Lack of support for the time-dependent molecular evolution hypothesis.

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Abstract

There is increasing momentum surrounding the hypothesis that rates of molecular evolution between individuals within contemporary populations are high, and that these rates decrease as a function of time, perhaps over several millions of years, before reaching stationarity. The implications of this are powerful, potentially reshaping our view of how climate history impacts upon both species distribution patterns and the geographic structuring of genetic variation within species. However, our assessment of the hypothesis reveals a lack of theoretical support and empirical evidence for hypothesized magnitudes of time-dependent rates of molecular evolution, with much of the apparent rate changes coming from artefacts and biases inherent in the methods of rate estimation. Our assessment also reveals a problem with how serial sampling is implemented for mutation rate estimation using ancient DNA samples, rendering published estimates unreliable.

38 **Overview of the hypothesis of time-dependent rates of molecular evolution**

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40 It has long been recognized that rates of molecular evolution vary, and we can think of this variance
41 as having two components. The first is inherent to the genome and presents itself as variation in the
42 rate of change among nucleotide positions within a gene within species, or among genes within a
43 genome, and one can expect to see relative similarity among species for this variance. The second
44 component occurs between species, with evidence suggesting that species characteristics such as
45 body size and generation time can explain some of this variance (Bromham 2011; Lanfear *et al.*
46 2010). It has recently been further hypothesised that variance of molecular rate within species may
47 also be explained by time-dependent processes (Ho *et al.* 2011a; Ho & Larson 2006; Ho *et al.* 2005;
48 Penny 2005), an idea that has captured the attention of many. Early estimates of mtDNA control
49 region mutation rate (μ - the instantaneous rate at which nucleotide changes occur along a genetic
50 lineage within a taxon) based on pedigree data (Howell *et al.* 2003; Parsons *et al.* 1997;
51 Sigurdardóttir *et al.* 2000), and the first implementation of Bayesian methods to estimate mutation
52 rate from heterochronously sampled DNA using Adelie penguin subfossil remains (Lambert *et al.*
53 2002) yielded rates of molecular evolution in excess of those derived from phylogenetic estimates.
54 In seeking to explain these rate estimate discrepancies, a more varied set of data were analysed and
55 presented as an exponential relationship for the rate of molecular change and time (Ho *et al.* 2005).
56 This echoed previous results for a subset of the same data (Garcia-Moreno 2004), but now provided
57 a formal hypothesis for the patterns observed – the time-dependency of molecular rates (hereafter
58 TDMR). The hypothesis predicts an exponentially declining rate estimate going back in time from
59 the present, in some cases extending to timescales measured in millions of years (Figure 1). The
60 primary explanation put forward for the hypothesis is an increasing role for purifying selection
61 removing novel mutant alleles with negative fitness consequences over time (Ho *et al.* 2005) as
62 observed polymorphisms transition from segregating intra-population variation to fixed

63 substitutions between populations. Although a more recent review acknowledges that different
64 types of estimation bias can also explain an apparent exponential decline of molecular rates with
65 time, these are seen as additional to, rather than an alternative to, an explanation of purifying
66 selection (Ho *et al.* 2011a).
67
68 A pattern of TDMR such as that depicted in Figure 1 can thus be explained either by: (i) real
69 biological phenomena (i.e. purifying selection); (ii) artefacts of data analysis, or; (iii) a combination
70 of (i) and (ii). Interpreting μ under a model of purifying selection has substantial implications for
71 our understanding of how climate impacts upon species distribution patterns (e.g. Lorenzen *et al.*
72 2011), the geographic structuring of genetic variation within species (e.g. Palkopoulou *et al.* 2013),
73 the timing of both population divergence or speciation events (e.g. Martínkova *et al.* 2013), and
74 estimating demographic change within species or populations (e.g. Crandall *et al.* 2012). With
75 increasing interest and reference to a model of purifying selection as an interpretative framework, it
76 is important and timely to evaluate both (i) support for the model, and (ii) alternative mechanistic
77 explanations for molecular rate estimation to suffer from time-dependent biases.
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80 **Per-generation mutation rate estimates from pedigree studies and mutation-accumulation**
81 **lines**
82
83 Pedigree rates provide the most direct window on non-lethal per generation μ along direct lineages
84 of descent. If rate estimates derived from pedigrees are found to exceed rate estimates from
85 comparable phylogenetic studies, then such discrepancies require explanation. The seminal paper
86 proposing the TDMR hypothesis contrasted human pedigree rates with comparable
87 phylogenetically derived rates from primates (Ho *et al.* 2005). However subsequent papers have

more generally invoked high mutation rates from pedigree analyses or mutation-accumulation lines as evidence for the TDMR hypothesis (e.g. Ho *et al.* 2007a; Ho *et al.* 2011a; Ho *et al.* 2011b). High and low are relative terms, and without a biologically meaningful comparative framework for their interpretation, isolated rate values lack context and offer no support for the TDMR hypothesis. As an example, a high μ derived from a *Caenorhabditis elegans* mutation-accumulation line is frequently presented as support for the TDMR hypothesis (e.g. Ho *et al.* 2007a; Ho *et al.* 2011a; Ho *et al.* 2011b). While the *C. elegans* μ may be high compared to other species when extrapolated over a timescale of millions of years (Denver *et al.* 2000), unless it exceeds phylogenetically derived substitution rates for *C. elegans* (which are unknown), it is not evidence for the TDMR hypothesis.

Thus, although high pedigree-derived estimates of μ and low phylogenetically inferred substitution rates are consistently cited as evidence for the TDMR hypothesis (e.g. Ho *et al.* 2007a; Ho *et al.* 2011a; Ho *et al.* 2011b; Ho & Larson 2006; Ho *et al.* 2005; Ho *et al.* 2007b; Penny 2005), it is important to point out that direct evidence only comes from a single species. Pedigree data from human studies are one of the two foundations for the TDMR hypothesis (Ho *et al.* 2005), and a number of human pedigree analyses using the hypervariable region I (HVRI) and HVRII of the mitochondrial D-loop region are cited (e.g. Howell *et al.* 2003; Parsons *et al.* 1997; Santos *et al.* 2005; Sigurdardóttir *et al.* 2000) in this way. However, compelling evidence is often lacking, with several human pedigree studies yielding estimates of μ that are comparable to phylogenetically derived estimates of substitution rates without the need to invoke a hypothesis of TDMR (Santos *et al.* 2005; Sigurdardóttir *et al.* 2000). Additionally, in their human pedigree analysis, Santos *et al.* (2005) offer a sensible explanation for why some human pedigree rates appear to yield high values when compared with phylogenetically derived estimates of μ. Without correction for both gender and the probability of intra-individual fixation, μ derived from pedigree data will be overestimated and in excess of those derived from phylogenetic studies. Estimates of μ from studies that use

mtDNA disease pedigrees (e.g. Howell *et al.* 2003) may also be compromised, and it has even been shown that disease associated mutations in the nuclear genome can be associated with enhanced variation within the mitochondrial genome (Annunen-Rasila *et al.* 2006).

