whereas we compared different demographic models in the coalescent prior. This has the potential to influence estimates of the phylogeny and of the substitution rate.

To address these concerns, we reanalyze most of the data sets used in our original study. We take a cautious approach when selecting data and calibration points, removing several of the data sets that Emerson considers contentious, including the Neandertals and mandrills. The data sets in this reanalysis were described in our previous study (Ho et al., 2005) and are listed in Table 1.

In this reanalysis, we focus our attention on data sets with calibration points of 1 Myr or less. A conservative value of 50 thousand years (kyr) is used to calibrate the rate estimate from the Amerindian data set, instead of the value of 24 kyr we used previously. This revised value, broadly based on archaeological evidence of eastward human migration in Asia (for a review, see Mellars, 2006), would be considered a conservatively old date for the coalescence of Nuu-Chah-Nulth lineages. Emerson chooses to discard this data point, citing Ward et al. (1991) in saying that “the magnitude of sequence divergence within the Nuu-Chah-Nulth tribe suggests that the origin of this diversity predates the entry of humans into the Americas.” This assertion is only correct if the time dependency of molecular rates does not hold, and as such his argument is circular. A coalescent-based approach to inferring the rate in the Nuu-Chah-Nulth tribe, which required an estimate of effective population size but did not necessitate the use of any independent calibration points, produced a rate estimate of 0.11 substitutions/site per 100 kyr (Lundstrom et al., 1992). This suggests that the high level of genetic diversity arises from an elevated mutation rate rather than a prolonged demographic history. We believe that useful information can still be extracted from the Amerindian data by using a conservative calibration of 50 kyr.

Calibration points for the Hawaiian honeycreepers, cranes, and sunbirds are taken from the studies by

TABLE 1. Estimates of mitochondrial mutation rates from pedigree and population studies of humans and birds made using calibration times of 1 Myr or less.

Species	Region	Rate ^a (substitutions/site/Myr)	Calibration	Method ^b	Best model ^c	Reference ^d
Human	D-loop	0.768 (0.164–1.398)	Pedigree	Pedigree	—	Santos et al. (2005)
Human	D-loop	0.475 (0.265–0.785)	Pedigree	Pedigree	—	Howell et al. (2003)
Human (Amerindian)	D-loop	0.197 (0.115–0.293)	80 kyr	Bayesian-E	HKY	This study
Human	Coding	0.150 (0.020–0.490)	Pedigree	Pedigree	—	Howell et al. (2003)
Rock partridge	D-loop	0.125	238 kyr	Coalescent	—	Randi et al. (2003)
Amakihi	<i>Cytb</i>	0.075 (0.036–0.111)	430 kyr	Bayesian-E	GTR+G+I	This study
Cranes	<i>Cytb</i>	0.005 (0.003–0.008)	1000 kyr	Bayesian-E	HKY	This study
Sunbirds	<i>ATP6, cytb, ND4</i>	0.108 (0.083–0.132)	125 kyr	Bayesian-E	TrN+G (<i>ATP6</i>),	This study
Sunbirds	<i>ATP6, ND4</i>	0.047 (0.016–0.095)	500 kyr	Bayesian-E	HKY (<i>cytb</i>),	This study
Sunbirds	<i>ATP6, cytb, ND4</i>	0.026 (0.015–0.049)	500 kyr	Bayesian-C	K81uf (<i>ND4</i>)	This study

^aNumbers in parentheses denote confidence intervals (pedigree-based estimates) or credibility intervals (Bayesian estimates). Pedigree- and coalescent-based rate estimates were taken directly from previous studies, with the remaining rates estimated using the Bayesian phylogenetic software BEAST (Drummond and Rambaut, 2003).

^bFor estimates made using Bayesian analysis, two population models were compared for the coalescent prior. Model selection was performed by comparison of average marginal posterior likelihoods. Estimates are given for the exponential growth model (Bayesian-E) when it was significantly better, with the remaining estimates produced by the constant-size model (Bayesian-C).

