

1

# The phylodynamic threshold of measurably evolving 2 populations

3 Ariane Weber<sup>1,\*</sup>, Julia Kende<sup>2,3</sup>, Camila Duitama González<sup>3,4</sup>, Sanni Översti<sup>1,‡</sup> and Sebastian  
4 Duchene<sup>3,4,5,‡,\*</sup>.

5

6 <sup>1</sup> Max Planck Institute of Geoanthropology, Jena, Germany.

7 <sup>2</sup> Bioinformatics and Biostatistics Hub, Institut Pasteur, Paris, France.

8 <sup>3</sup> Université Paris Cité, Paris, France.

9 <sup>4</sup> ED-ID unit, Dept of Computational Biology, Institut Pasteur, Paris, France.

10 <sup>5</sup> Peter Doherty Institute for Infection and Immunity, Dept of Microbiology and Immunology, University  
11 of Melbourne, Melbourne, Australia.

12 \*email: weber@gea.mpg.de, sduchene@pasteur.fr

13 ‡ Equal contribution to the supervision of this work.

14

15 **Abstract** The molecular clock is a fundamental tool for understanding the time and pace of evolution,  
16 requiring calibration information alongside molecular data. Sampling times are often used for calibration since  
17 some organisms accumulate enough mutations over the course of their sampling period. This practice ties  
18 together two key concepts: measurably evolving populations and the phylodynamic threshold. Our current  
19 understanding suggests that populations meeting these criteria are suitable for molecular clock calibration  
20 via sampling times. However, the definitions and implications of these concepts remain unclear. Using  
21 Hepatitis B virus-like simulations and analyses of empirical data, this study shows that determining whether  
22 a population is measurably evolving or has reached the phylodynamic threshold does not only depend on the  
23 data, but also on model assumptions and sampling strategies. In Bayesian applications, a lack of temporal  
24 signal due to a narrow sampling window results in a prior that is overly informative relative to the data,  
25 such that a prior that is potentially misleading typically requires a wider sampling window than one that  
26 is reasonable. In our analyses we demonstrate that assessing prior sensitivity is more important than the  
27 outcome of tests of temporal signal. Our results offer guidelines to improve molecular clock inferences and  
28 highlight limitations in molecular sequence sampling procedures.

29 **Keywords:** Measurably evolving population, phylodynamic threshold, molecular clock, Bayesian phylo-  
30 genetics, microbial evolution.

31

## 1 Introduction

32 Molecular sequence data have become nearly ubiquitous for studying the evolution of modern and ancient  
33 organisms. A fundamental concept in molecular evolution is the ‘molecular clock’, which posits that substitu-  
34 tions accumulate roughly constantly over time (Zuckerkandl and Pauling, 1965). An underlying assumption  
35 of the classic molecular clock is that selective constraints are negligible for most sites and over time. The  
36 development of molecular clock models as statistical processes relaxes this and other assumptions by allow-  
37 ing for rate variation among branches (and sometimes sites see Ho 2014) in phylogenetic trees (reviewed by  
38 Guindon (2020), Ho and Duchêne (2014)).

39 Molecular clock models necessarily involve two key quantities, the evolutionary timescale and the ‘evolu-  
40 tionary rate’, with the latter representing the combination of mutations and substitutions that accrue over  
41 time. However, evolutionary times and rates are unidentifiable (Dos Reis and Yang (2013), as reviewed by  
42 Bromham et al. 2018, Guindon 2020), and therefore cannot be jointly estimated using genetic sequence data  
43 alone. To make inferences from genetic sequences, all molecular clock methods require prior assumption  
44 about evolutionary times or rates, known as a ‘molecular clock calibration’. Three main calibrations exist:  
45 First, the age of the most recent common ancestor between two samples can be constrained to a given time  
46 point or interval, known as an ‘internal node calibration’. Second, a known estimate of the evolutionary rate  
47 can be incorporated (e.g. as a prior in Bayesian frameworks). Third, in cases where molecular sequences are  
48 sampled at different points in time (heterochronous sampling), the tips of the phylogeny can be anchored  
49 to these time points (‘tip calibration’; reviewed by Rieux and Balloux (2016)). The choice of calibration  
50 depends on the information available and its reliability (Duchêne et al., 2014, Warnock et al., 2012). For  
51 instance, it would be remiss to ignore evidence about when two lineages shared a common ancestor if the  
52 fossil record is compelling (Gavryushkina et al., 2017, Ronquist et al., 2016). Crucially, multiple sources of  
53 calibration information can be provided for the molecular clock.

## 54 1.1 Measurably evolving populations

55 Rapidly evolving organisms, notably many viruses and bacteria, have been found to accrue an appreciable  
56 number of substitutions over the sampling timescale. Influenza viruses, for example, have evolutionary rates  
57 of around  $6 \times 10^{-3}$  subs/site/year (substitutions per genomic site per year) (Ghafari et al., 2022, Sanjuán,  
58 2012). Assuming a genome size of 13,500 nucleotides, one would expect to observe one mutation every 4 to  
59 5 days ( $\frac{365 \text{ days/year}}{13,500 \text{ sites} \times 6 \times 10^{-3} \text{ subs/site/year}} \approx 4.5 \text{ days/subs}$ ). If genome samples are collected over the course of  
60 a few weeks, the sampling times themselves can be used to calibrate the molecular clock and tip calibration  
61 is therefore warranted. Data sets for which tip calibration is feasible are considered to have been sampled  
62 from a ‘measurably evolving population’ (Drummond et al., 2003b) and to have ‘temporal signal’.

63 Measurably evolving populations are typically characterised either by a sampling period that is long  
64 relative to the evolutionary rate, a sufficiently big data set (long molecular sequences or many samples), or  
65 both. Traditionally, such characteristics were mainly found in rapidly evolving organisms, typically RNA  
66 viruses. Nowadays, advances in sequencing technologies have dramatically expanded the range of organisms  
67 from which data sets can be considered to have been sampled from a measurably evolving population.  
68 Namely, ancient DNA techniques have effectively expanded the genome sampling window for many organisms  
69 (Duchene et al., 2020b, Spyrou et al., 2019a), and whole genome sequencing has meant that data sets of  
70 ‘slowly’ evolving microbes often carry sufficient information for calibrating the molecular clock (Biek et al.,  
71 2015) even when the sampling period covers only a few decades (Menardo et al., 2019).

## 72 1.2 The phylodynamic threshold

73 Genomic data sets collected during the early stages of an outbreak, for example, often pose two problems: low  
74 genetic diversity and a narrow sampling window. Both can lead to highly uncertain estimates of evolutionary  
75 rate and time of origin. The point in time when an organism has accumulated sufficient genetic changes since  
76 its emergence to allow for informative tip calibration is referred to as the ‘phylodynamic threshold’ (Duchene

77 et al., 2020a). At a minimum, tip calibration requires that one mutation has occurred over the sampling period  
78 for the method to be informative. For a given organism, the minimum sampling period can be calculated as the  
79 inverse of the product of genome size and the evolutionary rate (i.e.  $\frac{1}{\text{genome size (sites)} \times \text{evol. rate(subs/site/year)}} =$   
80 years to observe one mutation). We refer to this amount of time as the expected phylodynamic threshold.

81 The terms phylodynamic threshold and measurably evolving population are different, albeit related,  
82 concepts. A population is measurably evolving if the samples available are sufficiently informative as to allow  
83 for tip calibration. In contrast, the phylodynamic threshold is the amount of time over which we would need  
84 to draw samples after their emergence for them to behave as if being sampled from a measurably evolving  
85 population. For a recently evolving pathogen the phylodynamic threshold would simply correspond to the  
86 time until it can be considered a measurably evolving population, under the condition that the data have  
87 been collected constantly over time. In contrast, an organism that emerged further in the past may have  
88 accumulated considerable genetic diversity over time, effectively reaching its phylodynamic threshold, but if  
89 samples are drawn from a very short time window they may fail to capture a representative amount of such  
90 genetic diversity.

### 91 1.3 Tests of temporal signal

92 Our ability to extract information from a tip calibration framework can be assessed through tests of temporal  
93 signal. The importance of performing such tests arises from the observation that a lack of temporal signal  
94 is associated with unreliable evolutionary rate estimates (Duchêne et al., 2015, Rieux and Balloux, 2016),  
95 although the presence and direction of a potential bias remain poorly understood. However, it is important to  
96 note that a lack of temporal signal does not necessarily preclude estimating evolutionary rates and timescales  
97 because alternative sources of calibration, such as prior estimates of evolutionary rates or constraints on  
98 internal node ages, can still be used to inform analyses.

99 In principle, frameworks developed to test for temporal signal do not differentiate between recently  
100 emerging organisms (fig. 1a) and those with narrow sampling windows (fig. 1d), both of which may lack  
101 temporal signal. As most of these tests involve fitting a phylogenetic model to the data, they implicitly  
102 assume that the model adequately captures the evolutionary process and thus their performance also highly  
103 depends on model fit. Recent research, for example, suggests that the choice of tree prior and molecular  
104 clock model substantially impacts the sensitivity and specificity of temporal signal tests (Tay et al., 2024).  
105 Thus, temporal signal is not solely a property of the data but also depends on the choice of model.

106 Various methods exist for assessing temporal signal. The root-to-tip regression (Buonagurio et al., 1986,  
107 Drummond et al., 2003a, Gojobori et al., 1990) fits a regression to the distance from the root to the tips in a  
108 phylogenetic tree against sampling time. High  $R^2$  values of the regression suggest that phylogenetic distance  
109 can be sensibly modelled as a linear function of time and can therefore be used as an indication of informative  
110 tip-calibrations. Date-randomisation tests (Duchêne et al., 2015, Duchene et al., 2018, Ramsden et al., 2009,  
111 Trovão et al., 2015) compare evolutionary rate estimates using correct sampling times against those from  
112 permutations. Bayesian Evaluation of Temporal Signal (BETS; Duchene et al. (2020c)) evaluates whether  
113 a model with sampling times performs better than a model that assumes isochronous sampling using Bayes  
114 factors. Each method comes with a set of limitations and strengths, such that tests of temporal signal should  
115 rather be used in combination than being mutually exclusive (Duchene et al., 2020c, Rieux and Balloux,

116 2016).

117 **1.4 Concepts of measurably evolving populations, the phylodynamic threshold, and**  
118 **temporal signal in practice**

119 In fig. 1, we present four simple example cases to illustrate the relationships among the concepts of mea-  
120 surably evolving populations, the phylodynamic threshold, and temporal signal. The first example depicts  
121 an organism that has emerged recently and therefore has not yet reached its phylodynamic threshold (with  
122 a phylogenetic time-tree shown in panel (a)). Due to its recent origin, there has not been enough time for  
123 the accumulation of a sufficient number of substitutions (represented in the phylogram in panel (b)), and  
124 thus it is not possible to establish a statistical relationship between molecular evolution (i.e., substitutions)  
125 and time (as shown in panel (c)). A real-world example of such a case comes from the early phase of the  
126 SARS-CoV-2 outbreak: initial efforts to estimate the evolutionary rate and time of origin had substantial  
127 uncertainty due to a narrow sampling window and low genetic diversity (Boni et al., 2020). In Duchene et al.  
128 (2020a), Bayesian phylodynamic analyses were conducted on genome data as the outbreak unfolded. The  
129 number of available genomes and the width of the sampling window increased over time and ranged from  
130 22 genomes sampled over 31 days to 122 genomes sampled over 63 days. Although early estimates of the  
131 evolutionary rate and time of origin were highly uncertain, they quickly converged to stable values as more  
132 data became available (Ghafari et al., 2022).

133 The second example in fig. 1 illustrates a case in which an organism has evolved over a long period, but  
134 the available sequence data have been collected within a very narrow window of time, insufficient to treat the  
135 dataset as a measurably evolving population (time-tree in panel (d) and phylogram in panel (e)). This results  
136 in no temporal signal, as demonstrated by the lack of correlation in the root-to-tip regression in panel (f).  
137 The causative agent of tuberculosis, the bacterium *Mycobacterium tuberculosis*, was commonly considered to  
138 evolve too slowly for calibrating the molecular clock using samples collected over a few years Duchene et al.  
139 (2016). A range of studies have shown, however, that for *M. tuberculosis* a genome sampling window of a few  
140 decades might be sufficient for reliable clock calibration (Eldholm et al., 2015, Kühnert et al., 2018, Menardo  
141 et al., 2019, Merker et al., 2022).