The argument for high human mutation rates is more generally weakened by recent and thorough whole nuclear genome pedigree and population analyses, all of which converge on a human generational rate estimate that is actually less than the phylogenetic estimate (Altshuler *et al.* 2010; Awadalla *et al.* 2010; Lynch 2010; Nelson *et al.* 2012; O'Roak *et al.* 2012; Roach *et al.* 2010; Sanders *et al.* 2012). Scally and Durbin (2012) offer an insightful review of this data and the potential explanations for this discrepancy whereas Thomas and Hahn (2014) further point out the difficulties in extrapolating long term substitution rates from underlying short term and dynamic mutational parameters. But in the context of the TDMR hypothesis, it is sufficient to merely highlight that data rich analyses derived from next generation sequencing are so far in contradiction to one of the foundations of the TDMR hypothesis.

Temporally calibrated DNA sequences and the estimation of molecular rate

Substitution rate estimates derived from the coalescent-based phylogenetic analysis of temporally calibrated sequences comprise the second foundation of the TDMR hypothesis (Ho *et al.* 2005). Rate estimates can be generated from DNA sequences sampled from different time points because the known ages can be used as calibrations in the absence of other calibration points (Drummond *et al.* 2002; Rambaut 2000), and the most cited example in support of the TDMR hypothesis is that of Adélie penguins, *Pygoscelis adeliae* (Lambert *et al.* 2002). A Bayesian Markov chain Monte Carlo (MCMC) inferential framework that accounts for coalescent stochasticity in the times of co-ancestry between contemporary and ancient DNA (aDNA) lineages was used, under the

assumptions of a panmictic population for the entire sample of penguins. The analysis yielded a mean overall rate estimate of 0.96 mutations/site/Myr for the HVRI region, a value approximately five times higher than the fossil calibrated phylogenetically derived avian rate of 0.208 mutations/site/Myr. Rate estimates derived from aDNA are becoming the dominant empirical data set used to support the hypothesis (e.g. Ho *et al.* 2007a; Ho *et al.* 2007b), and are increasingly being seen as direct evidence for a long temporal persistence of transient polymorphisms within species. However, there are apparent contradictions. In contrast to Lambert *et al.*'s (2002) aDNA-based estimate of 0.96 mutations/site/Myr, a pedigree analysis of the HRVI region derived from 508 families of Adélie penguins with 915 chicks yields an estimate for μ of 0.55 mutations/site/Myr (Millar *et al.* 2008).

Substitution rate estimates from aDNA essentially use the mutational information contained across sampling time intervals (Drummond & Rodrigo 2000), and Lambert *et al.*'s (2002) original Adélie rate estimate of 0.96 mutations/site/Myr derives from an average aDNA sequence age of 3,014 yr BP. A reanalysis of Lambert *et al.*'s (2002) data by Ho *et al.* (2007a) yields a rate estimate in excess of 1.6 mutations/site/Myr, while a subsequent analysis by Millar *et al.* (2008) of a subset of the Lambert *et al.* (2002) data with several new sequences yielded a rate estimate of 0.86 mutations/site/Myr from an average aDNA sequence age of 4,279 yr BP (Millar *et al.* 2008). Against expectations from the TDMR hypothesis, all three estimates are higher than the pedigree rate for Adélie penguins, and in the case of Ho *et al.* (2007a), significantly so.

Concerns regarding the calculation and interpretation of rate estimates from aDNA have been raised because of the potentially confounding effects of demographic model misspecification, and information content limitation (Debruyne & Poinar 2009; Emerson 2007; Navascués & Emerson 2009; Ramakrishnan & Hadly 2009). Other factors may also confound rate estimates, such as the sampling of shared variation across time points, where sample sizes for different time points are limited (Figure 2). Several studies have employed simulation approaches to demonstrate

conditions under which the Bayesian estimation of rates from aDNA may or may not be reliable (Ho *et al.* 2007a; Navascués & Emerson 2009). However, while many studies have produced estimates from μ from aDNA, none have provided validation of their estimates independently of the Bayesian implementation from which they were derived. The endorsement of a given rate estimate from aDNA often seems to be that other rate estimates from aDNA are similarly high. One study, however, has presented an alternative validation of their aDNA rate estimate. By the progressive removal of older sequences for rate estimation, Ho *et al.* (2007b) demonstrate a pattern of increasing rate estimation with decreasing average ages of aDNA samples for *Bison bison* sequences, as predicted by the TDMR hypothesis. The result presented by Ho *et al.* (2007b) is compelling because all DNA sequences descend from an ancestral sequence at some unknown time in the past, but in common to all sampled alleles. That is to say, empirical values for both effective population size (*Ne*) and time of the most recent common ancestor (TMRCA) are identical across samples, assuming the standard coalescent panmictic model holds. While Ho *et al.* (2007b) address the temporal behaviour of μ with their sampling scheme, their study does not report posterior values for *Ne* and TMRCA, which are also estimated using the prior of θ, but not expected to be influenced by DNA sequence sampling times. We evaluate this expectation by reanalysing the data of Ho *et al.* (2007b), and reveal that the estimation of μ is confounded by prior distributions and posterior estimations of *Ne* and TMRCA (Box 1).