^cBest model as determined by hierarchical likelihood ratio tests using the software ModelTest. Model abbreviations follow those used by ModelTest.

^dDetails of all data sets are given in the original study by Ho et al. (2005), in the references cited here, and in the main text.

Fleischer et al. (1998), Krajewski and King (1996), and Warren et al. (2003), respectively. In the analyses of Hawaiian honeycreepers, we only use the calibration point that represents the divergence between the amakihi on the neighboring islands of Hawaii and Maui, which reduces the risk of calibration error due to lineage extinction. In the analyses of sunbirds, we concatenate the gene sequences and treat the alignment as a partitioned data set. Full details of each analysis can be found in the input files (Supplementary Material, available at <http://www.systematicbiology.org/>).

In addition, we take four published mutation rate estimates at face value: three pedigree-based estimates made from human mitochondria (Howell et al., 2003; Santos et al., 2005) and a coalescent-based estimate made from the D-loop of rock partridges (Randi et al., 2003). Emerson accepted the validity of the pedigree-based estimate of the mutation rate in human mitochondrial protein-coding genes (Howell et al., 2003), but his discussion of the estimate is somewhat misleading. Howell et al. (2003) presented two rate estimates from protein-coding data, the first of which was obtained from a pooled data set comprising their own data as well as those from a previous study (Howell et al., 1996). The rate was calculated by dividing the observed number of mutations (four) by the number of transmission events (170), then dividing the result by the assumed generation time (20 years) and the length of the sequence analyzed (approximately 7800 bp). This gave an estimated mutation rate of 0.15 mutations/site/Myr, which was remarkably high compared to previous phylogeny-based estimates. A third data set was then added to the pooled data (Cavelier et al., 2000), reducing the overall rate estimate to 0.06 mutations/site/Myr. This second rate estimate is extremely conservative, however, because the mutation count in the additional data set was obtained from a 500-bp mitochondrial segment but was still divided by 7800 bp to give the rate (Howell et al., 2003). In view of this, the first rate estimate should be more accurate. Additionally, contrary to Emerson's suggestion, both of these estimates are actually per-lineage mutation rates, not divergence rates.

In all analyses, we used the uncorrelated lognormal relaxed-clock model (described in detail by Drummond et al., 2006) in the Bayesian phylogenetic software BEAST 1.3 (Drummond and Rambaut, 2003) in order to match the model used by Emerson. Estimates were made using two different population models for the coalescent prior: constant size and exponential growth. Rate estimates were obtained under the better model, with coalescent model selection performed by comparison of average marginal posterior likelihoods (see Table 1). For all data sets, we compared substitution models using hierarchical likelihood ratio tests performed by ModelTest 3.7 (Posada and Crandall, 1998); the best models are shown in Table 1. For the parameters of each substitution model, with the exception of the proportion of invariant sites, we placed uniform priors of [0,500]. For the proportion of invariant sites, where applicable, we used a uniform prior of [0,1].

For each analysis, posterior distributions of parameters were approximated by Markov chain Monte Carlo (MCMC) sampling. Samples were drawn every 500 steps over a total of 5,000,000 steps, following a discarded burn-in of 500,000 steps. Mixing and convergence to the stationary distribution were investigated using Tracer 1.3 (Rambaut and Drummond, 2004); if the set of samples was found to be insufficient for a given data set, as determined by an effective sample size (ESS) less than 100, the analysis was repeated and the results combined with the first run.

The results indicate that there is support for time dependency among rates despite the conservative approach that has been used (Table 1). Four of the five rate estimates from the mitochondrial protein-coding regions of birds exclude the phylogenetic substitution rate of 0.01 substitutions/site/Myr from their 95% highest posterior densities (HPDs). If the elevated rate estimates are due to calibration error, as Emerson postulates, then several calibration times must have been understated by a factor of nearly 10 for the mean rate estimates to be reconciled with the traditional phylogenetic rate. Among the human data sets, the pedigree-based rates are significantly higher than other rate estimates. The Amerindian data set yielded a rate estimate of 0.197 substitutions/site/Myr (95% HPD: 0.115–0.293 substitutions/site/Myr), despite the use of a conservative calibration time.

