Prolonged decay of molecular evolutionary rates in metazoan mitochondrial DNA

Martyna Molak, Simon Y W Ho

Evolutionary timescales can be estimated from genetic data using the molecular clock, calibrated by fossil or other evidence. However, estimates of molecular rates in mitochondrial DNA appear to scale negatively with the age of the clock calibration. Although such a pattern has been observed in a limited range of data sets, it has not been studied on a large scale. In addition, there is uncertainty over the temporal extent of the time-dependent pattern in rate estimates. Here we present a meta-analysis of 239 rate estimates from metazoans, representing a range of timescales and taxonomic groups. We found evidence of time-dependent rates in both coding and non-coding mitochondrial markers, in every group of animals that we studied. The negative relationship between the estimated rate and time persisted across a much wider range of calibration times than previously suggested. This indicates that, over long timeframes, purifying selection gives way to mutational saturation as the main driver of the time-dependent biases in rate estimation. The results of our study stress the importance of accounting for time-dependent biases in estimating evolutionary rates using mitochondrial data regardless of the timescale over which they are inferred.

1 **Prolonged decay of molecular evolutionary rates in metazoan mitochondrial DNA**

2 Martyna Molak [1,2], Simon Y.W. Ho [1]

3 [1]*School of Biological Sciences, University of Sydney, Sydney, Australia*

4 [2]*Museum and Institute of Zoology, Polish Academy of Sciences, Warsaw, Poland*

6 Corresponding author: Martyna Molak, Museum and Institute of Zoology, Polish Academy

7 of Sciences, Wilcza 64, 00-679 Warszawa, Poland; Phone: +48226293221; Email:

8 martyna.molak@gmail.com

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11 dependent rates

14 **Abstract**

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16   calibrated by fossil or other evidence. However, estimates of molecular rates in mitochondrial

17   DNA appear to scale negatively with the age of the clock calibration. Although such a pattern

18   has been observed in a limited range of data sets, it has not been studied on a large scale. In

19   addition, there is uncertainty over the temporal extent of the time-dependent pattern in rate

20   estimates. Here we present a meta-analysis of 239 rate estimates from metazoans,

21   representing a range of timescales and taxonomic groups. We found evidence of time-

22   dependent rates in both coding and non-coding mitochondrial markers, in every group of

23   animals that we studied. The negative relationship between the estimated rate and time

24   persisted across a much wider range of calibration times than previously suggested. This

25   indicates that, over long timeframes, purifying selection gives way to mutational saturation as

26   the main driver of the time-dependent biases in rate estimation. The results of our study stress

27   the importance of accounting for time-dependent biases in estimating evolutionary rates using

28   mitochondrial data regardless of the timescale over which they are inferred.

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1. Introduction

Understanding the tempo and mode of the molecular evolutionary process is one of the fundamental goals of biological research. Determining rates and timescales of evolution allows us to explore such questions as the co-evolution of species (Reed et al. 2007), causes of extinction (Lorenzen et al. 2011), and drivers of diversification (Jetz et al. 2012). Molecular estimates of evolutionary timescales are made using the molecular clock, which assumes that DNA evolves at a constant rate among lineages (Zuckerkandl & Pauling 1962). With improving knowledge of the molecular evolutionary process, molecular-clock methods have undergone considerable development over the past five decades (Ho 2014; Kumar 2005), with various forms of rate variation being able to be taken into account (Ho & Duchêne In press).

To estimate evolutionary rates from DNA sequence data, molecular clocks need to be calibrated. This involves using independent information to constrain the age of one or more nodes in a phylogenetic analysis. There are several types of evidence that can provide calibrations for molecular clocks (for a recent review, see Hipsley & Müller 2014). One of the primary sources is the fossil record, which can provide an indication of the first appearance of a lineage and thus place a minimum bound on when it diverged from its sister lineage. The ages of nodes in the tree can also be estimated using biogeographic hypotheses. For example, the rise of barriers to gene flow or new habitats for colonization can provide age estimates for divergences between sister species (e.g., Fleischer et al. 1998).