Alternative drivers of rate overestimation and curvilinear time-rate relationships

Theoretical models cannot explain the TDMR hypothesis with only *de novo* mutation. Even under the unrealistic assumption that all mutations are in some way negative, unrealistically large population sizes are required (Woodhams 2006). However, by incorporating ancestral

188 polymorphism into the model, Peterson and Masel (2009) have successfully demonstrated that
189 patterns of rate elevation over extended timescales can be explained. They demonstrate that the
190 apparent acceleration of the molecular clock at short timescales can be explained by segregating
191 polymorphisms present at the time of the ancestral population, but not *de novo* mutations. Again, an
192 important distinction to be made here is that much of the TDMRA explored in Peterson and Masel
193 (2009) is in fact largely TDMRA as a methodological artefact as opposed to TDMRA being a
194 biological phenomena expected under purifying selection. In other words, ancestral polymorphism
195 can result in apparent TDMRA if one does not explicitly incorporate the coalescent into per lineage
196 estimates of μ (Charlesworth 2010).
197 Demographic factors that can further distort estimates of μ include historical population
198 subdivision and large ancestral population sizes that can both produce biases arising from ancestral
199 polymorphism. In the case of historical population subdivision, estimates of μ derived from serial
200 temporal samples that typically use a coalescent model that assumes historical panmixia will lead to
201 significant over-estimates to the degree that population samples come from a set of isolated
202 populations with limited genetic connectivity over time (Navascués & Emerson 2009). Whether or
203 not this is a common problem is an ecological question, as levels of subdivision and population
204 genetic structure vary widely across taxa and although species-specific dispersal ability is often
205 correlated with F_{ST} values (Bohonak 1999), there are many exceptions over broad taxonomic
206 groups (Selkoe *et al.* 2014; Weersing & Toonen 2009). While the assumption of historical panmixia
207 over large geographic scales might be met for many diverse taxa (e.g. Hellberg 2009), many
208 estimates of μ coming from serially sampled terrestrial species are likley to be over-estimates due to
209 various levels of un-modeled subdivision, especially in taxa where ancient DNA is availble, such as
210 Moas (Baker 2007), *Zea Maize* (Moeller *et al.* 2007), hyenas (Rohland *et al.* 2005), brown bears
211 (Hailer *et al.* 2012; Miller *et al.* 2012), bowhead whales (Alter *et al.* 2012) and ancestral
212 populations of horses and cattle (Bruford *et al.* 2003). Estimates from such datasets should not be

used as evidence for the TDMR model, and should be subjected to re-analysis after the development and availability of methods for rate estimation from serial DNA samples that explicity account for historical population substructure.

Another type of evidence used to evaluate the TDMR hypothesis is to derive rate estimates from different species pairs whose population splitting times are derived from geological evidence. However, especially in the case of large ancestral population sizes, a bias in rate estimation arises when one assumes a time of divergence between sister populations and then equates this time with the TMRCA between samples collected from the two sister populations. Unfortunately, the expected difference in splitting times between gene trees and species/population trees as well as the large stochastic variance in coalescent gene tree divergence times are often both ignored in these studies (but see Burridge *et al.* 2008). This oversight not only results in a strong upward bias in rate estimates as the assumed calibration time approaches zero (Charlesworth 2010), the apparent mutation rate is expected to approach infinity as the geologically-calibrated time of divergence approaches zero (Tuffley *et al.* 2012) (Figure 3). Although this artefactual problem has been recognized by the TDMR community (Ho *et al.* 2011a), phylogenetically-derived estimates of rates using models that ignore ancestral polymorphism are still commonly reported (Heath *et al.* 2012; Lukoschek *et al.* 2012). On the other hand there are a growing number of studies that derive estimates using biogeographical information under a coalescent phylogenetic model that accounts for gene trees being deeper than population trees (Obbard *et al.* 2012).

A similar bias can arise if one estimates mutation rates with a population growth model while assuming the known timing and demography of the historical population expansion. Specifically, one can constrain a population growth model by assumptions of post-LGM demographic expansions and use this model for mutation rate estimation (e.g. Crandall *et al.* 2012). However, by not accounting for the complex demographic history of admixture and size change of the entire species, the resulting mutation rate estimates could be biased and should be considered

provisional until more better fit models are used. For example, if a subset of samples are lineages coming from un-sampled populations or demes via historical migration, as would be a the case in a meta-population under the coalescent "scattering phase" (Wakeley 2004), using the panmictic model can result in biases such as spurious signals of population growth or compression and likely distort the mutation rate estimates that are extracted when constraining the timing of growth (Heller *et al.* 2013; Paz-Vinas *et al.* 2013).

Conclusions

We suggest that explanations of purifying selection for the TDMR hypothesis suffers from a number of problems that may have been overlooked, or disregarded, by adherents. The difference between generational mutation rates and those derived from long-term phylogenetic substitution rate estimates is at the heart of the TDMR hypothesis, as generational mutation rates are to be expected to exceed long-term phylogenetic estimates. However, there is at present a lack of data for the direct comparison of these two classes of rate estimate within the same taxon. Humans represent one example where comparisons can be made, but in contrast to previous assertions derived from aDNA (e.g. Ho *et al.* 2007a; Ho *et al.* 2011a; Ho *et al.* 2011b; Ho & Larson 2006; Ho *et al.* 2005; Ho *et al.* 2007b; Penny 2005), data suggests little difference between generational mutation rates and phylogenetic estimates (Altshuler *et al.* 2010; Awadalla *et al.* 2010; Lynch 2010; Nelson *et al.* 2012; O'Roak *et al.* 2012; Roach *et al.* 2010; Sanders *et al.* 2012), indicating that demographic history may exert a modulating influence. Theoretical models cannot explain exponential rate decay curves from the behaviour of *de novo* mutations (Peterson & Masel 2009; Woodhams 2006), but such curves can be explained by the segregation patterns of ancestral polymorphisms (Peterson & Masel 2009). Rate estimates derived from temporally sampled DNA sequence data have

increasingly been presented as evidence for rate decay relationships that may extend back several millions of years. It has already been shown that less than simple demographic histories can result in the overestimation of μ (Navascués & Emerson 2009). Here we have demonstrated that the estimation of μ from temporally sampled DNA sequences within a Bayesian MCMC inferential framework is confounded by the priors and estimates of *Ne* and TMRCA, meaning that such estimates are flawed.

Although clearly there is an important difference between intergenerational mutational rates and longer-term evolutionary substitution rates (Gibb & Hills 2013), the extent to which these vary within taxa, the temporal scale at which they change, and the direction of change, remain to be clearly understood. To address this we need: (i) more data sets sampled from within the same taxa to facilitate the direct comparison of intergenerational and longer-term evolutionary substitution rates; (ii) improved models and methodologies to extract rate information from temporally sampled DNA sequences; (iii) robust evaluation and validation of methodologies to extract rate information from temporally sampled DNA sequences (Hoban *et al.* 2012); and (iv) appropriate null models that take into account coalescent stochasticity, sampling deficiencies and spatial-temporal demographic structure. The TDMR hypothesis is elegant and simple, but both the hypothesis and the data that has been presented to support it can be explained by the behaviour or ancestral polymorphism, or non-biological phenomena, but not the behaviour of *de novo* mutations.