The 95% HPDs on our estimates, and those on the estimates made in our previous study (Ho et al., 2005), are noticeably wider than those obtained by Emerson. We believe that this is due to Emerson's adoption of an empirical Bayesian approach to the analysis, in which he has specified very narrow, bounded priors on parameter values based on maximum-likelihood estimates (MLEs) obtained using PAUP (Swofford, 1990). Many of the data sets in his analyses are small, so it is possible that these parameter MLEs are subject to substantial sampling errors (Yang, 2006). Emerson's use of an empirical Bayesian approach discards the uncertainty in the substitution model parameters without convincing justification, leading to artificially small HPDs on his rate estimates, which are therefore more likely to exclude the true rate than are our estimates. Therefore, in the present analyses, we feel that it is more appropriate to adopt a full Bayesian approach, especially as the software permits it and no substantial computational cost is incurred in doing so.

One of the data sets that was discarded in our reanalysis included four Neandertal sequences. In his reanalysis of this alignment, Emerson obtained a rate estimate consistent with traditional phylogenetic estimates, but his reanalysis suffers from a serious flaw, which has been warned against previously (Hasegawa et al., 1998; Ho and Larson, 2006). First, he estimates the age of the most recent common ancestor (MRCA) of Neandertals using two deep, phylogenetic calibration points, including a range of 4 to 6 Myr for the human-chimpanzee split. Emerson then uses this phylogenetically derived Neandertal MRCA estimate to calibrate his rate estimate in

an analysis of the same Neandertal sequences. Due to the manifest circularity of this approach, it is clear that his rate estimate (0.05 substitutions/site/Myr) is actually based on deep calibrations and not on the Neandertal MRCA and will therefore be consistent with traditional phylogenetic estimates *by construction*. We performed an analysis of the Neandertal sequences and were unable to replicate Emerson's results in full. When we fixed the age of the Neandertal MRCA at 295 kyr and treated all of the sequences as being contemporaneous, we obtained a mean rate estimate of 0.0537 substitutions/site/Myr (95% HPD: 0.0234–0.0870 substitutions/site/Myr), reflecting Emerson's estimate. When we added dates to the tips of the tree, the mean rate estimate was 0.0551 substitutions/site/Myr (95% HPD: 0.0248–0.0890 substitutions/site/Myr), whereas Emerson inferred a rate of 0.79 substitutions/site/Myr. In contrast with the results obtained by Emerson, our findings do not point to anything sinister with the operation of BEAST.

From the reanalyses of our original data sets, it is possible to conclude that the majority of rate estimates based on recent (<1 Myr) calibration points are significantly higher than phylogenetically derived substitution rates. There are, however, several limitations to the analyses described above and in our original paper. The lack of suitable calibration points precludes any detailed investigation of the critical region of time dependency. Additionally, the heterogeneous nature of the data sets inevitably introduces variation due to sampling, taxonomic, and stochastic factors. Some of these limitations can be circumvented using an ancient DNA data set, in which radiocarbon-dated sequences act as calibration points at the tips of the tree. Below, we present such an analysis of a large bison data set, in which the sequences range in age from 0 to approximately 60 kyr.

CASE STUDY: ANCIENT BISON SEQUENCES

An alignment of 182 mitochondrial control region sequences from Beringian bison was obtained from a published study (Shapiro et al., 2004). The data set comprises 22 modern and 160 ancient radiocarbon-dated individuals. A previous analysis of the complete data set, performed using BEAST with the HKY+G+I substitution model and with a four-parameter demographic model, produced a rate estimate of 0.32 substitutions/site/Myr (95% HPD: 0.23–0.42 substitutions/site/Myr; Shapiro et al. 2004).