142 The third example in fig. 1 describes a data set that may involve a wide sampling window of time and  
143 for which samples have been drawn from a population that has attained its phylodynamic threshold, but  
144 with substantial rate variation among lineages – i.e. overdispersed molecular clock -, resulting in a lack of  
145 temporal signal (panels (g) – (i)). This pattern appears to be the case in *Yersinia pestis*, the bacterium  
146 that causes the plague, for which some localised outbreaks display obvious temporal signal, but its long-term  
147 evolution has pervasive evolutionary rate variation (Andrades Valtueña et al., 2022, Eaton et al., 2023).

148 In the final example in fig. 1, a hypothetical organism has attained its phylodynamic threshold, has been  
149 sampled for sufficiently long time, and evolutionary rate variation among lineages is low. These conditions  
150 together produce a clear relationship between molecular evolution and time, thus providing unequivocal  
151 temporal signal (panels (j) – (l)). The long term evolution of *Vibrio cholerae*, the causative agent of cholera,  
152 and H3N2 influenza virus are exemplar microbes whose molecular evolution has been fairly constant across  
153 long periods of time (Devault et al., 2014, Rambaut et al., 2016).

154 In summary, the concepts of measurably evolving population, phylodynamic threshold and temporal signal

155 describe the information that can be drawn from a sampled population about its evolutionary timescale.  
156 Because populations that are not measurably evolving have been observed to yield biased estimates, they  
157 remain important to consider (Gharbi et al., 2024). In Bayesian inference, such biases can be the result  
158 of complex interactions between prior distributions or model settings that do not align with the true data  
159 generating model, as these drive the inference in the absence of informative data. Traditionally, potential  
160 biases due to prior interactions (Tay et al., 2024) or model misspecification (Möller et al., 2018) have been  
161 found through simulations studies, while data analyses often involve little validation of the results (Mendes  
162 et al., 2025). Here, we illustrate through a range of examples the degree to which differing levels of temporal  
163 signal in a data set can interact with prior settings and model assumptions, both on simulated and empirical  
164 data.

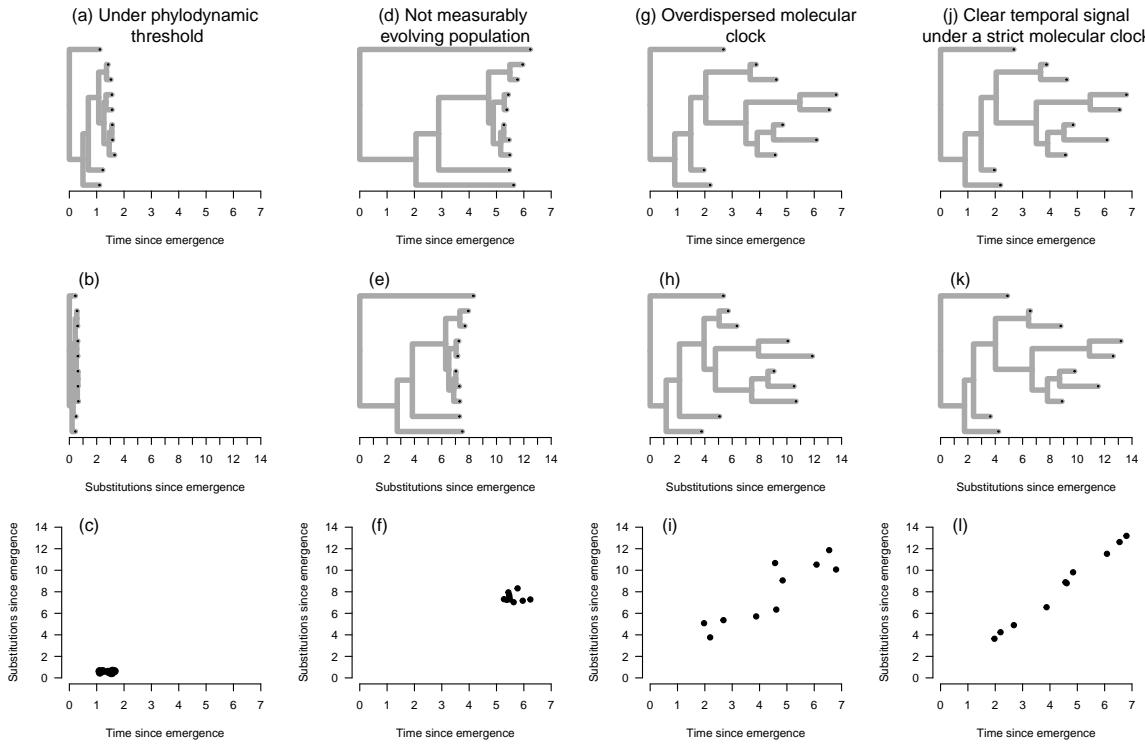


Figure 1: Examples of situations where temporal signal may or may not be detected. An organism that has not attained its phylodynamic threshold has a recent time of emergence (with a phylogenetic time-tree shown in (a)) because it has not had sufficient time to accrue an appreciable number of substitutions (phylogenetic tree with branch lengths in subs/site, i.e. a ‘phylogram’, shown in (b)), such that it is not possible to establish a statistical relationship between molecular evolution (substitutions) and time (shown in (c)). Sequence data from an organism that has evolved for a substantial amount of time may have been sampled over a very narrow window of time that is not sufficient to treat it as a measurably evolving population (time-tree in (d) and phylogram in (e)), which results in no temporal signal (root-to-tip regression in (f)). A data set may involve a wide sampling window of time and from a population that has attained its phylodynamic threshold, but an overdispersed molecular clock (substantial rate variation among lineages; panels (g) - (i)) may result in a lack of temporal signal. In (j) through (l) we show the situation where an organism has attained its phylodynamic threshold, it has been sampled for sufficiently long, and where evolutionary rate variation among lineages is negligible, as to produce a clear relationship between molecular evolution and time, and thus unequivocal temporal signal.

165 **2 Results**

166 We sought to pinpoint the impact of sampling strategies on molecular clock estimates. We focused our attention  
167 on two major problems for emerging microbes and studies involving ancient DNA. First, we conducted  
168 simulations varying the sampling window of a population that had attained its phylodynamic threshold, and  
169 which was analysed with a range of prior distributions on the evolutionary rate. In the second simulation  
170 scenario, we subsampled a population over time to vary the number of ancient samples, leading to a temporal  
171 sampling bias. Finally, we illustrate these results in an empirical data set of Hepatitis B virus (HBV) that  
172 includes a large number of ancient samples (Kocher et al., 2021). This virus has been the subject of intense  
173 research due to its close association with human populations and complex evolutionary dynamics (Kahila  
174 Bar-Gal et al., 2012, Paraskevis et al., 2013, Ross et al., 2018).

175 **2.1 Sampling windows relative to the phylodynamic threshold**

176 We simulated sequence data that resembled the evolution of HBV, a double-stranded DNA (dsDNA) virus  
177 that has evolved in humans for at least ten thousand years (Kocher et al., 2021). Our synthetic data had a  
178 genome length of 3,200 nucleotides and an evolutionary rate of  $1.5 \times 10^{-5}$  subs/site/year (Kocher et al., 2021,  
179 Mühlemann et al., 2018) with a moderate amount of rate variation among lineages (see Materials and meth-  
180 ods). Under these conditions we expect to observe one mutation every 20 years ( $\frac{1}{3,200 \text{ sites} \times 1.5 \times 10^{-5} \text{ subs/site/year}} \approx$   
181 20 years/subs). This number is important for the design of our simulation experiments: 20 years is the ex-  
182 pected phylodynamic threshold, as introduced above, and typically serves as a good reference point from  
183 when to expect temporal signal. We analysed the data under a Bayesian phylogenetic framework and con-  
184 sidered whether the posterior contained three performance statistics: coverage (whether the 95% credible  
185 interval, CI, contains the true value used to generate the data), uncertainty (the width of the posterior 95%  
186 CO divided by its mean), and bias (the difference between the posterior mean and the truth divided by the  
187 truth).

188 We conceived a simulation process under which the evolutionary timescale had an expectation of ten  
189 thousand years and with a sampling window of 0, 10, 20, 200 , or 2,000 years. A sampling window spanning 0  
190 years results in ultrametric trees with the sampling times providing no calibration information (all calibration  
191 information comes from the tree prior and the prior on the evolutionary rate). In contrast, a sampling window  
192 of 10 years is half of the expected phylodynamic threshold and is likely to have weak temporal signal (see fig.  
193 1(d)-(f)). Sampling windows of 20 years (the expected phylodynamic threshold) or wider are more likely to  
194 behave as measurably evolving populations with increasingly strong temporal signal (see fig. 1(j)-(l)). Our  
195 synthetic data sets were analysed under Bayesian phylogenetic framework, as implemented in the BEAST 2  
196 platform (Bouckaert et al., 2019).

197 To investigate the impact of the prior we considered several configurations for the prior on the mean  
198 evolutionary rate. In our analyses the molecular clock model is a uncorrelated relaxed molecular clock model  
199 with an underlying lognormal distribution, with mean  $M$ . For this parameter we set nine possible prior  
200 Gamma distributions, for which the prior mean could be the value used to generate the data, or one order of  
201 magnitude higher or lower. We also included three degrees of uncertainty in this prior (see fig. 2 and table  
202 6). In this respect, a prior with low uncertainty, and a mean that is much higher or lower than the true

203 should result in more bias than one that has higher uncertainty or is centred on the true value.

204 Our simulations for which the prior on  $M$  was centred on the true value had very high coverage. At  
 205 least 94 out of 100 simulation replicates across sampling windows included the true value of  $M$  within their  
 206 95% CI (table 1 and fig. 2). However, when the prior was highly precise (95% CI/mean=1.0) but biased  
 207 downwards, even the simulations with a sampling window of  $100 \times$  the phylodynamic threshold (i.e. 2,000  
 208 years before present) still had low coverage (only 1 out of 100 simulation replicates included the true value  
 209 in the 95% credible interval, table 1).

Table 1: Coverage of the mean evolutionary rate,  $M$ . We consider coverage as whether a posterior distribution contains the value used to generate the data within its 95% credible interval (CI) and we show the number of simulation replicates out of 100 for which we found coverage. Data were simulated under trees with five possible sampling window depths relative to the phylodynamic threshold, where  $Sw = \times D$  is for simulations with a sampling window of  $D$  times the phylodynamic threshold (20 years in our data; see fig. 2). Each column is a sampling window depth, and the rows denote the configuration of the prior on the mean rate,  $M$ , for which we show the prior mean and the width of the 95% CI divided by the mean (larger values imply higher uncertainty). The true value of  $M$  is  $1.5 \times 10^{-5}$  subs/site/year. The horizontal lines separate prior configurations with different prior means.