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288 **References**

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425

426 **Author contributions**

427 Both authors contributed equally to the preparation of this manuscript.

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429 **Data Accessibility**

430 All DNA sequence data sets and parameter values for BEAST analyses presented in this manuscript

431 are provided as online supporting information.

432

433 **Supporting Information**

434 Additional Supporting Information may be found in the online version of this article:

435 **Appendix S1** Values reported in Figure I.

436 **Appendix S2** BEAST input files from Ho *et al.* (2007b) with cataclysm model, and modifications

437 of these used to generate Figure I.

438

Box 1: Temporally sampled DNA, and the estimation of μ, *Ne* and TMRCA

Here we present a reanalysis of the *Bison bison* data of Ho *et al.* (2007b) for the estimation of μ, where we also report posterior values for contemporary *Ne* and TMRCA (Figure I). The original analyses of Ho *et al.* (2007b) implemented a 12-category Bayesian skyline plot model, however the authors were unable to provide these original files for reanalysis. We were instead provided with files implementing a cataclysm model, which exhibit essentially the same rate trend as that reported by Ho *et al.* (2007b). We also include an additional data set not included by Ho *et al.* (2007b), sequences sampled from between 5,000 years ago and the present. Of immediate note is a clear positive relationship between sequence calibration time and estimates for both *Ne* (contemporary effective population size) and TMRCA (tree root height in years). As older DNA sequences are pruned from the DNA sequence matrix, the estimation of both *Ne* and TMRCA decrease. In contrast, estimates of μ increase, meaning that the apparent increase in μ with calibration time is confounded by coincident decreases of *Ne* and TMRCA, all of which are controlled by θ. To control for the confounding effects of *Ne* and TMRCA for time calibrated estimation of μ, we have undertaken new analyses that control for both *Ne* and TMRCA by placing strong priors on both parameters, both separately and jointly. We arbitrarily selected the posteriors for the full data set (all sequences sampled over the last 60,000 years) to parameterise all data sets (Figure I). Thus, all data sets were reanalysed with (i) a uniform root height prior between 159,000 and 161,000 years, (ii) a uniform effective population size prior between 400,000 and 410,000 and (iii) both priors implemented simultaneously (Figure I). In contrast to the results presented by Ho *et al.* (2007b), all three analyses result in a decrease in estimation of μ with decreasing calibration time.

The results of our reanalyses would appear to suggest that, in contrast to the TDMR hypothesis, μ increases as a function of time, consistent with human generational rate estimates that are less than phylogenetic estimates (Altshuler *et al.* 2010; Awadalla *et al.* 2010; Lynch 2010; Nelson *et al.* 2012; O'Roak *et al.* 2012; Roach *et al.* 2010; Sanders *et al.* 2012). However, we

caution against conclusions of both absolute value of μ, and trends in μ derived from temporally sampled sequences, until a clearer understanding of how estimates of μ are derived. While our reanalyses demonstrate shortcomings when temporally sampled sequences are used for the estimations of μ, we do not consider out results to themselves provide meaningful estimates of μ.

Figure I. Estimates of TMRCA (black), effective population size (*N*; blue) and DNA mutation rate (red) given temporally calibrated DNA sampled at intervals ranging from the present to an upper limit ranging from 5,000 to 60,000 years before the present. Solid lines depict estimates from Ho *et al.* 2007 and dashed lines depict estimates from reanalysis under different priors: Panel A: Pr(TMRCA)=159,000-161,000 years; Panel B: Pr(*Ne*)=400,000-410,000; Panel C: Pr(TMRCA)=159,000-161,000 years and Pr(*Ne*)=400,000-410,000. Values are presented in Appendix S1, Supporting Information. BEAST input files from Ho *et al.* (2007b) with cataclysm model and modifications of these used to prepare Figure I are provided in Appendix S2, Supporting Information.

Figure 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. From Ho *et al.* (2011a).