Beginning with the full alignment, 10 further data sets were created by progressive removal of older sequences in increments of 5000 years, so that the eleventh and smallest data set comprised 66 sequences, all of which were less than 10 kyr in age (see Table 2). For each data set, phylogenetic analysis was performed using BEAST with a strict molecular clock and using the HKY+G+I model of nucleotide substitution. A 12-category general Bayesian skyline plot model, which was previously shown to offer the best fit to the data (Drummond et al., 2005), was used to estimate the population demographic history. Substitution parameters were coestimated along

TABLE 2. Details of bison data sets used to estimate rates. All alignments are 615 bp in length.

Age range (kyr)	Sequences		Distinct variable sites	Estimated mutation rate (substitutions/site/Myr)	
	Number			Mean	95% HPD
0–60	182		236	0.291	0.212–0.384
0–55	176		228	0.316	0.234–0.402
0–50	173		219	0.342	0.249–0.440
0–45	162		214	0.373	0.273–0.491
0–40	157		200	0.397	0.270–0.522
0–35	146		187	0.442	0.312–0.585
0–30	132		171	0.527	0.379–0.707
0–25	118		156	0.503	0.334–0.694
0–20	105		130	0.543	0.330–0.768
0–15	95		117	0.457	0.272–0.654
0–10	66		85	0.502	0.250–0.767

with demographic model parameters and genealogical divergence times. For each data set, the MCMC was run for 30,000,000 steps with the initial 3,000,000 steps discarded as burn-in. Trees and model parameters were sampled every 3000 steps thereafter. Substitution rate and population size parameters were well sampled and their posterior distributions were unimodal. Convergence was assessed by manual inspection of traces and all ESSs were larger than 100.

Estimated substitution rates from the eleven data sets showed a pattern of increasing rate with decreasing calibration time (Fig. 1 and Table 2), consistent with the hypothesis of time dependency. Further investigation, using a consistent sample size from each age category, revealed that this was not an artefact of decreasing sample size (results not shown). The rate pattern can be explained by considering the ratio of terminal branches to the total tree height. If transient polymorphisms are the cause of elevated rates towards the present, then terminal branches are predicted to bear an excess of mutations (Williamson and Orive, 2002). With increasing calibration age, deeper nodes are being added to the tree, thereby reducing the amount of terminal branches as a proportion of the tree. The corollary of this is a decrease in the mean estimated rate.

Some component of the pattern could be due to a bias because as the calibrations move closer to the present, there is a decreasing amount of information in the sequences. Consequently, there is greater uncertainty on the inferred rates, so that the posterior distribution spreads out and the mean estimate increases. In general, estimates of scale parameters (parameters bounded at zero but with no upper bound) are upwardly biased, whether they are estimated by maximum likelihood or Bayesian MCMC. This estimation problem is well known and not limited to BEAST. Provided that the sequences are informative and that the dated tips are sufficiently distinct in age, the impact of this estimation bias will be small, but this requires further investigation. It should also be noted that this bias in no way affects the coverage of the credible interval on the estimate. So, although the means may be upwardly biased, the 95% HPDs will still accurately reflect the uncertainty in the estimate.