Prior configuration (mean, 95%CI width)	$Sw=0\times$	$Sw=0.5\times$	$Sw=1\times$	$Sw=10\times$	$Sw=100\times$
$1.5 \times 10^{-5}, 3.04$	100	100	100	96	95
$1.5 \times 10^{-5}, 6.33$	100	100	99	95	94
$1.5 \times 10^{-5}, 1.00$	100	100	100	94	94
$1.5 \times 10^{-4}, 3.04$	100	97	97	93	93
$1.5 \times 10^{-4}, 6.33$	100	100	99	94	92
$1.5 \times 10^{-4}, 1.00$	0	0	0	77	91
$1.5 \times 10^{-6}, 3.04$	0	0	0	68	91
$1.5 \times 10^{-6}, 6.33$	100	88	85	95	94
$1.5 \times 10^{-6}, 1.00$	0	0	0	0	1

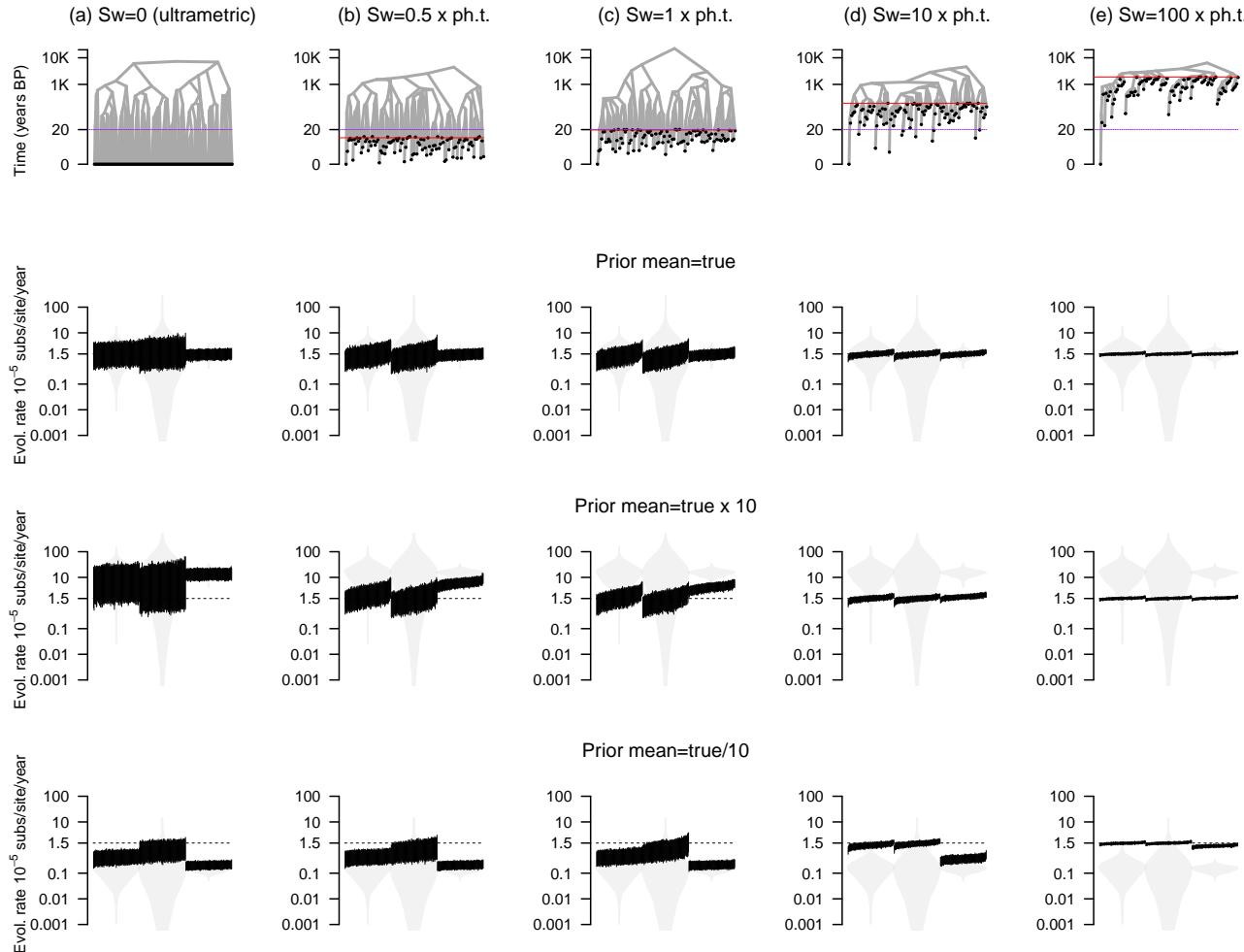


Figure 2: Estimates of evolutionary rates,  $M$  for simulations of varying sampling window widths. Each column corresponds to a simulation setting: (a) is for ultrametric trees where all samples are collected at the same point in time (sampling window,  $sw=0$ ), (b) is for the situation where the sampling window is 10 years (half the expected phylodynamic threshold; sampling window,  $sw=0.5 \times \text{ph.t.}$ ), (c) is where the sampling window is exactly the expected phylodynamic threshold of 20 years ( $sw=1 \times \text{ph.t.}$ ). Scenarios (d) and (e) denote sampling windows that are 10 and 100  $\times$  the expected phylodynamic threshold ( $sw=10 \times \text{ph.t.}$  and  $sw=100 \times \text{ph.t.}$ , respectively). The rows denote example phylogenetic trees and prior configurations where the mean is set to the correct value (first row), an order of magnitude higher (second row), or an order of magnitude lower, last row). The prior distributions are shown with the grey violins and each black bar is the 95% credible interval of the posterior. The dashed line in each case denotes the correct value.

210 The uncertainty in estimates of evolutionary rates was associated with the width of the sampling window,  
 211 but also with the uncertainty in the prior. In the situation where the sampling window was  $1\times$  the  
 212 phylodynamic threshold or less we consistently found that uncertainty in the prior was commensurate with  
 213 uncertainty in the posterior. Estimates using a prior uncertainty of 1.00 (the width of the 95% CI is the  
 214 same as the mean) are narrower than those using a prior of uncertainty of 3.04 or 6.33 (see table 2 and fig.  
 215 2).

216 When the sampling window was  $10\times$  the phylodynamic threshold or more we found a more complicated  
 217 picture. When the prior had a downward bias (mean= $1.5 \times 10^{-6}$  subs/site/year) and a low uncertainty of  
 218 1.00, the posterior estimate was wider than when this prior was less uncertain (table 2 and fig. 2). This  
 219 finding can be explained because a very wide sampling window provides a large amount of information for  
 220 inferring the evolutionary rate, which can yield high uncertainty if the prior stands in conflict. Importantly,  
 221 the widest sampling window in our experiments, of  $100\times$  the phylodynamic threshold produced consistently  
 222 low uncertainty, although it is important to note that when the prior is highly biased downward (mean of  
 223  $1.5 \times 10^{-6}$  subs/site/year and uncertainty of 1.00) coverage is very low, with only one simulation replicate  
 224 containing the true value used to generate the data within the 95% CI.

Table 2: Average uncertainty of the mean evolutionary rate,  $M$  across 100 simulation replicates in each case. We quantify uncertainty as the posterior width of the 95% credible interval (CI) divided by the mean. The rows and columns match those from table 1, with the posterior for each simulation replicate shown in fig. 2.

Prior configuration (mean, 95%CI width)	Sw=0×	Sw=0.5×	Sw=1×	Sw=10×	Sw=100×
$1.5 \times 10^{-5}$ , 3.04	2.24	1.87	1.55	0.48	0.21
$1.5 \times 10^{-5}$ , 6.33	3.06	2.01	1.72	0.48	0.22
$1.5 \times 10^{-5}$ , 1.00	0.96	0.94	0.91	0.45	0.21
$1.5 \times 10^{-4}$ , 3.04	1.36	1.83	1.50	0.48	0.22
$1.5 \times 10^{-4}$ , 6.33	5.39	2.11	1.70	0.49	0.21
$1.5 \times 10^{-4}$ , 1.00	1.04	0.87	0.80	0.43	0.21
$1.5 \times 10^{-6}$ , 3.04	1.36	1.37	1.38	0.52	0.22
$1.5 \times 10^{-6}$ , 6.33	1.99	1.90	1.72	0.48	0.22
$1.5 \times 10^{-6}$ , 1.00	0.75	0.75	0.76	0.75	0.28

225 To understand the directionality of posterior evolutionary rate estimates relative to the value used to  
 226 generate the data, we quantified the amount of bias, as the difference between the true evolutionary rate  
 227 and the posterior mean divided by the true value (i.e.  $\frac{\text{true value} - \text{posterior mean}}{\text{true value}} = \frac{1.5 \times 10^{-5} - \text{posterior mean}}{1.5 \times 10^{-5}}$ ).  
 228 As expected, when the prior was centred in the true value, we observed no or minimum bias, with values  
 229 ranging from an average of 0.00 and 8.31 (a posterior estimate that was on average 8 times higher than the  
 230 truth). The most marked average bias was found for our prior with mean  $1.5 \times 10^{-4}$  subs/site/year and an  
 231 uncertainty of 1.00, which could be as high as 8.31 (table 3). Increasingly wide sampling windows had lower  
 232 amounts of bias. A sampling window of  $100\times$  the phylodynamic threshold had a maximum average bias of  
 233 -0.22 for the prior with downward bias and low uncertainty, with other prior configurations resulting in a  
 234 bias of at most -0.22.

Table 3: Average bias of the mean evolutionary rate,  $M$  across 100 simulation replicates in each case. Bias is calculated as the true evolutionary rate,  $1.5 \times 10^{-5}$  subs/site/year - posterior mean, divided by the true value.

Prior configuration (mean, 95%CI width)	Sw=0×	Sw=0.5×	Sw=1×	Sw=10×	Sw=100×
$1.5 \times 10^{-5}, 3.04$	0.16	0.06	0.01	0.00	0.01
$1.5 \times 10^{-5}, 6.33$	0.28	0.03	-0.03	0.00	0.01
$1.5 \times 10^{-5}, 1.00$	0.00	-0.01	-0.01	0.00	0.01
$1.5 \times 10^{-4}, 3.04$	4.93	0.67	0.30	0.02	0.01
$1.5 \times 10^{-4}, 6.33$	3.37	0.27	0.08	0.00	0.01
$1.5 \times 10^{-4}, 1.00$	8.31	3.22	1.84	0.17	0.04
$1.5 \times 10^{-6}, 3.04$	-0.71	-0.70	-0.69	-0.16	-0.02
$1.5 \times 10^{-6}, 6.33$	-0.45	-0.43	-0.37	-0.04	0.00
$1.5 \times 10^{-6}, 1.00$	-0.86	-0.86	-0.86	-0.75	-0.22

Overall, these simulations demonstrate that increasingly wide sampling windows result in evolutionary rate estimates that are more accurate, precise, and less biased, than those from data sets with narrow sampling windows. Although increasingly wide sampling windows are more robust to prior misspecification, we emphasise the importance of choosing the prior for the evolutionary rate carefully because a highly biased prior can overcome the information from data with wide sampling windows. Contrary to the expectation that low temporal signal necessarily results in an underestimation of the evolutionary rate and an overestimation of the tree height (Duchêne et al., 2015), we find that a lack of temporal signal due to narrow sampling windows may simply lend more influence to the prior.

For all trees that were non ultrametric (i.e. those with a sampling window width of at least  $0.5 \times$  the phylodynamic threshold) a downward bias in the evolutionary rate prior appears to be more detrimental than one with an upward bias. In our simulations with sampling windows of width  $100 \times$  the phylodynamic threshold a highly biased prior (mean= $1.5 \times 10^{-6}$  subs/site/year and uncertainty of 1.00) still had very low coverage. In contrast, a prior with a similar upward bias (mean= $1.5 \times 10^{-4}$  and uncertainty of 1.00) produced much higher coverage for sampling windows from  $10 \times$  the phylodynamic threshold (table 1).

These results indicate that priors with high uncertainty should be advised for practical studies where a researcher is unsure about the evolutionary rate of the population. In our simulations posterior estimates using a prior uncertainty of 6.33 seemed to produce a good trade-off between uncertainty and accuracy. Such a prior means that the 95% CI spans just over six orders of magnitude. For a sampling window of  $1 \times$  the phylodynamic threshold, the posterior distribution had an average uncertainty of around 1.7, which may be sufficient for biological interpretation of estimates of evolutionary rates and timescales.

## 2.2 Hierarchical priors and the phylodynamic threshold

An attractive approach for specifying prior distributions for parameters that are largely unknown is to use a hierarchical structure, where the parameters that govern the prior have priors themselves (sometimes known as hyperpriors). In the context of Bayesian molecular clocks, such hierarchical structure has been used to specify uncertainty in time calibrations (Heath et al., 2014). We employed this method by setting a prior

260 for the shape and rate parameters of the Gamma distribution that governs the evolutionary rate,  $M$  (see  
261 Materials and methods).

262 Under the hierarchical structure, the marginal prior for  $M$  is very uncertain and is biased upwards,  
263 relative to the true value (fig 3). However, for sampling windows of at least  $1\times$  the phylodynamic threshold  
264 its performance in terms of coverage, average uncertainty, and average bias were comparable to those using  
265 the standard prior centred on the correct value (see first three rows under  $SW=1\times$  in tables 1, 2, and 3,  
266 compared to table 4). This result demonstrates the effect of Bayesian regularisation and suggests that such  
267 hierarchical structure is likely preferable to using a prior that is potentially highly biased.

Table 4: Coverage, average uncertainty and average bias of the mean evolutionary rate,  $M$  using a hierarchical prior on  $M$ . Statistics are reported over 100 simulation replicates under each of the five sampling window widths ( $Sw$ ) relative to the phylodynamic threshold (of 20 years).