Over short timescales, molecular clocks can be calibrated by heterochronous sampling or when the ancestry of the samples is known through a pedigree. Heterochronous sampling is common in studies of viruses and of ancient DNA. If the ages of the samples are sufficiently distinct from one another, they can be used to calibrate estimates of evolutionary rates

56 (Drummond et al. 2003; Molak et al. 2013). In pedigree studies, the relationships among

57 samples have been documented, and their divergence times might be known exactly.

58 The most significant challenge to the molecular clock has been widespread evidence of rate

59 variation among lineages. These patterns of rate variation can be partly explained by

60 differences in generation time, longevity, and other life-history characters (Bromham 2009).

61 Recently, however, there has been growing evidence of heterogeneity in rate estimates across

62 timescales. For example, mitochondrial rates estimated in phylogenetic studies are about an

63 order of magnitude lower than those estimated in analyses of pedigrees (Ho & Larson 2006;

64 Howell et al. 2003). More generally, estimated rates appear to be time-dependent, showing a

65 negative relationship with the timescale over which they are measured (Ho et al. 2011; Ho et

66 al. 2005). This pattern has been observed in coding and non-coding mitochondrial DNA from

67 a range of taxa, including humans (Endicott et al. 2009; Henn et al. 2009; Rieux et al. 2014),

68 fish (Burridge et al. 2008; Genner et al. 2007), birds (García-Moreno et al. 2006; Ho et al.

69 2005), and invertebrates (Crandall et al. 2012; Gratton et al. 2008; Ho & Lo 2013;

70 Papadopoulou et al. 2010).

71 Various explanations have been proposed for time-dependent biases in rate estimation. These

72 include natural selection, calibration errors, model misspecification, sequence errors, and

73 biases in phylogenetic estimation and sampling. The first three factors are likely to be the

74 most important (see Ho et al. 2011 for a review). Transient deleterious mutations tend to

75 inflate estimates of rates over short timescales, but they are removed by selection over longer

76 timescales (Duchêne et al. 2014; Soares et al. 2009; Woodhams 2006). Calibration errors

77 mostly derive from the assumption that the genetic split between lineages coincides with the

78 divergence of populations. The impact of calibration error is more severe in short-term rate

79 estimates, where the discrepancy between genetic and population split time constitutes a large

80 proportion of the overall timescale (Ho et al. 2005; Peterson & Masel 2009). Additionally,

81 estimates of rates can be misled if they are obtained using models that provide a poor

82 reflection of real evolutionary and demographic processes (Emerson 2007; Navascues &

83 Emerson 2009; Soubrier et al. 2012). Misspecified models of nucleotide substitution can lead

84 to underestimates of saturation, causing underestimates of rates over long evolutionary

85 timescales (Duchêne et al. 2014; Ho et al. 2007b).

86 The time-dependent biases in molecular rate estimation have important implications for

87 studies of recent evolutionary timescales. For example, the ages of coalescence events within

88 populations will tend to be overestimated if they are based on long-term rates calibrated using

89 fossil data (Ho et al. 2008). This time-dependence, together with substantial variation in rates

90 among different taxa, also calls into question the application of so-called 'standard'

91 mitochondrial rates, such as those estimated in studies of lizards (Macey et al. 1998), birds

92 (Shields & Wilson 1987; Weir & Schluter 2008), mammals (Brown et al. 1979), and

93 arthropods (Brower 1994) and used in many subsequent studies. Time-dependent biases in

94 rate estimation might also contribute to a wider discussion about discrepancies between

95 molecular and palaeontological estimates of divergence times (Benton & Ayala 2003).

96 Although there is a clear discrepancy between rates estimated on short and long timescales,

97 there remains uncertainty about how rates scale with time. A major hindrance has been the

98 paucity of reliable calibrations on intermediate timescales. In this regard, ancient DNA data

99 can be particularly useful in bridging the gap between short- and long-term rate estimates (Ho

100 et al. 2007a). Because ages are assigned directly to sequences in the analysis, no assumptions

101 need to be made about the timing of population splits and their correspondence to genetic

102 divergences (Rambaut 2000). Unfortunately, ancient DNA analyses are constrained by the

103 post-mortem degradation of DNA and the limited reach of radiocarbon dating.

104   Accurate characterization and quantification of time-dependent biases in rate estimation is

105   crucial for understanding their causes and accounting for their effects. Studies of time-

106   dependence in rate estimates have typically involved limited data sets, comprising small

107   numbers of taxa or sequences. This leaves open questions concerning the prevalence and

108   ubiquity of these biases, the timescales across which rate estimates are time-dependent, and

109   the rate of decay between high pedigree rates and low phylogenetic rate estimates.