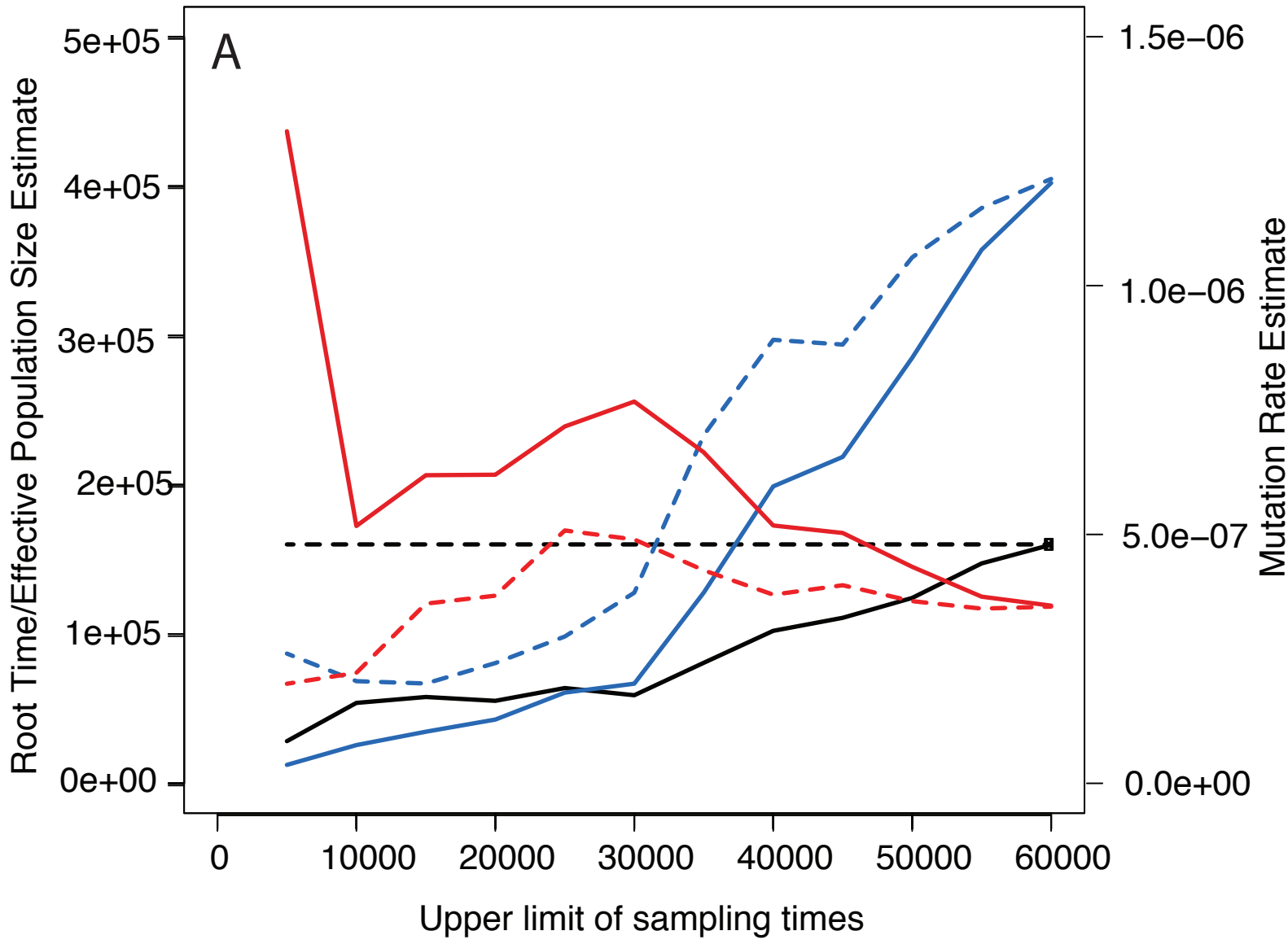
Figure 2. Shared variation across sampling times and the estimation of molecular rate. DNA sequences sampled from different time points may differ because of mutation events occurring between time points, or because of sampling effects. Panel A summarises the mutational relationships and sampling times for DNA sequences sampled from voles of the Orkney archipelago, used for the estimation of μ (Martínkova *et al.* 2013). Black indicates contemporary sequences, red indicates ancient sequences, and the white asterisk indicates the root of the network (the inferred ancestral sequence, based on relatedness to continental sequences). Shared variation across time points is demonstrated by the presence of both ancestral and derived sequences (circles shaded equally in black and red) in both contemporary and ancient samples, and statistically non-significant differences between mean mutational differences from the ancestral sequence for contemporary (4.1) and ancient (3.6) sequences ($p = 0.28$). Sampling of shared variation across different time points may have consequences for the estimation of μ. Panel B summarises hypothetical mutational relationships among 11 haplotypes (A-K), with blue bars indicating mutational events along branches. Panels C and D represent two hypothetical sampling scenarios, over three time points, across which all 11 haplotypes are available for sampling, but for which no subsequent mutations (and thus no new haplotypes) occur. Both scenarios will yield non-zero estimations of μ, yet *Ne* and μ cannot be co-estimated when no mutational events have occurred across the sampling intervals due to unidentifiability of these two component parameters of θ.

505

506 **Figure 3. Coalescent drivers of a curvilinear time and rate relationship**. Panels A, B, C and D
507 depict that the common ancestor of a sample (MRCA) from two sister populations are decreasingly
508 a proportion of the population age (τ). By ignoring this expectation predicted by the coalescent in
509 the estimates of mutation rates given geologically calibrated sister population divergence times, an
510 artifactual curvilinear relationship arises between divergence times and rate estimates.