Statistic	$Sw=0\times$	$Sw=0.5\times$	$Sw=1\times$	$Sw=10\times$	$Sw=100\times$
Coverage	100	100	99	96	94
Uncertainty	57.69	2.44	1.86	0.49	0.22
Bias	1.01	0.09	0.01	0.00	0.01

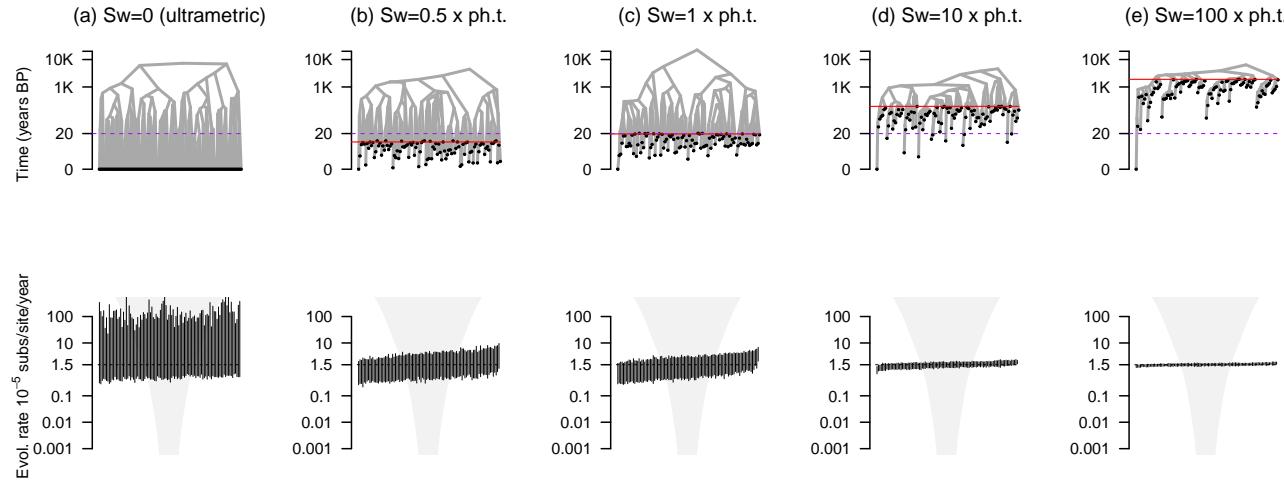


Figure 3: Estimates of evolutionary rates,  $M$  for simulations of varying sampling window widths. Each column corresponds to a simulation setting: (a) is for ultrametric trees where all samples are collected at the same point in time (sampling window,  $sw=0$ ), (b) is for the situation where the sampling window is 10 years (half the expected phylodynamic threshold; sampling window,  $sw=0.5 \times ph.t.$ ), (c) is where the sampling window is exactly the expected phylodynamic threshold of 20 years ( $sw=1 \times ph.t.$ ). Scenarios (d) and (e) denote sampling windows that are 10 and 100  $\times$  the expected phylodynamic threshold ( $sw=10 \times ph.t$  and  $sw=100 \times ph.t.$ , respectively). The first row denotes example phylogenetic trees, while the second row is the corresponding posterior estimates. The prior is shown with the grey violins (a hierarchical prior configuration) and each black bar is the 95% credible interval of the posterior. The dashed line in each case denotes the correct value.

268 **2.3 Temporal sampling bias**

269 We investigated the impact of temporal sampling bias on the quality of molecular clock estimates. For this  
270 purpose we simulated data with the same genomic characteristics as HBV and where genome sampling was  
271 conducted over five periods of time uniformly distributed between the present and the root of the tree (fig.  
272 4(a)). The fully sampled trees contained 500 genome samples, with 100 for each of the five sampling times.  
273 Such stratified sampling is expected in ancient DNA studies, for example when a set of samples are drawn  
274 from archaeological sites (e.g. Spyrou et al. 2019b). We sampled the complete data sets either by randomly  
275 selecting 20 samples from each strata, which we refer to as ‘time-uniform’ sampling, or by sampling with  
276 a probability that is inversely proportional to the age of the strata, referred to as ‘time-biased’. The time-  
277 uniform and time-biased sampling strategies both contain 100 samples ( $1/5^{th}$  of the complete data), but the  
278 time-biased only includes a small number of ancient samples.

279 The coverage of the evolutionary rate estimate was comparable across simulation treatments (table 5). It  
280 is worth noting that the coverage and other performance metrics were lower than for our simulation treatment  
281 where samples are uniformly drawn between the phylodynamic threshold and the present, which likely occurs  
282 because in this case samples with identical sampling times form monophyletic groups, reducing the amount  
283 of temporal information in the data (see (Murray et al., 2016) for a detailed investigation). Our measures of  
284 coverage and average bias did not differ substantially between sampling treatments.

Table 5: Coverage, average uncertainty and average bias of the mean evolutionary rate,  $M$ , for data with temporal sampling bias. Statistics are reported over 100 simulation replicates three possible scenarios, shown in fig. 4(a).

Statistic	Complete data (n=500)	Time-uniform sampling (n=100)	Time-biased sampling (n=100)
Coverage	88	83	89
Uncertainty	0.32	0.36	0.55
Bias	0.03	0.03	0.03

285 A striking result of the temporal sampling strategies was its impact in uncertainty of the posterior. Both,  
286 the time uniform and time-biased sampling treatments resulted in posterior distributions that were wider  
287 than with the complete data, which is to be expected because they are effectively smaller data sets with less  
288 information, but with no systematic bias (fig. 5(a)). However, the time-biased sampling data sets almost  
289 invariably have posterior distributions with higher uncertainty than those from the time-uniform sampling  
290 (fig. 5(b)), implying that the distribution of samples, and not just the number, is important for estimation  
291 uncertainty.

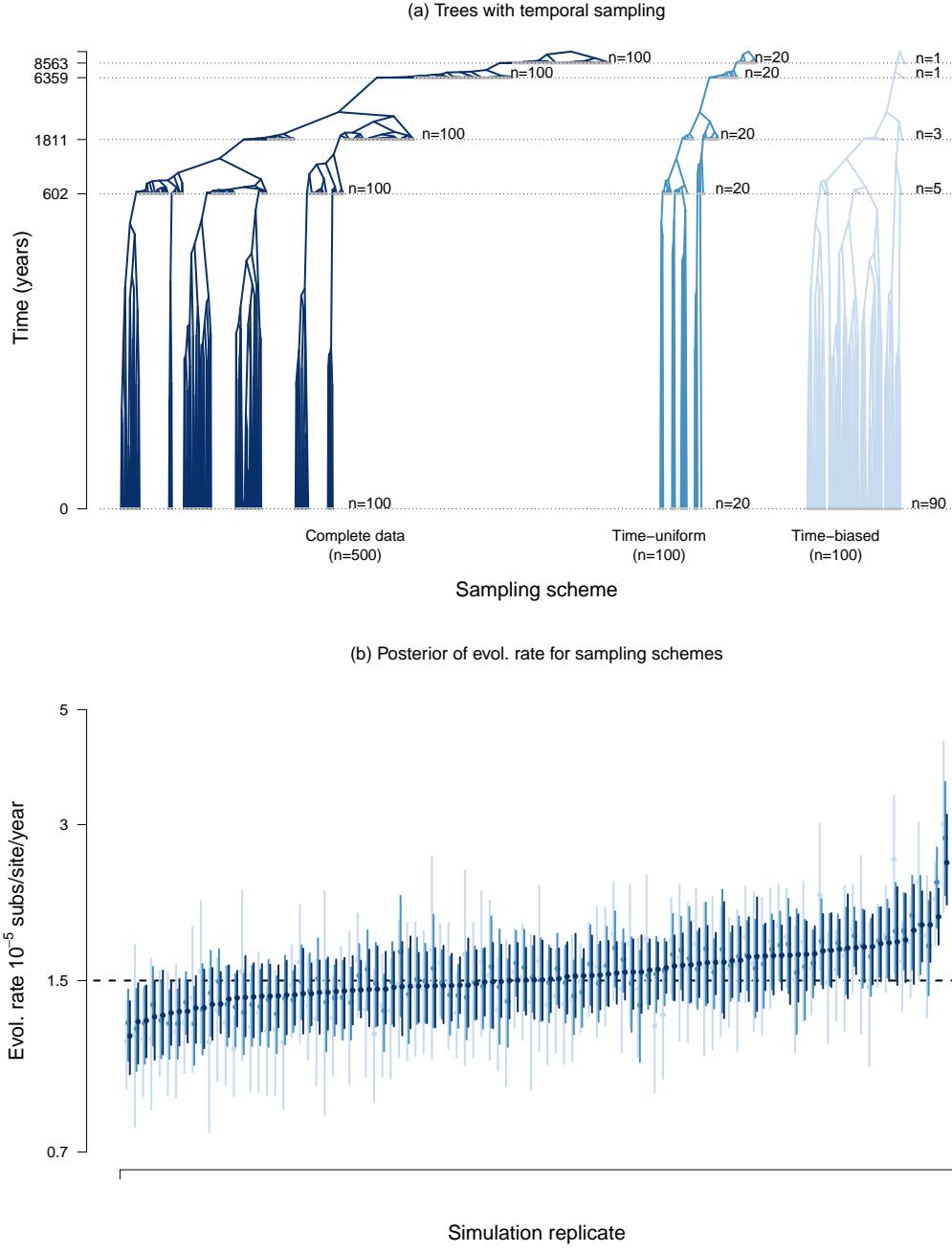


Figure 4: Analyses under sampling treatments over time. In (a) we show an example of the trees for a simulation replicate, with branch lengths and time in  $\log_{10}$  scale. The complete data set consists of 500 genome samples, collected in five points in time, with an equal number of samples per time point ( $n=100$ ). The first sampling strategy is unbiased, where 20 samples are drawn from each time point, and is known here as ‘time-uniform’. The ‘time-biased’ regime is where sampling intensity decreases over time. Note that the total number of samples in the time-uniform and time-biased treatments is identical. In (b) we show the posterior estimates of the evolutionary rate for each treatment. Each simulation replicate is represented by three error bars: dark blue for the complete data, and lighter shades of blue for the estimates from the time-uniform and time-biased sampling treatments. The width of the error bars denotes the 95% quantile range and the dots are the mean value. The dashed line shows the true value used to generate the data.

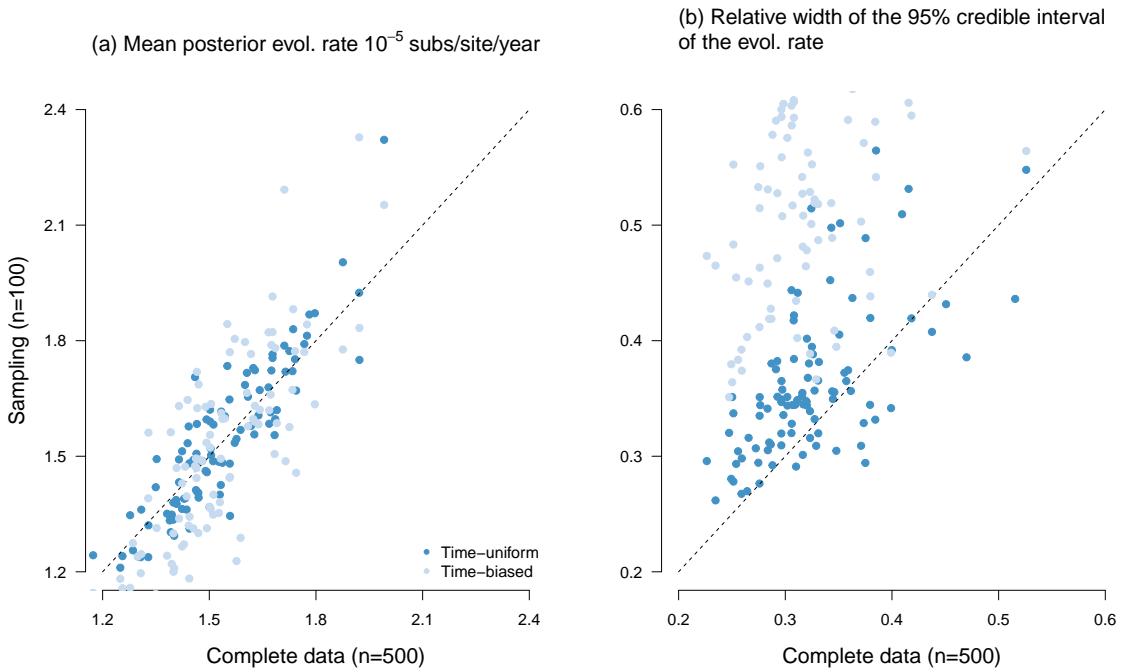


Figure 5: Comparison of posterior evolutionary rate estimates between complete data (x-axis) and two sampling treatments (y-axis): time-uniform (dark blue) and time-biased (light blue). Each dot is a simulation replicate. In (a) we show the mean posterior evolutionary rate,  $M$ , estimate. Points that fall along the  $y=x$  line (dashed line) represent identical mean posterior for the sampling treatment and the complete data, while those above or below represent higher or lower estimates, respectively, relative to the complete data. In (b) we show the uncertainty, calculated as the width of the lower 95% credible interval divided by the mean value. Values that fall along the  $y=x$  line denote those for which the complete data and either sampling strategies are equally uncertain, while those above and below the  $y=x$  line are more or less uncertain, respectively.