110   In particular, quantifying the extent of time-dependent rates is important because it can

111   provide insights into its causes. For example, purifying selection is only expected to operate

112   over relatively short timescales, whereas mutational saturation tends to become a problem at

113   greater time depths (Duchêne et al. 2014; Subramanian et al. 2009). On the other hand, if

114   saturation has only a small impact on rate estimates, there should be an upper limit to the

115   timescale over which the biases can be observed. If these limits and the mode of decay of rate

116   estimates can be identified and incorporated into phylogenetic models, the accuracy of the

117   inference of evolutionary processes will be substantially improved.

118   In this study, we aim to characterize the taxonomic breadth and temporal depth of time-

119   dependent rates by performing a meta-analysis of mitochondrial rates estimated from a wide

120   range of amniote and insect taxa. We investigate how these rate estimates depend on the age

121   of the calibrations used to estimate them, how they vary among taxonomic groups and

122   molecular markers, and whether time-dependent biases persist across a broad temporal scale.

123

124   **2. Methods**

125   Our data set comprised published estimates of molecular evolutionary rates, along with the

126   ages of their corresponding calibration points (Table S1). We collected rate estimates for

127 amniotes and insects from recent publications in several leading journals in molecular

128 evolution (Table S1). Because methods used to estimate rates varied widely among studies,

129 we chose the oldest calibration (which we refer to here as the "calibration time") as a proxy

130 for the timescale over which the rate had been estimated.

131 Thirty novel estimates and re-estimates of rates were obtained using published ancient DNA

132 sequences from 16 species (Tables S2). Twelve of these estimates were obtained using

133 ancient DNA as the sole source of calibration. The remaining 18 estimates were obtained

134 from ancient DNA data sets, but with the addition of a single sequence from a sister species

135 and a fossil-based estimate of the divergence time as calibration. Identification of sister

136 species and divergence times was done with reference to published studies (Table S2). One

137 additional rate estimate was obtained using a mitochondrial data set from modern humans

138 and an orangutan, calibrated using fossil evidence of the divergence time between these two

139 species (Table S2).

140 The best-fitting model of nucleotide substitution was chosen for each data set according to

141 the Bayesian Information Criterion using ModelGenerator 0.85 (Keane et al. 2006).

142 Substitution rates were estimated using the Bayesian phylogenetic software BEAST 1.7.2

143 (Drummond & Rambaut 2007). We used a strict clock calibrated only by the ages of the

144 sequences in the analyses of intraspecific data (Brown & Yang 2011; Drummond et al. 2006)

145 and an uncorrelated lognormal relaxed clock when analysing the data sets that included sister

146 species. A uniform prior of 0 to 10^{-4} substitutions/site/year was specified for the mean

147 substitution rate, whereas a 1/x prior was used for the population size in the coalescent prior

148 for the tree. In analyses of data sets that included sister species, an informative prior

149 distribution was specified for the age of the root (Table S2). Posterior distributions of

150 parameters were estimated using Markov chain Monte Carlo sampling, with samples drawn

151 every 10^3 steps over a total of at least 10^7 steps. Some chains were extended to ensure

152 sufficient sampling and effective sample sizes above 100 for all parameters.

153 For each data set the analysis was repeated using Bayesian skyride model with a default

154 gamma precision prior. The fit of the two population models were compared using Bayes

155 factors calculated in Tracer v1.5 (Rambaut & Drummond 2007) and the rate estimate for the

156 model with better fit was used. Median estimates of the strict clock rate for the intraspecific

157 analyses and of the mean rate over the whole inferred phylogeny for the analyses including

158 sister species were taken as the "rate estimates" to be included in all further analyses.

159 We conducted a meta-analysis of all of the collected rate estimates (previously published and

160 generated in this study) by plotting log-transformed rate estimates against log-transformed

161 calibration times. To avoid biasing the regression analysis with multiple estimates for single

162 species, estimates from human pedigree studies were pooled together and used as a single

163 data point during meta-analysis (Table S1). For the same reason, we took the average of the

164 human rate estimates calibrated using the *Homo-Pan* divergence time (Table S1). When a

165 study reported rate estimates from a number of mitochondrial markers for a single species,

166 we took the average of the estimates.