511

Supplementary Table 1. Temporally sampled *Bison bison* DNA sequences and the estimation of μ, *Ne* and TMRCA. In all three panels mean estimations of μ, *Ne* (effective population size) and TMRCA (root height) derived from the analysis conditions of Ho *et al.* (2007b) are shaded in grey. Sequence sampling intervals are the same as those of Ho *et al.* (2007b), with the addition of a sampling interval of 5,000 bp until the present. In panel A the root height is constrained to be equal for the reanalysis of all data sets. In panel B the effective population size is constrained to be equal for the reanalysis of all data sets. In panel C both the root height and effective population size are constrained to be equal for the reanalysis of all data sets. Constraint priors were chosen to be consistent with mean posterior values obtained from unconstrained analyses of the complete sequence data set spanning the full 60,000-year sampling interval.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Root Time/Effective Population Size Estimate
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1.0e−06
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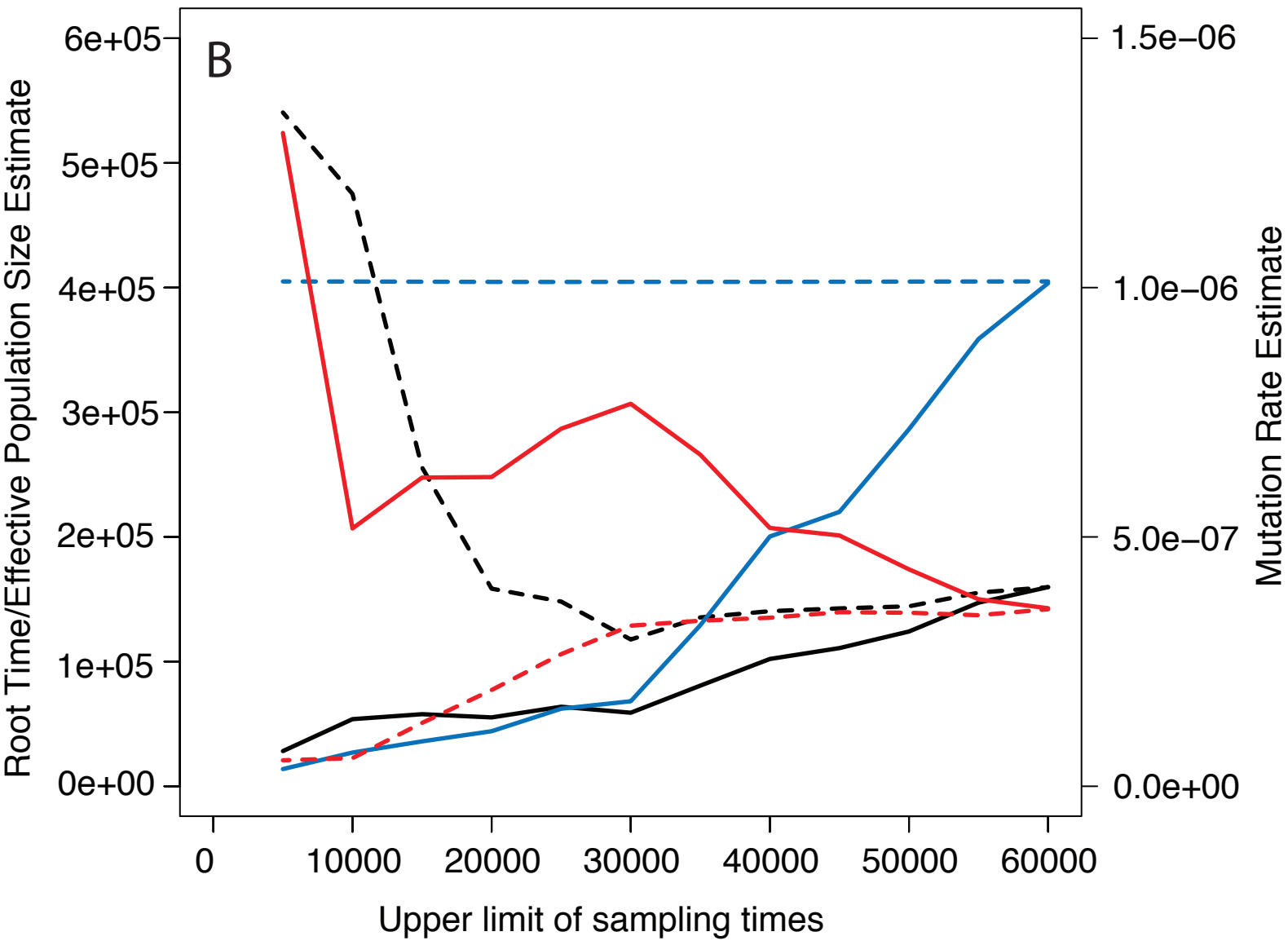
Mutation Rate Estimate
0
10000
20000
30000
40000
50000
60000
Upper limit of sampling times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5e+05
4e+05
3e+05
2e+05
1e+05
0e+00
Root Time/Effective Population Size Estimate
1.5e−06
1.0e−06
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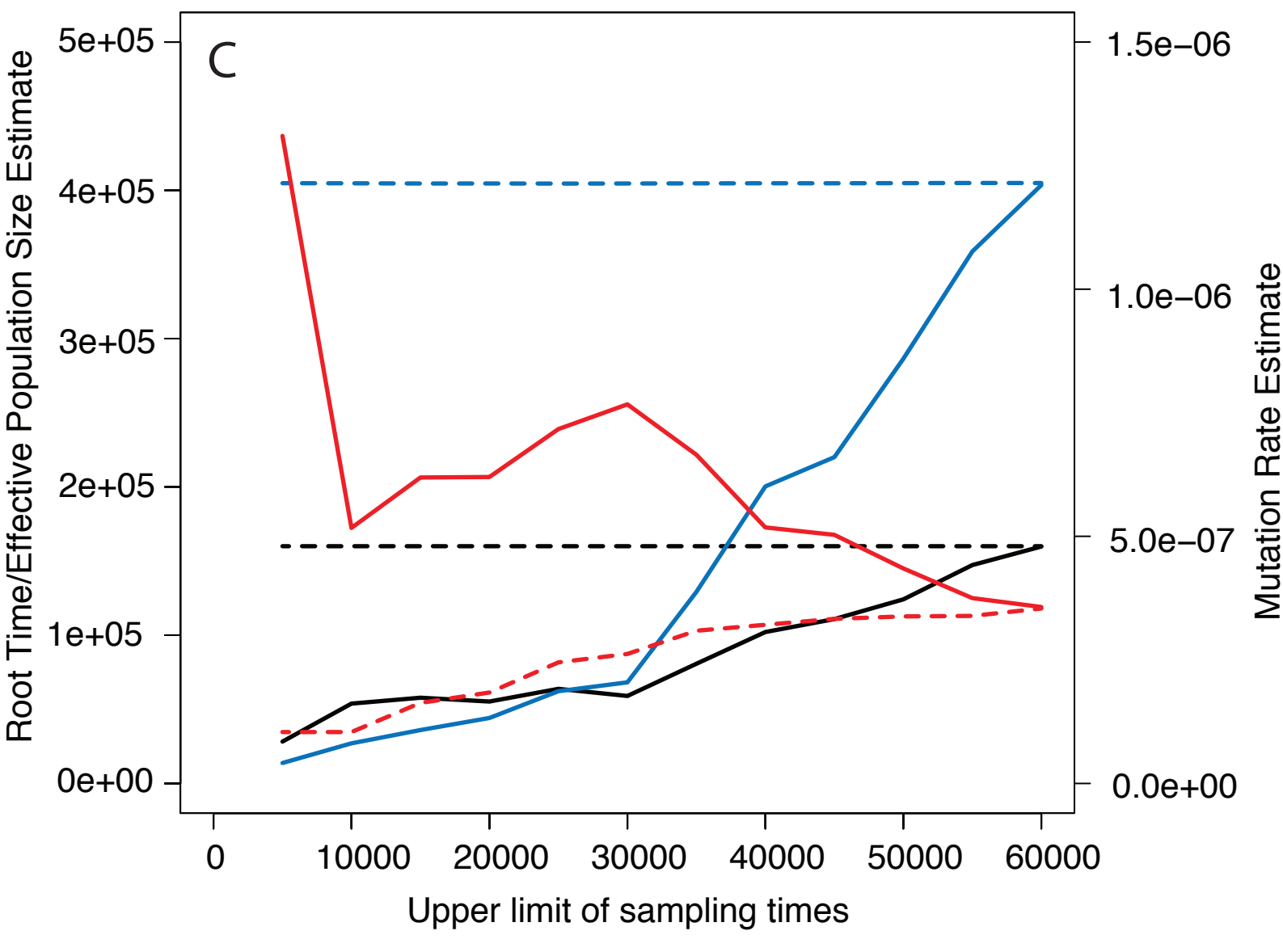
Mutation Rate Estimate
0
10000
20000
30000
40000
50000
60000
Upper limit of sampling times