## 292 2.4 Empirical analyses of Hepatitis B virus (HBV) ancient and modern genomes

293 To explore the impact of the width of the sampling window and the temporal sampling bias on the estimates  
 294 of evolutionary rates and times, we performed analyses of a HBV data set that includes modern and ancient  
 295 genomes, from Kocher et al. (2021). The complete data set consisted of 232 genomes of length 3,344 nu-  
 296 cleotides and with a sampling window of 10,535 years. HBV is an ancient pathogen that has likely codiverged  
 297 with human populations for thousands of years (Locarnini et al., 2021, Mühlmann et al., 2018, Paraskevis  
 298 et al., 2013, Zehender et al., 2014), and thus its phylodynamic threshold has been reached while it has not  
 299 been empirically established if it can be considered to be a measurably evolving population, as is the case  
 300 for recent outbreaks, like SARS-CoV-2 (Duchene et al., 2020a).

301 For our first set of analyses we varied the width of the sampling window. We drew 100 genomes with  
 302 different sampling window widths: 0 (only modern samples), up to 500, 1,000, or 5,000 years before present.  
 303 Increasing the sampling window resulted in estimates of the evolutionary rate that were more precise and  
 304 closer to the estimate from the complete data set (fig. 6). Here we find that the evolutionary rate is estimated  
 305 to be higher for shorter sampling windows, with correspondingly younger estimates for the tree height. This  
 306 pattern can be due to one or a combination of other factors influencing the inference, for example the vagaries

307 of evolutionary rate variation in this virus, particularly time-dependency (Vrancken et al., 2017). Similarly,  
 308 population structure that is unaccounted for has been shown to produce an overestimation of the evolutionary  
 309 rate, because under the tree prior samples that are genetically linked are expected to have been sampled at  
 310 the same point in time (Möller et al., 2018).

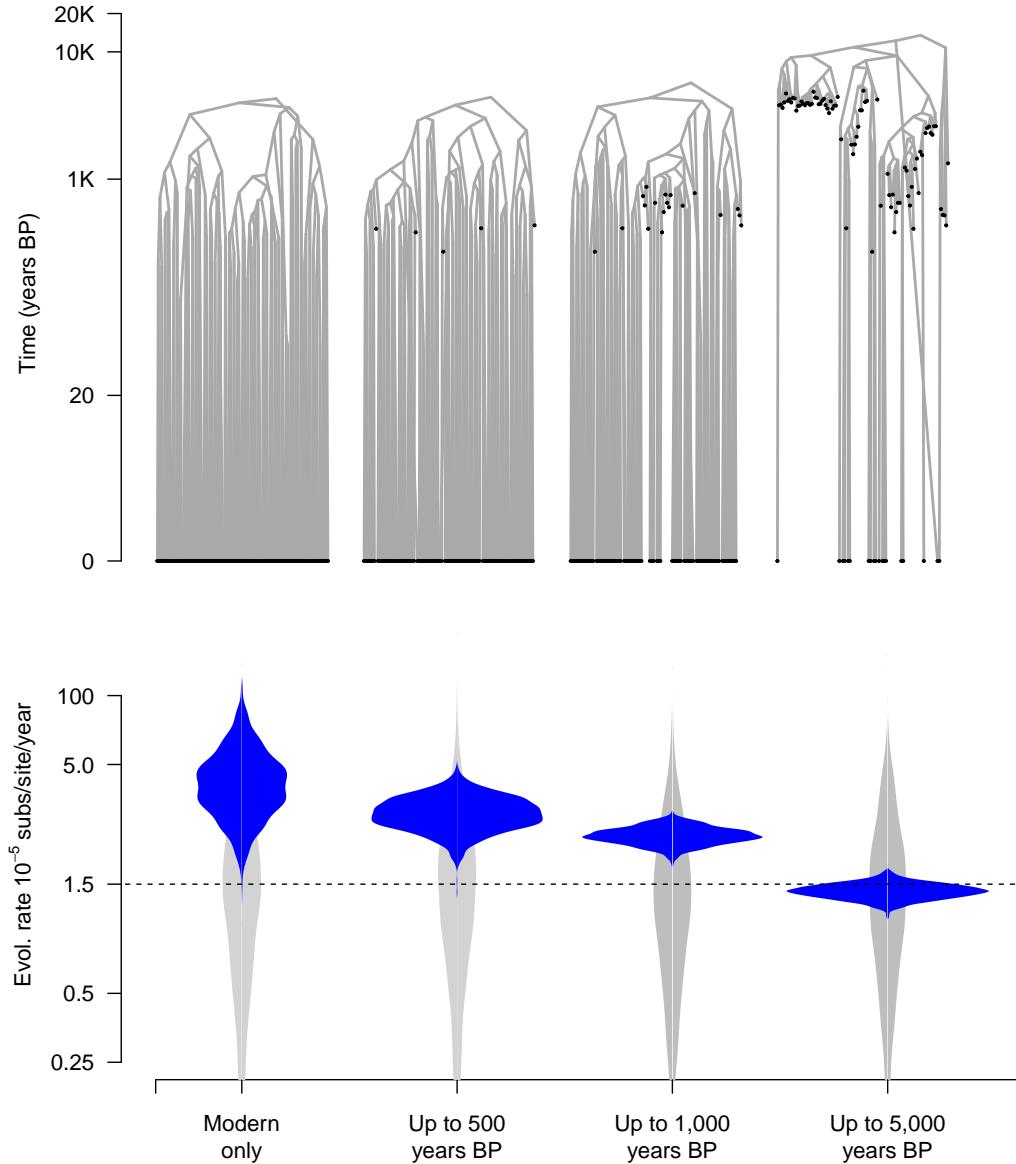


Figure 6: Results from empirical analyses of Hepatitis B virus (HBV) ancient DNA data. The phylogenetic trees correspond to highest clade credibility trees from three analyses where the data were subsampled to increase the width of the sampling window progressively. First, we only consider modern samples, then those up to 500, 1,000, and 5,000 years before present. In all cases the data sets consist of 100 genome samples. The violin plots show the posterior distribution of the evolutionary rate,  $M$  in blue and its corresponding prior in orange. The dashed line shows the mean  $M$  estimate from the complete data set.

311 For our second set of empirical analyses, we subsampled the data to produce a range of temporally biased

312 sampling scenarios. We varied the proportion of modern samples, from 95% to 10%. Here the sampling  
313 window is constant because we always retain the most ancient samples. Each data set consisted of 100  
314 genomes, such that they only differ in the distribution of modern and ancient samples (fig. 7).

315 The impact of temporal sampling bias was less clear than in our simulations above (figs 4 and 5). The data  
316 set with 95% modern genomes had the highest uncertainty in the evolutionary rate estimate, but uncertainty  
317 did not decrease monotonically with the proportion of modern genomes. Moreover, the posterior estimate of  
318 the evolutionary rate from the data set with 50% modern genomes deviated the most from the that obtained  
319 using the full data set. These results demonstrate that the exact impact of temporal sampling bias may be  
320 difficult to predict in practice, but that generally increasing the number of ancient samples results in data  
321 sets that are more informative, in that the difference between the prior and posterior is more pronounced  
322 than when only a few ancient samples are available.

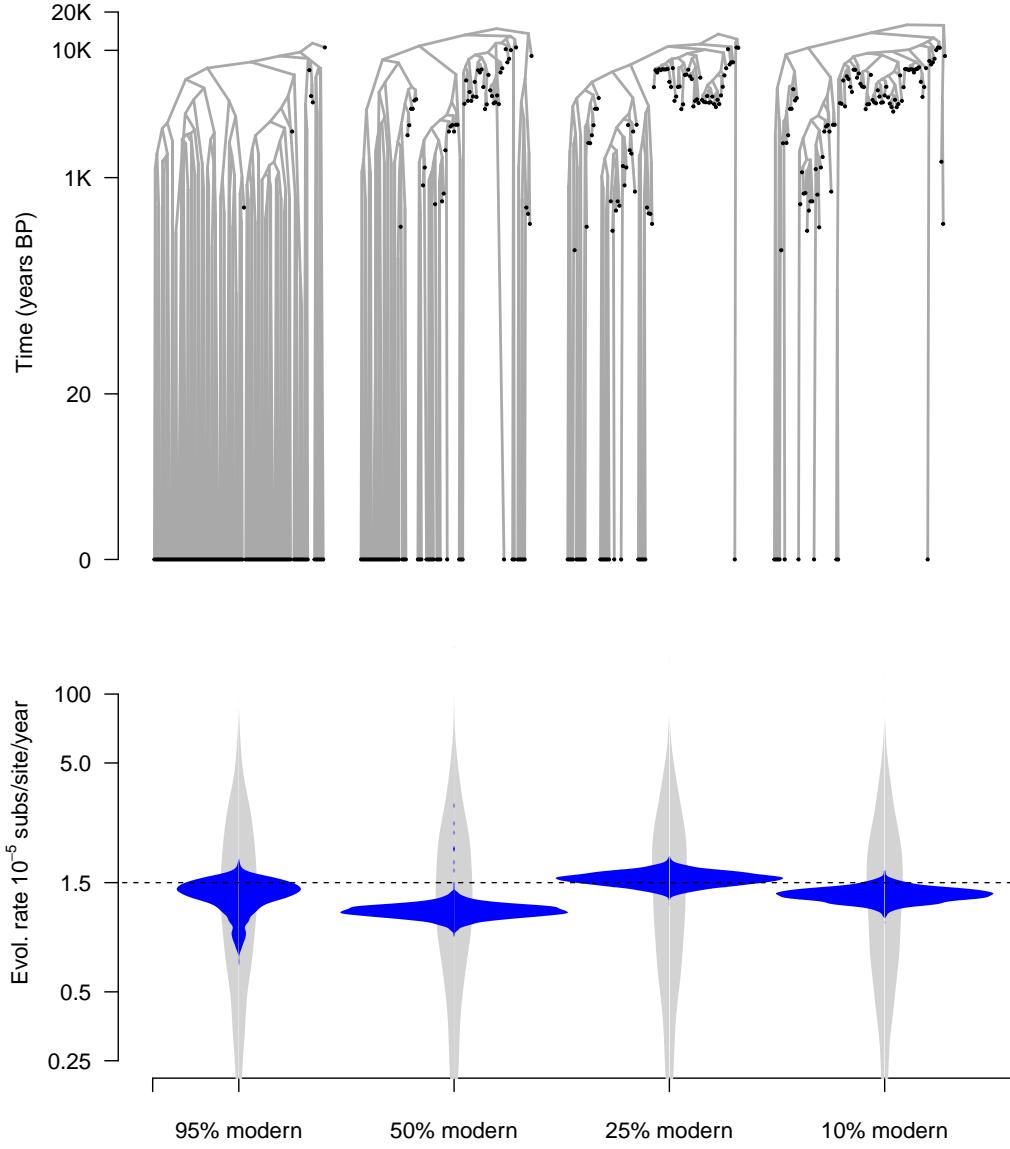


Figure 7: Results from empirical analyses of Hepatitis B virus (HBV) ancient DNA data. The phylogenetic trees correspond to highest clade credibility trees from three analyses where the data were subsampled to include an increasing number of ancient samples. First, we consider a data set for which the samples are 95% modern and the remaining 5% being the most ancient. Then, we reduce the number of modern samples to 50%, 25% and 10%, and the rest being ancient. Note that the sampling window is constant because we always retain the most ancient samples. In all cases the data sets consist of 100 genome samples. The violin plots show the posterior distribution of the evolutionary rate,  $M$  in blue and its corresponding prior in orange. The dashed line shows the mean  $M$  estimate from the complete data set.