167 We did separate linear regressions for coding and non-coding markers, and for subsets based

168 on taxonomic classes (insects, reptiles, birds, and mammals). We also performed an

169 intraspecific regression on the human data to test for time-dependent rates at the intraspecific

170 level (Table S1). All linear regressions were performed in R (R Development Core Team

171 2013).

172 To test whether the strength of time-dependence varied across timescales, we performed

173 regression analyses for subsets of the data, chosen according to calibration times. We used a

174 sliding window with a width of four orders of magnitude (10^0–10^4 years, 10^1–10^5 years, …,

175   10^5–10^9 years). To investigate the timescale over which time-dependent biases in rate

176   estimation can be observed, we performed regression analyses on subsets of data based on

177   calibrations of 10^6–10^9, 10^7–10^9, and 10^8–10^9 years.

178   For all of our regression analyses, we investigated the effects of spurious self-correlation

179   (Kenney 1982; Sheets & Mitchell 2001) using a randomization test (Jackson & Somers

180   1991). Genetic distances, calculated as substitution rate multiplied by calibration time, were

181   randomized. We recalculated substitution rates using these randomized distances, then

182   performed regression analyses against calibration times. This procedure was repeated 10,000

183   times for each subset of data for which regression analysis was performed. We then tested

184   whether the estimate of the regression slope for the original data fell within the distribution of

185   the estimated slopes for the randomized data. For all data subsets tested, the randomized data

186   produced an average slope of -1. Therefore, we performed an additional test against spurious

187   self-correlation by repeating the regression analysis for each subset of data using a null

188   hypothesis of slope = -1.

189

3. Results

191   Our analyses were based on a data set comprising 239 published, re-estimated, and novel

192   estimates of evolutionary rates in metazoan mitochondrial DNA (Table S1). Rate estimates

193   ranged from 1.78×10^{-10} substitutions/site/year (cytochrome *b* and tRNA genes in woolly

194   mammoth; Rohland et al., 2010) to 2.31×10^{-6} substitutions/site/year (the control region in

195   Adélie penguins; Lambert et al., 2002). Calibration ages ranged from 6 years (pedigree study

196   of Adélie penguins; Millar et al., 2008) to 4×10^8 years (fossil calibration for *Bacillus* stick

197   insects; Plazzi et al., 2011).

198   To test the relationship between mitochondrial rate estimates and calibration ages, we

199   performed separate linear-regression analyses of coding and non-coding regions. All linear

200   regressions were performed using log-transformed values of both estimated rates and

201   calibration times. We found significant correlations between these two variables in coding

202   markers (slope = -0.28, p = 1.58×10^{-12}, R^2 = 0.24) as well as non-coding markers (slope = -

203   0.43, p = 1.38×10^{-12}, R^2 = 0.60) (Figure 1, Table S3).

204   To test whether time-dependent biases were present across the entire timescale that we

205   studied, we performed additional regression analyses for subsets of the data that represented

206   different calibration ages. We found significant non-zero relationships for all time slices

207   containing more than 12 data points (Table 1, Table S4). This includes time depths of $>10^7$

208   years for coding and $>10^6$ years for non-coding markers.

209   We found significant negative relationships between mitochondrial rate estimates and

210   calibration ages in our separate analyses of coding and non-coding markers in mammals,

211   birds, reptiles, and insects (Figure 2, Table S3). However, there were too few non-coding data

212   points for reptiles (2 data points) and insects (0 data points) to allow regression analysis. We

213   also found support for time-dependent rates in our intraspecific analysis of mitochondrial

214   sequence data from humans (Figure S1, Table S3), corroborating results previously published

215   for a smaller data set (Henn et al. 2009).

216   Each of the significant regressions reported above was significantly different from a

217   corresponding regression that was calculated after randomizing the data. In addition, we

218   found that each of our original regressions was significant for a null hypothesis of slope = -1,

219   which was the average slope produced by randomized data (Tables 1, S3 and S4) and

220   matches the expectation for a linear regression between a ratio and its denominator (Kenney

221   1982).