C
5e+05
4e+05
3e+05
2e+05
1e+05
0e+00
Root Time/Effective Population Size Estimate
1.5e−06
1.0e−06
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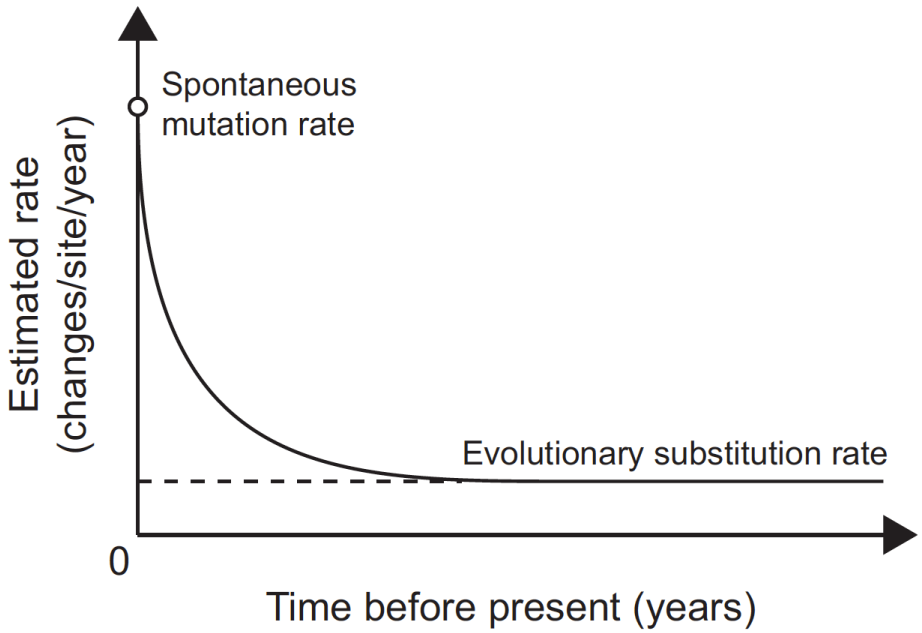
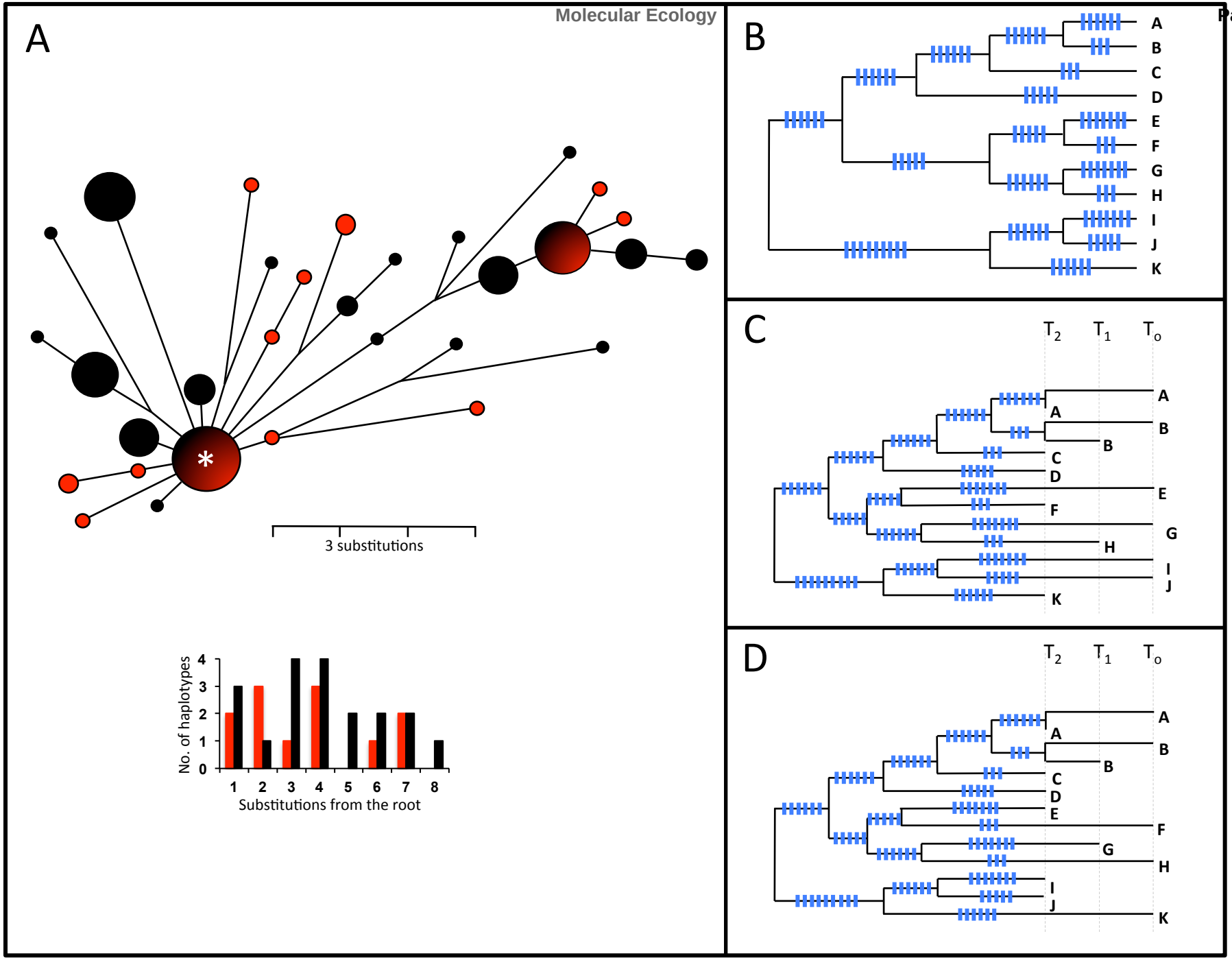
Mutation Rate Estimate
0
10000
20000
30000
40000
50000
60000
Upper limit of sampling times


Estimated rate
(changes/site/year)
Spontaneous
mutation rate
Evolutionary substitution rate
0
Time before present (years)