323 **3 Discussion**

324 The concepts of the phyloodynamic threshold, measurably evolving populations, and temporal signal are  
325 helpful for our understanding of rapidly evolving organisms or data sets with ancient DNA. Our analyses  
326 help us disentangle the definition of these concepts and their practical implications. Our study concentrates  
327 on the estimates of the evolutionary rate. Beyond its biological implications, this parameter is essential for  
328 estimating evolutionary timescales and our performance metrics for the age of the root-node are comparable  
329 to those of the evolutionary rates (see Supplementary material online).

330 The phylodynamic threshold and measurable evolution are not discrete bounds. Increasing the sampling  
331 window generally reduces uncertainty and improves accuracy (bias and coverage), but there is no clear cut-off  
332 for when the estimates become accurate and objectively ‘precise’. Notably, the prior on the phylogenetic tree  
333 and the evolutionary rate are particularly influential for estimating evolutionary rates and timescales (for a  
334 detailed investigation see Tay et al. (2024)).

335 In fact, data sampled from a population that has attained its phylodynamic threshold does necessarily  
336 mean that the resulting estimates will be correct. In these circumstances the phylodynamic threshold simply  
337 means that there is a measurable amount of genetic diversity within the sampling window. However,  
338 our measures of coverage, uncertainty and bias for data with sampling windows of  $0.5 \times$  the phylodynamic  
339 threshold are often comparable to those obtained with much wider sampling windows, given that the prior  
340 on the evolutionary rate prior is not misleading.

341 In the case that the prior is substantially biased a sampling widow of  $100 \times$  the phylodynamic threshold  
342 may be needed to reduce such bias. The extent to which we can draw inferences from data that have attained  
343 the phylodynamic threshold or that are measurably evolving depends on the extent to which the data can  
344 inform the posterior, which is ultimately a measure of the relative contribution of the prior and the data  
345 (via the likelihood function). Our results suggest that using hierarchical priors may be an effective means  
346 of minimising the biases due to a poorly chosen prior. We emphasise that a prior that imposes a downward  
347 bias appears to be more problematic than one that favours high evolutionary rates. We attribute this to the  
348 fact that the upper bound on the evolutionary rate is effectively bracketed by the sampling window. The  
349 maximum evolutionary rate is the total genetic divergence divided by the sampling window width, whereas in  
350 principle there is no bound on the age of a tree and thus the evolutionary rate can effectively reach zero (i.e. if  
351 time tends to infinity). We note that the default prior on the evolutionary rate in popular Bayesian phylogenetic  
352 packages (e.g. BEAST2 and BEASTX (Baele et al., 2025)) is either a uniform distribution between 0 and  
353 infinity or a CTMC rate reference prior (Ferreira and Suchard, 2008, Wang and Yang, 2014), both of which  
354 tend to favour low evolutionary rate values, and should thus be considered carefully, particularly when the  
355 sampling window is narrower than the phylodynamic threshold.

356 Ideally, the prior and posterior of key parameters, such as the height of the root or the evolutionary rate  
357 should overlap, while the posterior should be narrower than the prior (i.e. more informative), meaning that  
358 the data and the prior are not in conflict (for recent work on quantifying prior-data conflict see: Nott et al.  
359 2020). In this vein, assessing the adequacy of the model and prior via predictive checks can be illuminating  
360 (McElreath, 2018), especially in situations where the joint prior is poorly understood (see Baele and Lemey  
361 2014, Wang and Yang 2014 for discussions about the prior in Bayesian phylogenetics). Recent years have seen  
362 the development of a range of methods for assessing phylogenetic model adequacy (Brown and Thomson,

363 2018, Duchêne et al., 2018, Duchene et al., 2019, McElreath, 2018), for instance one can simulate phylogenetic  
364 trees under the posterior estimates to verify whether the height of the root node and the topology could have  
365 been generated by the model in question.

366 Measurably evolving populations are those for which the phylodynamic threshold has been attained and  
367 the sampling window is *sufficiently* wide. The criteria for determining the phylodynamic threshold and  
368 whether a population is measurably evolving are the same, and are typically assessed via temporal signal.  
369 Statistical tests for this purpose quantify the strength of the association between sampling times and genetic  
370 distance (Duchêne et al., 2015, Featherstone et al., 2024, Murray et al., 2016, Rambaut et al., 2016, Rieux  
371 and Balloux, 2016). That is, the degree to which the sampling times on their own constitute an informative  
372 molecular clock calibration. We contend that assessing prior sensitivity is more important than the outcome  
373 of tests of temporal signal for obtaining reliable molecular clock estimates. In fact, a poor choice of prior  
374 can mislead tests of temporal signal (Tay et al., 2024). If data are drawn from a sampling window that  
375 spans the expected phylodynamic threshold, the presence of temporal signal is likely supported by most  
376 tests, suggesting accurate estimates. Yet, if the prior used for the estimation is misleading and informative,  
377 it might actually obscure the ‘correct’ signal from the data. In contrast, if the data are drawn from a narrow  
378 sampling window but the prior is reasonable then the estimates may be still be reliable, despite a lack of  
379 strong temporal signal. It also has to be noted that an increasing sampling effort does not necessarily lead  
380 to increasingly correct inferences, because misspecification not only in prior distributions of parameters, but  
381 also in the underlying model, can introduce biases (Ferretti et al., 2024, Möller et al., 2018).

382 An obvious concern about molecular clock calibrations using sequence sampling times is sampling bias.  
383 We find that temporal sampling bias, where data are overwhelmingly collected at a particular period of  
384 time does not have a substantial impact in estimation accuracy on a simple coalescent model, but that  
385 increasing the number of ancient sequences can reduce uncertainty. An other form of sampling bias is when  
386 genetic diversity is not uniformly sampled or the underlying population is structured. Previous work has  
387 demonstrated that in such cases, the evolutionary rate and the age of the root node tend to be overestimated  
388 (Möller et al., 2018), a problem that diminishes when sequence data are increasingly informative or by using  
389 a tree prior that explicitly models population structure (e.g. Kühnert et al. 2016, Müller et al. 2017).

390 Our study has a few limitations that have been partly addressed elsewhere. The number of sequences  
391 is fixed in most of our experiments, but it is well known that increasing the number of sequences generally  
392 means that data are more informative and thus the estimates are more precise (see Möller et al. (2018) and  
393 our fig. 4), and therefore it is likely that the width of the sampling window needed to obtain reliable estimates  
394 also depends on the number of sequences. Moreover, our simulation experiments involved a low degree of  
395 evolutionary rate variation among lineages. In this respect, it is expected that the width of the sampling  
396 window scales with the amount of dispersion in the molecular clock. In addition, our simulations are based  
397 on a simple population dynamic model, the constant coalescent. The impact of the sampling scheme and  
398 width is likely to be more complex for models with more parameters. We also assume the correct model of  
399 evolution and population growth in all simulation-based inferences. With the empirical analysis, we, however,  
400 highlight how conclusions drawn from these do not directly extend to real-world data. Rather, the isolated  
401 effects found therein describe only one of many elements impacting the inference from real data. Further  
402 scrutiny of these factors is warranted, but the main implication is that the necessary sampling time window

403 is combination of the data set, the organism, and the model at hand.

404 Overall, our study elucidates some of the fundamental intricacies of molecular clock calibration strategies.  
405 We urge researchers to carefully question their model and its underlying prior assumptions, not only via tests  
406 of temporal signal, but also through careful choice of the prior, an understanding of the information content  
407 in the data, and the implications of model misspecification.

## 408 4 Materials and methods

### 409 4.1 Simulations

#### 410 4.1.1 Data generation

411 We simulated phylogenetic trees and the evolution of nucleotide sequences to assess the impact of varying  
412 the sampling window and temporal sampling bias. We parameterised our simulations to resemble an HBV  
413 population evolving over 10,000 years before present, as described by Kocher et al. (2021).

414 We generated phylogenetic trees under a coalescent process in which the population size has been constant  
415 over time using the package ReMaster (Vaughan, 2024), part of the BEAST 2 (version 2.7) software. We set  
416 the population size to 5,000, which results in trees with an average age of about 10,000 time units (years).  
417 The number of samples (i.e. tips) drawn from the trees and their ages was defined in ReMaster, according  
418 to the simulation scenario described below.

419 The simulation of sequence data requires trees with branch lengths in units of subs/site, instead of time.  
420 For this purpose, we multiplied the branch lengths of the simulated time-tree with the rate of evolution, a  
421 lognormally distributed random variable with parameters  $\mu = \log(1.5 \times 10^{-5}) - \frac{0.25^2}{2}$  and  $\sigma = 0.25$ . This  
422 procedure equates to simulating an uncorrelated relaxed molecular clock model with an underlying lognormal  
423 distribution (Drummond et al., 2006) with mean,  $M$  of  $1.5 \times 10^{-5}$  subs/site/year and a standard deviation  
424 of 0.25 subs/site/year (following that  $\mu = \log(M) - \frac{\sigma^2}{2}$ ). Because we multiply the branch lengths in units of  
425 years by a variable in subs/site/year, the resulting trees have branch lengths in units of subs/site, formally  
426 known as phylogenograms, in contrast to time-trees or chronograms where the branch lengths correspond to time.  
427 We obtained sequence alignments using the R package phangorn (v2.8.1) (Schliep, 2011), according to a  
428 HKY+ $\Gamma_4$  substitution model, with parameters  $\kappa = 2$ ,  $\alpha = 4$ , and equal base frequencies. The alignments  
429 consisted of 3,200 nucleotides to match the average genome size of HBV.

430 We considered the expected phylodynamic threshold of our data to be about 20 years. For our simulations  
431 where we varied the sampling window, we set the ages of 100 tips to be sampled at present (all have an age  
432 of 0), or to be drawn from a uniform distribution between 0 and 10 (1/2 of the expected phylodynamic  
433 threshold), 0 and 20, 0 and 200, or 0 and 2,000.

434 To investigate the impact of temporal sampling bias we initially simulated trees with 500 tips with  
435 sampling times distributed in 5 time points, with 100 tips per time point. The distribution of sampling times  
436 followed an exponential distribution with mean of 4,000, such that sampling times were concentrated towards  
437 the present. We simulated these complete trees in ReMaster and we followed the procedure above to simulate  
438 a molecular clock model and sequence alignments.

439 We conducted two sampling schemes for the trees with 500 tips: the ‘time-uniform’ scheme consisted  
440 of drawing 20 samples from each time point, whereas the ‘time-biased’ scheme included of mostly modern

441 samples (90 from the present, and 5, 3, 1, and 1 from the remaining time points). For each simulation  
442 scenario we generated 100 replicates.

#### 443 4.1.2 Analysis of simulated data

444 We analysed all data sets in BEAST 2 under a model that matched that used to generate the data: the  
445 HKY+ $\Gamma_4$  substitution model, a uncorrelated relaxed molecular clock model with an underlying lognormal  
446 distribution, and a constant size coalescent tree prior. We used the default prior configuration in the program,  
447 except for the mean evolutionary rate ( $M$ ) and the population size of the coalescent ( $N$ , where  $N = N_e \times \tau$ ,  
448 and where  $N_e$  is the effective population size and  $\tau$  is generation time). For the population size we assumed  
449  $N \sim \text{Exponential}(\text{mean} = 5,000)$ , which is centred in the value used to generate the data. The expected  
450 height of the root node is roughly 10,000 years (expected time to coalescent =  $2 \times N$  for an ultrametric tree  
451 (Arbisser et al., 2018, Nordborg, 2019)).

452 For the mean evolutionary rate we considered a range of priors with different degrees of information  
453 content (uncertainty) and for which the mean was either the value used to generate the data, or one order of  
454 magnitude higher or lower, as shown in table 6. We also included three degrees of uncertainty in such prior  
455 distributions, where the 95% CI width was equal to the mean, or around three or six times as wide. In all  
456 cases we set the sampling times for calibration.