222

4. Discussion

224 Our analyses demonstrate a consistent pattern of time-dependent biases in rate estimates in

225 mitochondrial DNA across metazoan taxa. We observed this pattern across the entire

226 timescale that was analysed, beyond at least 10^6 years in non-coding and 10^7 years in coding

227 markers (Tables 1 and S4). This is in contrast with previous estimates of the temporal depth

228 of time-dependence, which has been variously estimated at a few hundred years (Richards M.

229 in Gibbons 1998), around 50 kyr (Henn et al. 2009), 200 kyr (Burridge et al. 2008), or 1-2

230 Myr (Ho et al. 2005; Papadopoulou et al. 2010). However, our results are consistent with a

231 recent large-scale analysis of substitution rates in viruses, which found evidence of a time-

232 dependent bias in rate estimates across a temporal scale spanning multiple orders of

233 magnitude (Duchêne et al. 2014). Together, these results have considerable implications for

234 studies of divergence times.

235 Our finding of a prolonged time-dependent bias in rate estimates sheds some light on the

236 causes of time-dependent biases in rate estimation. Specifically, the temporal breadth of the

237 relationship between rate and calibration time suggests that more than one factor is

238 responsible. The effects of purifying selection decline over time and thus are not expected to

239 be detectable over long timescales (Nei 1971; Phillips 2009), even though the lack of

240 recombination in mitochondrial DNA can potentially extend the life of deleterious mutations

241 through genetic hitchhiking (Ballard & Kreitman 1995; Birky & Walsh 1988). In turn,

242 mutational saturation is expected to increase over time and, if the available nucleotide

243 substitution models do not correct for multiple hits sufficiently, the rates estimated over

244 longer timescales will be more severely underestimated. Therefore, we propose that purifying

245   selection and mutational saturation are the main drivers of these biases. This is consistent

246   with the patterns found in sequence data from viruses (Duchêne et al. 2014).

247   Signs of a time-dependent bias in rate estimation persisting for more than 10 Myr and in a

248   wide range of taxa also allow us to challenge some other hypotheses about the causes of time-

249   dependent rates. Population fluctuations and other demographic factors (Balloux & Lehmann

250   2012; Henn et al. 2009), which vary among species, cannot fully explain the patterns that we

251   have observed across a diverse range of taxa. Given the temporal depth of time-dependent

252   biases in rate estimation, calibration errors are unlikely to represent a sufficient explanation

253   (Ho et al. 2011; Peterson & Masel 2009). Our data-randomization analyses suggest that the

254   time-dependent pattern of rates is unlikely to be a mathematical artefact. Even if this were the

255   case, however, accounting for time-dependent biases in rate estimates would still be

256   necessary when estimating evolutionary timescales using the molecular clock.

257   Our results also support a gradual decay from high pedigree to low phylogenetic rate

258   estimates through time. Intermediate rate estimates, such as those obtained from ancient DNA

259   studies, have previously been attributed to various methodological biases (Debruyne &

260   Poinar 2009; Navascues & Emerson 2009). However, our results do not indicate that rate

261   estimates based on ancient DNA are anomalous. Additionally, estimates of rates calibrated

262   using biogeographic events in the late Pleistocene and Holocene are comparable to those

263   based on ancient DNA data of similar age. This can be seen, for example, in our intraspecific

264   analysis of rate estimates from humans (Figure S1, Table S1).

265   One of the disadvantages of our meta-analysis is the paucity of mitochondrial rate estimates

266   based on young calibrations for coding markers and for insects. Although the uneven

267   representation of taxonomic classes on different timescales might affect the regression

268   analysis that includes all data points, our analyses of class-specific subsets of data yielded

269 significant evidence of time-dependent trends. One other potential source of bias in the meta-

270 analysis is that studies of evolutionary processes on short timescales tend to focus on fast-

271 evolving taxa, whereas studies of longer timescales have a greater focus on slowly evolving

272 organisms (Sheets & Mitchell 2001). This, however, can be rejected as a major cause for the

273 biases based on the observed intraspecific time-dependent variation of estimated rates, which

274 was shown previously (Duchêne et al. 2014; Henn et al. 2009) and in this study (Figures S1,

275 Table S3).

276 Our results confirm that considerable caution needs to be exercised in molecular studies of

277 evolutionary timescales. The need to correct for time-dependent biases in rate estimates has