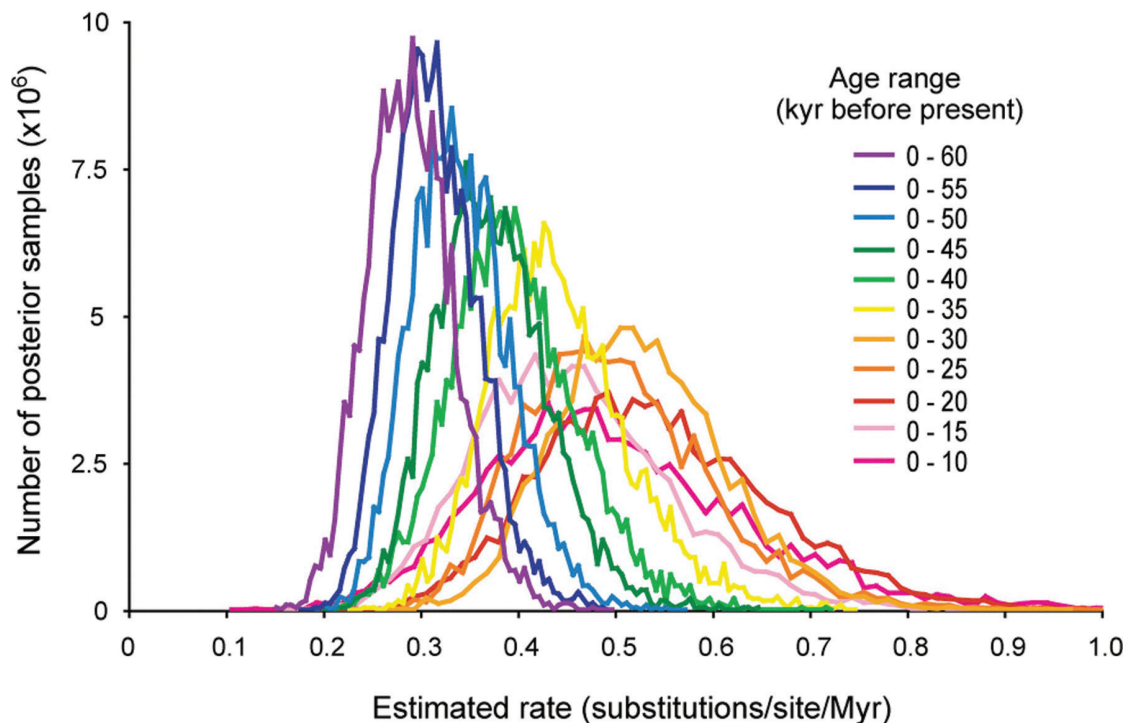


FIGURE 1. Posterior distributions of substitution rates for 11 data sets of mitochondrial control region sequences from Beringian bison. Each data set is a subset of a published alignment of 184 taxa (Shapiro et al., 2004), with sequences ranging in age from 0 to approximately 60 kyr. The 11 data sets used in this analysis were produced by the progressive removal of older sequences from the complete data set in increments of 5 kyr, with an associated decrease in both the number of sequences and in total tree length. The resulting data sets ranged from 184 sequences spanning 60 kyr to 66 sequences spanning only 10 kyr. Each data set was analyzed using the Bayesian phylogenetic software BEAST, with 30,000,000 MCMC steps. The first 10% of the MCMC was discarded as burn-in and parameters were sampled every 3000 steps thereafter. Further details are given in the main text.

Therefore, given the HPDs associated with our estimates, we suggest that the magnitude of the estimation bias is not sufficient to explain the rate patterns obtained in our study.

OTHER EVIDENCE FOR TIME DEPENDENCY

From the analyses and discussion above, the difficulty in acquiring an adequate number of diverse calibration points is evident. For this reason, the hypothesis of the time dependency of rates, despite being a relatively unsurprising pattern from a theoretical perspective, has only recently received directed empirical attention. Here we provide a review of the current evidence and suggest potential avenues for further experimental examination.

As demonstrated by the bison case study above, one particularly promising source of evidence is ancient DNA. Dated ancient sequences act as calibration points on the tips of the phylogenetic tree, permitting mutation and substitution rates to be estimated over time spans of tens to hundreds of thousands of years (Drummond et al., 2002, 2003). All published rate estimates from population-level studies of ancient DNA have been substantially higher than estimated phylogenetic substitution rates (Table 3), providing strong support for the time dependency hypothesis.