457 For ultrametric trees, sampling times are set to the present, such that all calibration information is  
458 provided by the tree prior. Concretely, let the tree length in time (sum of all branch lengths  $t_i$  in units  
459 of time) be  $T_t$ , the tree height in units of time  $T_h$ , the branch rates  $r_i$  (the vector of branch rates  $\vec{R} \in$   
460  $\{r_1, \dots, r_{\text{num. branches}}\}$ , see Douglas et al. 2021), and  $T_d$  is the tree length in genetic divergence (sum of branch  
461 lengths  $d_i$  in subs/site).

462 In most Bayesian phylogenetic frameworks branch lengths in genetic distance are the product of rates  
463 and times (Douglas et al., 2021, Drummond et al., 2006, Höhna et al., 2016), such that  $T_d = \sum d_i = \sum r_i t_i$ .  
464 Importantly,  $T_d$  is given by the phylogenetic likelihood and only depends on genetic distance and substitution  
465 model parameters. The average evolutionary rate (an estimate of parameter  $M$ ) is  $\bar{r} = \frac{\sum r_i t_i}{\sum t_i} = \frac{T_d}{T_t}$ . However,  
466  $T_t$  is a function of population size  $N$  through  $\mathbb{E}[T_t] = 2 \times N \times \log(n)$ , where  $n$  is the number of sampled lineages  
467 (Arbisser et al., 2018, Tavaré et al., 1997). Thus, the prior on  $N$  affects  $T_h$  (through  $\mathbb{E}[T_h] = 2 \times N(1 - \frac{1}{n}) \approx$   
468  $2 \times N$ , for large  $n$ ),  $T_t$ , and the average evolutionary rate ( $\bar{r} = \frac{T_d}{2 \times N \times \log(n)}$ , where  $T_d$  is independent of time).

Table 6: Prior configuration for the mean evolutionary rate,  $M$  of the lognormal distribution of branch rates. Note that the mean of the *Gamma* distribution here is *shape/rate* and that the true value used to generate the data is  $1.5 \times 10^{-5}$  subs/site/year.

Mean value of $M$	Prior configuration	95% quantile width / mean $M$
$1.5 \times 10^{-5}$	$\text{Gamma}(\text{shape} = 1.5, \text{rate} = 1 \times 10^5)$	3.04
$1.5 \times 10^{-5}$	$\text{Gamma}(\text{shape} = 0.3, \text{rate} = 2 \times 10^4)$	6.33
$1.5 \times 10^{-5}$	$\text{Gamma}(\text{shape} = 15, \text{rate} = 1 \times 10^6)$	1.00
$1.5 \times 10^{-4}$	$\text{Gamma}(\text{shape} = 1.5, \text{rate} = 1 \times 10^4)$	3.04
$1.5 \times 10^{-4}$	$\text{Gamma}(\text{shape} = 0.3, \text{rate} = 2 \times 10^3)$	6.33
$1.5 \times 10^{-4}$	$\text{Gamma}(\text{shape} = 15, \text{rate} = 1 \times 10^5)$	1.00
$1.5 \times 10^{-6}$	$\text{Gamma}(\text{shape} = 1.5, \text{rate} = 1 \times 10^6)$	3.04
$1.5 \times 10^{-6}$	$\text{Gamma}(\text{shape} = 0.3, \text{rate} = 2 \times 10^5)$	6.33
$1.5 \times 10^{-6}$	$\text{Gamma}(\text{shape} = 15, \text{rate} = 1 \times 10^7)$	1.00

We used an additional configuration for the prior  $M$  using a hierarchical structure as follows:

$$M \sim \text{Gamma}(\text{shape}, \text{rate})$$

$$\text{shape} \sim \text{LogNormal}(1, 5)$$

$$\text{rate} \sim \text{LogNormal}(1, 5)$$

469 To set up this model, we simply treat the *shape* and *rate* as parameters that are sampled in the model.

470 We used Markov chain Monte Carlo (MCMC) to sample the posterior distribution for all analyses. We  
471 set the chain length to  $10^8$  steps, sampling every  $5 \times 10^4$  steps. We deemed sufficient sampling by verifying  
472 that the effective sample size was at least 200, by using the R package CODA (version 0.19) (Plummer et al.,  
473 2006). When this criterion was not met we extended the chain length to  $5 \times 10^8$  steps.

474 To visualise the prior on  $M$  in our model with a hierarchical prior we drew MCMC samples from the  
475 marginal prior of  $M$ . That is, the prior integrating over the hyperprior distributions and all other parameters.  
476 We obtained such samples by setting the option `sampleFromPrior="true"` in the input xml files in BEAST  
477 2, which conducts the MCMC while ignoring the phylogenetic likelihood.

#### 478 4.1.3 HBV empirical data

479 We selected a complete genome data set of HBV published by Kocher et al. (2021). The complete alignment  
480 included 232 genomes of length 3,344 nucleotides, with 1,807 variable sites, and 1,498 site patterns. The  
481 sampling times ranged from the present to 10,535 years before present. To investigate the impact of varying  
482 the sampling window and on temporal sampling bias we subsampled the data as described in our Results  
483 section. We analysed each data set using the same model and prior settings as in our simulations, including  
484 the use of the reasonable and misleading prior configuration.

485 **5 Data availability**

486 Computer code, analysis files, and data sets in this study are available at:

487 [https://github.com/sebastianduchene/phylo\\_threshold\\_code\\_data](https://github.com/sebastianduchene/phylo_threshold_code_data)

488 **6 Competing interests**

489 None.

490 **7 Acknowledgments**

491 Pending.

492 **8 Funding**

493 This work received funding from the Inception program (Investissement d’Avenir grant ANR-16-CONV-0005  
494 awarded to SD) and the Australian National Health and Medical Research Council (2017284 awarded to SD),

495 **References**

496 A. Andrades Valtueña, G. U. Neumann, M. A. Spyrou, L. Musralina, F. Aron, A. Beisenov, A. B. Belinskiy,  
497 K. I. Bos, A. Buzhilova, M. Conrad, et al. Stone age yersinia pestis genomes shed light on the early  
498 evolution, diversity, and ecology of plague. *Proceedings of the National Academy of Sciences*, 119(17):  
499 e2116722119, 2022.

500 I. M. Arbisser, E. M. Jewett, and N. A. Rosenberg. On the joint distribution of tree height and tree length  
501 under the coalescent. *Theoretical population biology*, 122:46–56, 2018.

502 G. Baele and P. Lemey. Bayesian model selection in phylogenetics and genealogy-based population genetics.  
503 In M. Chen, K. L, and L. PO, editors, *Bayesian phylogenetics, methods, algorithms, and applications*,  
504 chapter 4, pages 59–93. CPC Press, Boca Raton (Florida), 2014.

505 G. Baele, X. Ji, G. W. Hassler, J. T. McCrone, Y. Shao, Z. Zhang, A. J. Holbrook, P. Lemey, A. J. Drummond,  
506 A. Rambaut, et al. Beast x for bayesian phylogenetic, phylogeographic and phylodynamic inference. *Nature  
507 Methods*, 22(8):1653–1656, 2025.

508 R. Biek, O. G. Pybus, J. O. Lloyd-Smith, and X. Didelot. Measurably evolving pathogens in the genomic  
509 era. *Trends in ecology & evolution*, 30(6):306–313, 2015.

510 M. F. Boni, P. Lemey, X. Jiang, T. T.-Y. Lam, B. W. Perry, T. A. Castoe, A. Rambaut, and D. L. Robertson.  
511 Evolutionary origins of the sars-cov-2 sarbecovirus lineage responsible for the covid-19 pandemic. *Nature  
512 microbiology*, 5(11):1408–1417, 2020.

- 513 R. Bouckaert, T. G. Vaughan, J. Barido-Sottani, S. Duchêne, M. Fourment, A. Gavryushkina, J. Heled,  
514 G. Jones, D. Kühnert, N. De Maio, et al. Beast 2.5: An advanced software platform for bayesian evolu-  
515 tionary analysis. *PLoS computational biology*, 15(4):e1006650, 2019.
- 516 L. Bromham, S. Duchêne, X. Hua, A. M. Ritchie, D. A. Duchêne, and S. Y. Ho. Bayesian molecular dating:  
517 opening up the black box. *Biological Reviews*, 93(2):1165–1191, 2018.
- 518 J. M. Brown and R. C. Thomson. Evaluating model performance in evolutionary biology. *Annual Review of  
519 Ecology, Evolution, and Systematics*, 49(1):95–114, 2018.
- 520 D. A. Buonagurio, S. Nakada, J. D. Parvin, M. Krystal, P. Palese, and W. M. Fitch. Evolution of human  
521 influenza a viruses over 50 years: rapid, uniform rate of change in ns gene. *Science*, 232(4753):980–982,  
522 1986.
- 523 A. M. Devault, G. B. Golding, N. Waglechner, J. M. Enk, M. Kuch, J. H. Tien, M. Shi, D. N. Fisman, A. N.  
524 Dhody, S. Forrest, et al. Second-pandemic strain of vibrio cholerae from the philadelphia cholera outbreak  
525 of 1849. *New England Journal of Medicine*, 370(4):334–340, 2014.
- 526 M. Dos Reis and Z. Yang. The unbearable uncertainty of bayesian divergence time estimation. *Journal of  
527 Systematics and Evolution*, 51(1):30–43, 2013.
- 528 J. Douglas, R. Zhang, and R. Bouckaert. Adaptive dating and fast proposals: Revisiting the phylogenetic  
529 relaxed clock model. *PLoS computational biology*, 17(2):e1008322, 2021.
- 530 A. Drummond, O. G. Pybus, and A. Rambaut. Inference of viral evolutionary rates from molecular sequences.  
531 *Adv Parasitol*, 54:331–358, 2003a.
- 532 A. J. Drummond, O. G. Pybus, A. Rambaut, R. Forsberg, and A. G. Rodrigo. Measurably evolving popu-  
533 lations. *Trends in ecology & evolution*, 18(9):481–488, 2003b.
- 534 A. J. Drummond, S. Y. W. Ho, M. J. Phillips, and A. Rambaut. Relaxed phylogenetics and dating with  
535 confidence. *PLoS Biology*, 4(5):e88, 2006.
- 536 D. A. Duchêne, S. Duchêne, and S. Y. Ho. Phylomad: efficient assessment of phylogenomic model adequacy.  
537 *Bioinformatics*, 34(13):2300–2301, 2018.
- 538 S. Duchêne, R. Lanfear, and S. Y. Ho. The impact of calibration and clock-model choice on molecular  
539 estimates of divergence times. *Molecular phylogenetics and evolution*, 78:277–289, 2014.
- 540 S. Duchêne, D. Duchêne, E. C. Holmes, and S. Y. Ho. The performance of the date-randomization test in  
541 phylogenetic analyses of time-structured virus data. *Molecular Biology and Evolution*, 32(7):1895–1906,  
542 2015.
- 543 S. Duchene, K. E. Holt, F.-X. Weill, S. Le Hello, J. Hawkey, D. J. Edwards, M. Fourment, and E. C. Holmes.  
544 Genome-scale rates of evolutionary change in bacteria. *Microbial genomics*, 2(11):e000094, 2016.