A
3 substitutions
No. of haplotypes
0
1
2
3
4
1 2 3 4 5 6 7 8
Substitutions from the root
B
A
B
C
D
E
F
G
H
I
J
K
C
T2
T1
To
A
B
C
D
E
F
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B
C
D
E
F
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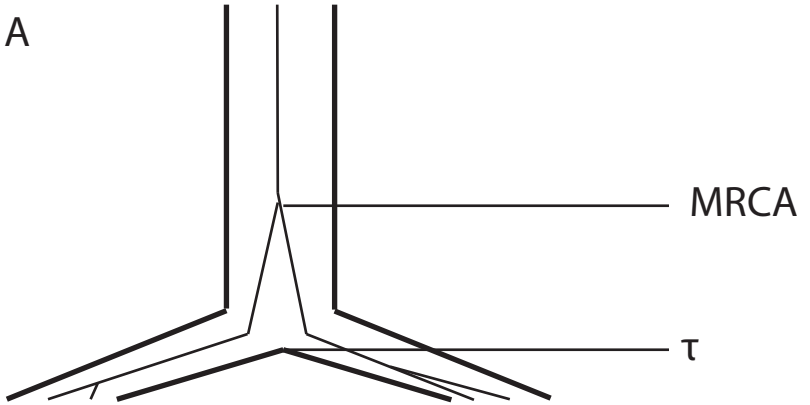
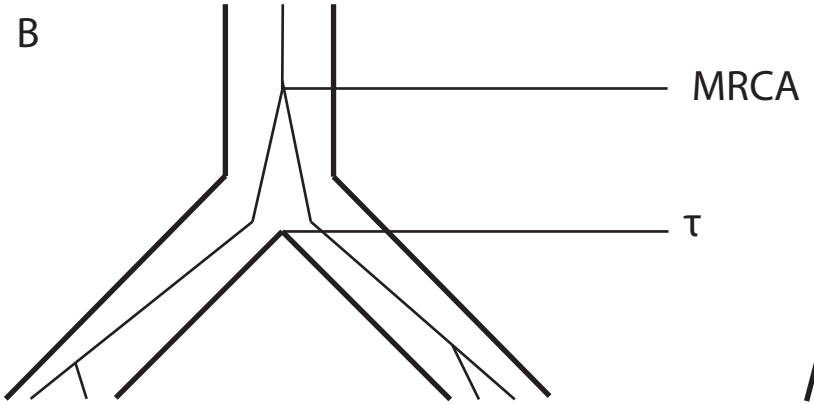
H
I
J
K


A
MRCA
τ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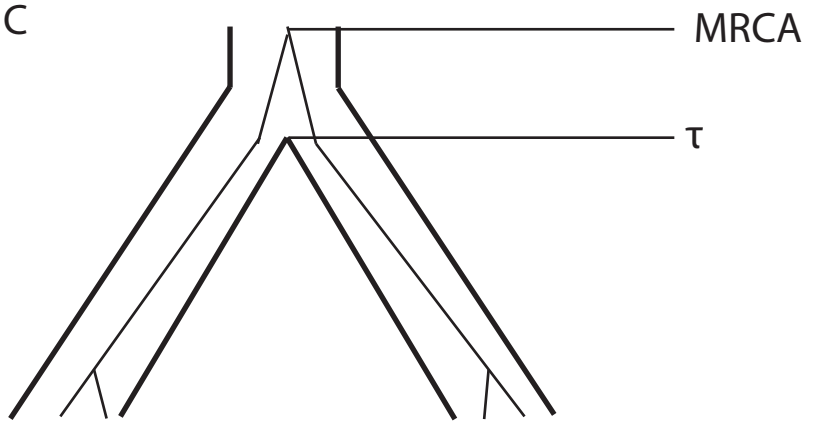
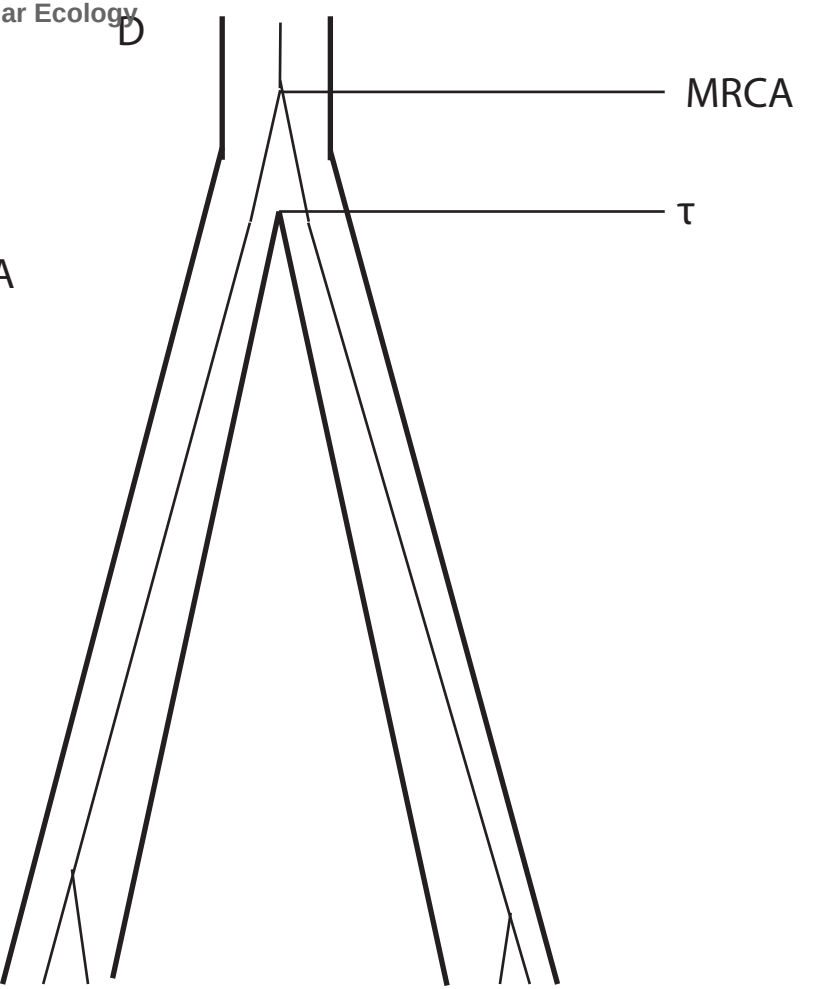
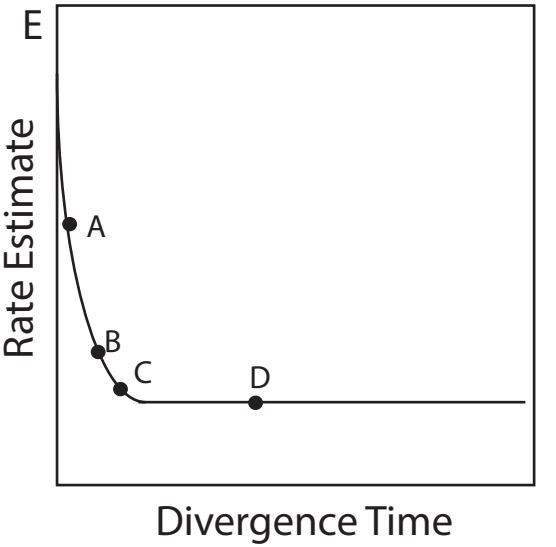
MRCA
τ


C
MRCA
τ


D
MRCA
τ


E
Rate Estimate
A
B
C
D
Divergence Time