Studies of human evolution have produced large amounts of genetic and archaeological data on time-

scales of tens to hundreds of thousands of years. Detailed pedigree studies have yielded high estimates of the mitochondrial mutation rate (Bendall et al., 1996; Heyer et al., 2001; Howell et al., 1996, 2003; Mumm et al., 1997; Parsons et al., 1997; Sigurdardóttir et al., 2000), whereas comparisons with sequences from the chimpanzee have provided estimates of the long-term substitution rate (Kumar et al., 2005). Between these extremes, known colonization times for different continents and islands offer valuable calibration points for rate estimates from humans, and these should be exploited in future studies.

Indirect evidence for the time dependency of rates is offered by analyses of selection in human populations. On its own, purifying selection will produce a time-dependent effect in substitution rate estimates because of the removal of polymorphisms over time. There has been abundant evidence of purifying selection, especially in the terminal branches of the human tree (e.g., Hasegawa et al., 1998; Ruiz-Pesini et al., 2004; Wise et al., 1998). Kivisild et al. (2006) found an excess of nonsynonymous mutations on deeper branches, indicative of incomplete purifying selection. These patterns are consistent with the prolonged survival of transient polymorphisms.

Similar relationships have been observed for bacteria and RNA viruses despite their patent biological differences. Specifically, the ratio of nonsynonymous to

TABLE 3. Published rate estimates from studies of ancient mitochondrial DNA, made using Bayesian analysis.

Species	Region	Rate (substitutions/site/Myr)	Oldest tip (kyr)	Reference
Adélie penguin (<i>Pygoscelis adeliae</i>)	D-loop	0.96 (0.53–1.43)	6	Lambert et al. (2002)
Aurochs (<i>Bos primigenius</i>)	D-loop	0.69 (0.15–1.30)	12	Edwards et al. (2007)
Bison (<i>Bison bison</i>)	D-loop	0.32 (0.23–0.41)	60	Shapiro et al. (2004)
Brown bear (<i>Ursus arctos</i>)	D-loop	0.30 (0.13–0.48)	59	Saarma et al. (2007)
Cave bear (<i>Ursus spelaeus</i>)	D-loop	0.26 (0.10–0.53)	80	Saarma et al. (2007)
Cave lion (<i>Panthera leo spelaea</i>)	D-loop	0.20 (0.03–0.40) ^a	62	Ho et al. (2007)
Horse (<i>Equus caballus</i>)	D-loop	0.11 (0.02–0.31) ^a	28	Ho et al. (2007)
Mappin's moa (<i>Pachyornis mappini</i>)	D-loop	0.67 (0.01–2.09) ^a	6	Ho et al. (2007)
Ox (<i>Bos taurus</i>)	D-loop	0.13 (0.00–0.41) ^a	8	Ho et al. (2007)
Social tuco-tuco (<i>Ctenomys sociabilis</i>)	<i>cytb</i>	0.05 (0.02–0.09)	10	Chan et al. (2006)

^aThese estimates were made while simultaneously implementing a Bayesian model of ancient DNA damage, which treats some of the observed polymorphisms as sequence damage. Therefore, the values given here may be underestimates.

synonymous mutations (dN/dS) has been found to decrease as the observational timescale increases. This was discovered in a study of 26 complete genomes from closely related bacteria (Rocha et al., 2006). In Dengue virus, intrahost variation was found to be much higher than interhost variation, reflecting the action of strong purifying selection (Holmes, 2003). A high deleterious mutation rate was measured in vesicular stomatitis virus (Elena and Moya, 1999), whereas compelling evidence of purifying selection has been identified in West Nile virus (Jerzak et al., 2005), hepatitis B virus (Chain and Myers, 2005), human influenza A virus (Suzuki, 2006), and HIV-1 (Edwards et al., 2006). For HIV-1 sequences, Sharp et al. (2001) observed an excess of nonsynonymous mutations on the terminal branches of the inferred phylogeny. They discovered that dN/dS declined in an exponential manner with increasing time depth, before eventually reaching a plateau representing the long-term dN/dS value. This closely mirrors the results that we obtained from birds and primates.