278 been pointed out previously (Ho et al. 2005). As the variability of regression slopes in our

279 study shows, however, there is no universal function that can be applied across taxa and

280 markers. Although a few studies have proposed corrections for specific taxa (Cutter 2008;

281 Gignoux et al. 2011; Obbard et al. 2012; Soares et al. 2013; Soares et al. 2009), they focused

282 on the effects of purifying selection. Additionally, it is not clear to what extent they can be

283 extrapolated to other taxa. Ultimately, it might be best to only use calibrations that are

284 reasonably close in time to the evolutionary events of interest (Ho & Larson 2006).

285 In view of the prevalence of time-dependent biases in estimates of mitochondrial rates,

286 neutrally evolving nuclear loci might be a preferable alternative to the mitochondrial genome

287 for estimating evolutionary timescales. However, there has been compelling evidence of

288 time-dependent biases in estimates of nuclear rates in some organisms, including

289 *Caenorhabditis elegans* (Denver et al. 2004) and *Drosophila melanogaster* (Haag-Liautard et

290 al. 2007). In contrast, studies of human nuclear genomes have yielded lower estimates of

291 rates from pedigrees than from fossil-calibrated comparisons with chimpanzees (Roach et al.

292 2010; Xue et al. 2009). This represents a prominent anomaly against the broad time-

293 dependent biases seen in other estimates of evolutionary rates. It remains to be seen whether

294 is the results from human genomes are representative of a broader trend among nuclear

295 genomes (Scally & Durbin 2012), and further work will elucidate its impacts on the use of

296 nuclear markers in molecular-clock analyses.

297

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301

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504   Figure captions:

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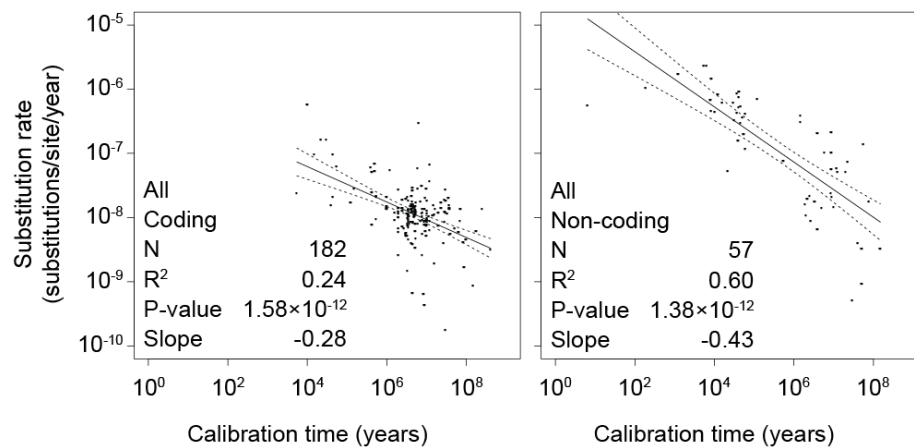
506   **Figure 1** Linear regressions of log-transformed rate estimates from mitochondrial markers in

507   a range of metazoan taxa against the log-transformed calibration times that were used to

508   estimate the rates. Separate analyses were performed for coding (left) and non-coding (right)

509   markers.