- 545 S. Duchene, D. A. Duchene, J. L. Geoghegan, Z. A. Dyson, J. Hawkey, and K. E. Holt. Inferring demographic  
546 parameters in bacterial genomic data using bayesian and hybrid phylogenetic methods. *BMC evolutionary*  
547 *biology*, 18:1–11, 2018.
- 548 S. Duchene, R. Bouckaert, D. A. Duchene, T. Stadler, and A. J. Drummond. Phylodynamic model adequacy  
549 using posterior predictive simulations. *Systematic biology*, 68(2):358–364, 2019.
- 550 S. Duchene, L. Featherstone, M. Haritopoulou-Sinanidou, A. Rambaut, P. Lemey, and G. Baele. Temporal  
551 signal and the phylodynamic threshold of sars-cov-2. *Virus evolution*, 6(2):veaa061, 2020a.
- 552 S. Duchene, S. Y. Ho, A. G. Carmichael, E. C. Holmes, and H. Poinar. The recovery, interpretation and use  
553 of ancient pathogen genomes. *Current Biology*, 30(19):R1215–R1231, 2020b.
- 554 S. Duchene, P. Lemey, T. Stadler, S. Y. Ho, D. A. Duchene, V. Dhanasekaran, and G. Baele. Bayesian  
555 evaluation of temporal signal in measurably evolving populations. *Molecular Biology and Evolution*, 37  
556 (11):3363–3379, 2020c.
- 557 K. Eaton, L. Featherstone, S. Duchene, A. G. Carmichael, N. Varlik, G. B. Golding, E. C. Holmes, and  
558 H. N. Poinar. Plagued by a cryptic clock: insight and issues from the global phylogeny of yersinia pestis.  
559 *Communications Biology*, 6(1):23, 2023.
- 560 V. Eldholm, J. Monteserin, A. Rieux, B. Lopez, B. Sobkowiak, V. Ritacco, and F. Balloux. Four decades of  
561 transmission of a multidrug-resistant mycobacterium tuberculosis outbreak strain. *Nature communications*,  
562 6(1):7119, 2015.
- 563 L. A. Featherstone, A. Rambaut, S. Duchene, and W. Wirth. Clockor2: Inferring global and local strict  
564 molecular clocks using root-to-tip regression. *Systematic biology*, 73(3):623–628, 2024.
- 565 M. A. Ferreira and M. A. Suchard. Bayesian analysis of elapsed times in continuous-time markov chains.  
566 *Canadian Journal of Statistics*, 36(3):355–368, 2008.
- 567 L. Ferretti, T. Golubchik, F. Di Lauro, M. Ghafari, J. Villabona-Arenas, K. E. Atkins, C. Fraser, and M. Hall.  
568 Biased estimates of phylogenetic branch lengths resulting from the discretised gamma model of site rate  
569 heterogeneity. *bioRxiv*, pages 2024–08, 2024.
- 570 A. Gavryushkina, T. A. Heath, D. T. Ksepka, T. Stadler, D. Welch, and A. J. Drummond. Bayesian total-  
571 evidence dating reveals the recent crown radiation of penguins. *Systematic biology*, 66(1):57–73, 2017.
- 572 M. Ghafari, L. du Plessis, J. Raghwani, S. Bhatt, B. Xu, O. G. Pybus, and A. Katzourakis. Purifying selec-  
573 tion determines the short-term time dependency of evolutionary rates in sars-cov-2 and ph1n1 influenza.  
574 *Molecular Biology and Evolution*, 39(2):msac009, 2022.
- 575 N. Gharbi, E. Rousseau, and T. Wirth. Clock rates and bayesian evaluation of temporal signal. In *Phyloge-  
576 nomics*, pages 153–175. Elsevier, 2024.
- 577 T. Gojobori, E. N. Moriyama, and M. Kimura. Molecular clock of viral evolution, and the neutral theory.  
578 *Proceedings of the National Academy of Sciences*, 87(24):10015–10018, 1990.

- 579 S. Guindon. Rates and rocks: strengths and weaknesses of molecular dating methods. *Frontiers in Genetics*,  
580 11:526, 2020.
- 581 T. A. Heath, J. P. Huelsenbeck, and T. Stadler. The fossilized birth–death process for coherent calibration of  
582 divergence-time estimates. *Proceedings of the National Academy of Sciences*, 111(29):E2957–E2966, 2014.
- 583 S. Y. Ho. The changing face of the molecular evolutionary clock. *Trends in Ecology & Evolution*, 29(9):  
584 496–503, 2014.
- 585 S. Y. Ho and S. Duchêne. Molecular-clock methods for estimating evolutionary rates and timescales. *Molecular  
586 Ecology*, 23(24):5947–5965, 2014.
- 587 S. Höhna, M. J. Landis, T. A. Heath, B. Boussau, N. Lartillot, B. R. Moore, J. P. Huelsenbeck, and  
588 F. Ronquist. Revbayes: Bayesian phylogenetic inference using graphical models and an interactive model-  
589 specification language. *Systematic biology*, 65(4):726–736, 2016.
- 590 G. Kahila Bar-Gal, M. J. Kim, A. Klein, D. H. Shin, C. S. Oh, J. W. Kim, T.-H. Kim, S. B. Kim, P. R.  
591 Grant, O. Pappo, et al. Tracing hepatitis b virus to the 16th century in a korean mummy. *Hepatology*, 56  
592 (5):1671–1680, 2012.
- 593 A. Kocher, L. Papac, R. Barquera, F. M. Key, M. A. Spyrou, R. Hübner, A. B. Rohrlach, F. Aron, R. Stahl,  
594 A. Wissgott, et al. Ten millennia of hepatitis b virus evolution. *Science*, 374(6564):182–188, 2021.
- 595 D. Kühnert, T. Stadler, T. G. Vaughan, and A. J. Drummond. Phylodynamics with migration: a computa-  
596 tional framework to quantify population structure from genomic data. *Molecular biology and evolution*, 33  
597 (8):2102–2116, 2016.
- 598 D. Kühnert, M. Coscolla, D. Brites, D. Stucki, J. Metcalfe, L. Fenner, S. Gagneux, and T. Stadler. Tuber-  
599 culosis outbreak investigation using phylodynamic analysis. *Epidemics*, 25:47–53, 2018.
- 600 S. A. Locarnini, M. Littlejohn, and L. K. Yuen. Origins and evolution of the primate hepatitis b virus.  
601 *Frontiers in Microbiology*, 12:653684, 2021.
- 602 R. McElreath. *Statistical rethinking: A Bayesian course with examples in R and Stan*. Chapman and  
603 Hall/CRC, 2018.
- 604 F. Menardo, S. Duchêne, D. Brites, and S. Gagneux. The molecular clock of mycobacterium tuberculosis.  
605 *PLoS pathogens*, 15(9):e1008067, 2019.
- 606 F. K. Mendes, R. Bouckaert, L. M. Carvalho, and A. J. Drummond. How to validate a bayesian evolutionary  
607 model. *Systematic Biology*, 74(1):158–175, 2025.
- 608 M. Merker, J.-P. Rasigade, M. Barbier, H. Cox, S. Feuerriegel, T. A. Kohl, E. Shitikov, K. Klaos, C. Gaudin,  
609 R. Antoine, et al. Transcontinental spread and evolution of mycobacterium tuberculosis w148 euro-  
610 pean/russian clade toward extensively drug resistant tuberculosis. *Nature Communications*, 13(1):5105,  
611 2022.

- 612 S. Möller, L. du Plessis, and T. Stadler. Impact of the tree prior on estimating clock rates during epidemic  
613 outbreaks. *Proceedings of the National Academy of Sciences*, 115(16):4200–4205, 2018.
- 614 B. Mühlmann, T. C. Jones, P. d. B. Damgaard, M. E. Allentoft, I. Shevnina, A. Logvin, E. Usmanova, I. P.  
615 Panyushkina, B. Boldgiv, T. Bazartseren, et al. Ancient hepatitis b viruses from the bronze age to the  
616 medieval period. *Nature*, 557(7705):418–423, 2018.
- 617 N. F. Müller, D. A. Rasmussen, and T. Stadler. The structured coalescent and its approximations. *Molecular  
618 biology and evolution*, 34(11):2970–2981, 2017.
- 619 G. G. Murray, F. Wang, E. M. Harrison, G. K. Paterson, A. E. Mather, S. R. Harris, M. A. Holmes,  
620 A. Rambaut, and J. J. Welch. The effect of genetic structure on molecular dating and tests for temporal  
621 signal. *Methods in Ecology and Evolution*, 7(1):80–89, 2016.
- 622 M. Nordborg. Coalescent theory. *Handbook of Statistical Genomics: Two Volume Set*, pages 145–30, 2019.
- 623 D. J. Nott, X. Wang, M. Evans, and B.-G. Englert. Checking for prior-data conflict using prior-to-posterior  
624 divergences. *Statistical Science*, 35(2):234–253, 2020.
- 625 D. Paraskevis, G. Magiorkinis, E. Magiorkinis, S. Y. Ho, R. Belshaw, J.-P. Allain, and A. Hatzakis. Dating  
626 the origin and dispersal of hepatitis b virus infection in humans and primates. *Hepatology*, 57(3):908–916,  
627 2013.
- 628 M. Plummer, N. Best, K. Cowles, K. Vines, et al. Coda: convergence diagnosis and output analysis for  
629 mcmc. *R news*, 6(1):7–11, 2006.
- 630 A. Rambaut, T. T. Lam, L. Max Carvalho, and O. G. Pybus. Exploring the temporal structure of hetero-  
631 rochronous sequences using tempest (formerly path-o-gen). *Virus evolution*, 2(1):vew007, 2016.
- 632 C. Ramsden, E. C. Holmes, and M. A. Charleston. Hantavirus evolution in relation to its rodent and  
633 insectivore hosts: no evidence for codivergence. *Molecular biology and evolution*, 26(1):143–153, 2009.
- 634 A. Rieux and F. Balloux. Inferences from tip-calibrated phylogenies: a review and a practical guide. *Molecular  
635 ecology*, 25(9):1911–1924, 2016.
- 636 F. Ronquist, N. Lartillot, and M. J. Phillips. Closing the gap between rocks and clocks using total-evidence  
637 dating. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 371(1699):20150136, 2016.
- 638 Z. P. Ross, J. Klunk, G. Fornaciari, V. Giuffra, S. Duchêne, A. T. Duggan, D. Poinar, M. W. Douglas, J.-S.  
639 Eden, E. C. Holmes, et al. The paradox of hbv evolution as revealed from a 16th century mummy. *PLoS  
640 pathogens*, 14(1):e1006750, 2018.
- 641 R. Sanjuán. From molecular genetics to phylodynamics: evolutionary relevance of mutation rates across  
642 viruses. *PLoS pathogens*, 8(5):e1002685, 2012.
- 643 K. P. Schliep. phangorn: phylogenetic analysis in r. *Bioinformatics*, 27(4):592–593, 2011.

- 644 M. A. Spyrou, K. I. Bos, A. Herbig, and J. Krause. Ancient pathogen genomics as an emerging tool for  
645 infectious disease research. *Nature Reviews Genetics*, 20(6):323–340, 2019a.
- 646 M. A. Spyrou, M. Keller, R. I. Tukhbatova, C. L. Scheib, E. A. Nelson, A. Andrades Valtueña, G. U.  
647 Neumann, D. Walker, A. Alterauge, N. Cartey, et al. Phylogeography of the second plague pandemic  
648 revealed through analysis of historical yersinia pestis genomes. *Nature communications*, 10(1):4470, 2019b.
- 649 S. Tavaré, D. J. Balding, R. C. Griffiths, and P. Donnelly. Inferring coalescence times from dna sequence  
650 data. *Genetics*, 145(2):505–518, 1997.
- 651 J. H. Tay, A. Kocher, and S. Duchene. Assessing the effect of model specification and prior sensitivity on  
652 bayesian tests of temporal signal. *PLoS Computational Biology*, 20(11):e1012371, 2024.
- 653 N. S. Trovão, G. Baele, B. Vrancken, F. Bielejec, M. A. Suchard, D. Fargette, and P. Lemey. Host ecology  
654 determines the dispersal patterns of a plant virus. *Virus evolution*, 1(1):vev016, 2015.
- 655 T. G. Vaughan. Remaster: improved phylodynamic simulation for beast 2.7. *Bioinformatics*, 40(1):btae015,  
656 2024.
- 657 B. Vrancken, M. A. Suchard, and P. Lemey. Accurate quantification of within-and between-host hbv evolu-  
658 tionary rates requires explicit transmission chain modelling. *Virus Evolution*, 3(2):vex028, 2017.
- 659 Y. Wang and Z. Yang. Priors in bayesian phylogenetics. *Bayesian phylogenetics: methods, algorithms, and*  
660 *applications*, pages 5–24, 2014.
- 661 R. C. Warnock, Z. Yang, and P. C. Donoghue. Exploring uncertainty in the calibration of the molecular  
662 clock. *Biology letters*, 8(1):156–159, 2012.
- 663 G. Zehender, E. Ebranati, E. Gabanelli, C. Sorrentino, A. L. Presti, E. Tanzi, M. Ciccozzi, and M. Galli.  
664 Enigmatic origin of hepatitis b virus: an ancient travelling companion or a recent encounter? *World*  
665 *Journal of Gastroenterology: WJG*, 20(24):7622, 2014.
- 666 E. Zuckerkandl and L. Pauling. Evolutionary divergence and convergence in proteins. In *Evolving genes and*  
667 *proteins*, pages 97–166. Elsevier, 1965.