The major hindrance to progress in research on the time dependency of rates has been the considerable difficulty in acquiring suitable data. In particular, there is a conspicuous paucity of calibration points for investigating rates on genealogical and population-level time scales. There is also a need for pedigree-based estimates of the mutation rate from diverse taxonomic groups, because they provide an important benchmark for other rate estimates. Unfortunately, these short timescales represent the critical region of the time dependency curve. Notwithstanding this impediment, the numerous lines of evidence presented above already demonstrate that the time dependency phenomenon is being observed in a diverse range of taxa. Additionally, in the absence of calibration points, sequence divergence in intergenic or non-coding regions can be used as a proxy for time (Rocha et al., 2006), a possibility that is becoming particularly inviting with the continual growth of available sequence data.

CAUSES OF TIME DEPENDENCY

The observation that mutation rates are significantly higher than substitution rates, at least in many of the organisms studied thus far, is not particularly surprising.

The current debate centers on the intermediate phases, specifically the persistence of elevated rates over tens or hundreds of thousands of years. In his critique, Emerson is prepared to accept the validity of the high pedigree-based rate estimates but not of other elevated short-term mutation rates. This would necessitate an extremely rapid transition in rates over time.

Simulation studies have suggested that incomplete purifying selection produces patterns that are consistent with observations of time-dependent dN/dS (Rocha et al., 2006). If purifying selection is the sole cause of the time dependency of rates, however, the large disparity between pedigree-based rate estimates and phylogenetic substitution rates requires the condition that most mutations are significantly deleterious (Woodhams, 2006). For such mutations to persist over millions of years, however, it is necessary to postulate very large effective population sizes (Woodhams, 2006). Therefore, a better understanding of whether purifying selection alone is a satisfactory explanation for the observed rate patterns will require a more accurate picture of the exact timescale of the rate curves.

The persistence of transient polymorphisms can be explained by more complex selective processes. In populations of fluctuating size, for example, even moderately deleterious mutations can be fixed stochastically while the population is small and eventually replaced when the population size increases. Transient polymorphisms can also be maintained and protected when the environment is fluctuating, because mutations are periodically selected depending on the immediate conditions (Dean, 2005; Dempster, 1955; Gillespie, 1972; Gillespie, 1973; Haldane and Jayakar, 1963). Recently, it was suggested that positive selection is the driving force behind mitochondrial evolution, causing the fixation of hitchhiking sites through "genetic draft" (Bazin et al., 2006; Gillespie, 2000); this could provide an alternative explanation for the rate decline. It is unclear whether or not this explanation could be extended to the nuclear genome, for which time dependency patterns are also beginning to emerge (e.g., Armour et al., 1996; Kayser et al., 2004; Nachman and Crowell, 2000; Zhivotovsky et al., 2006).

As suggested previously, the time-dependent pattern of rates could partly be the spurious result of errors

in sequences (Ho et al., 2005), although a recent study demonstrated that high substitution rates estimated from ancient DNA data sets are unlikely to be an artefact of sequence damage (Ho et al., 2007). Errors in calibration could also lead to biases in estimates of substitution rates. Substitution rate estimates from intraspecific data sets are particularly susceptible to calibration errors due to incomplete lineage sorting and ancestral population subdivision, but these errors would need to be made systematically and substantially in order to explain the time dependency of rates. The presence of mutation hot spots in the mitochondrial genome, which would become heavily saturated over phylogenetic time scales, could also contribute to an apparent decline in rates with time if there is an insufficient correction for multiple hits (Galtier et al., 2006; García-Moreno, 2004). These factors would also be applicable to the nuclear genome. It is likely that a combination of these factors, along with selection, lies behind the time dependency of molecular rate estimates.

The time dependency of rates may be problematic for the estimation of recent divergence times, but the weight of evidence suggests that it should not be ignored. Nevertheless, we expect that debate will persist until additional data are available to complete the gaps in the time dependency curve.

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