510
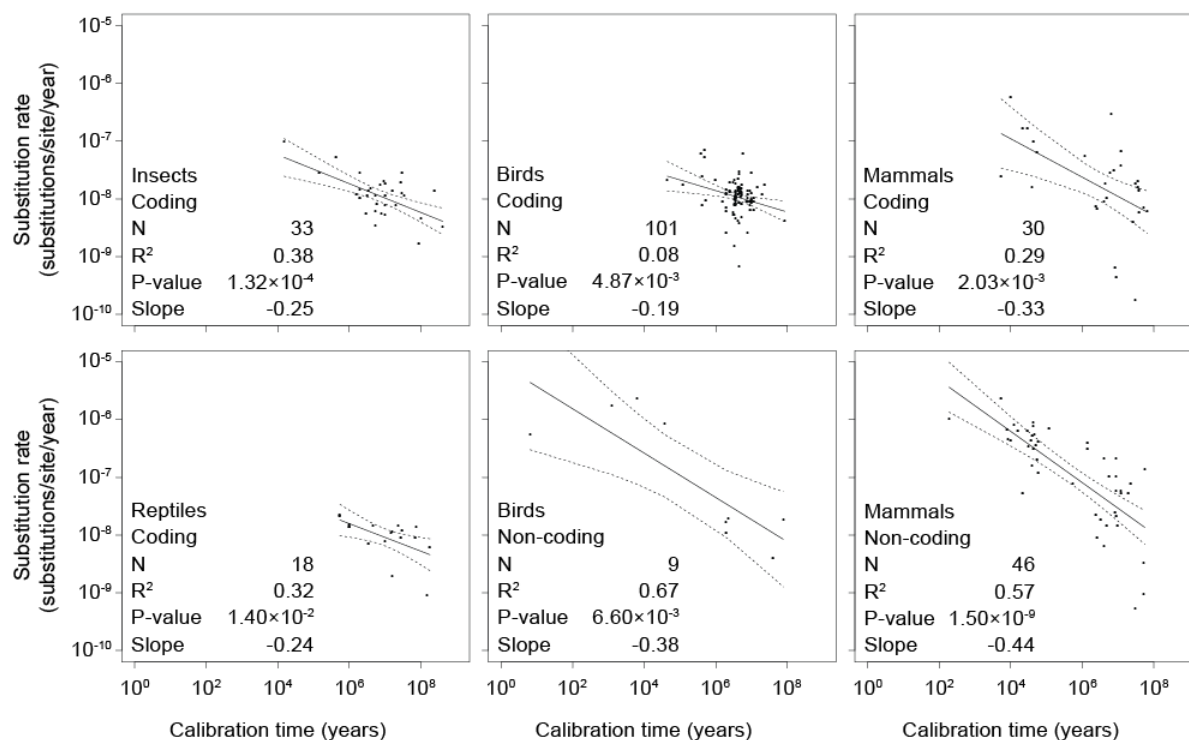
Figure 2 Linear regressions of log-transformed rate estimates against log-transformed

calibration times used for their estimation for different taxonomic groups (insects, reptiles,

birds, and mammals) and mitochondrial marker types (coding and non-coding). There were

insufficient data for regression analyses of non-coding markers in reptiles and insects.

518   Tables:

519   **Table 1** Linear-regression analysis of data points from different time slices using a sliding

520   window with a width of four orders of magnitude, or with a shrinking window for the oldest

521   calibrations. Significant regression results (p≤0.05) are shown in bold. A more detailed

522   version of this table is available as supplementary material (Table S4).

		calibration ages								
		10^0-10^4 years	10^1-10^5 years	10^2-10^6 years	10^3-10^7 years	10^4-10^8 years	10^5-10^9 years	10^6-10^9 years	10^7-10^9 years	10^8-10^9 years
coding markers	N	1	9	20	132	176	173	162	50	5
	R^2	0	0.05	0.24	0.31	0.23	0.09	0.04	0.11	0.04
	P-value	n/a	5.67×10^{-1}	**2.67×10^{-2}**	**3.43×10^{-12}**	**1.48×10^{-11}**	**4.79×10^{-5}**	**1.26×10^{-2}**	**1.76×10^{-2}**	7.6×10^{-1}
	Slope	n/a	-0.33	-0.34	-0.37	-0.29	-0.20	-0.15	-0.34	0.36
	Non-SSC[a]	n/a	-	***	***	***	***	***	***	-
non-coding markers	N	8	25	27	43	48	31	29	12	1
	R^2	0.12	0.27	0.33	0.62	0.55	0.27	0.17	0.16	0
	P-value	4.09×10^{-1}	**7.41×10^{-3}**	**1.80×10^{-3}**	**4.47×10^{-10}**	**1.41×10^{-9}**	**2.58×10^{-3}**	**2.55×10^{-2}**	2.00×10^{-1}	n/a
	Slope	0.08	-0.34	-0.36	-0.48	-0.49	-0.58	-0.53	-0.89	n/a
	Non-SSC[a]	**	***	***	***	***	*	*	-	n/a

523   [a]Results of tests against spurious self-correlation (non-SSC). Higher p-value from the two

524   tests is shown: * p<0.05, ** p<0.005, *** p<0.0005.

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