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Molecular clock calibration and evolutionary rate variation among vertebrate mitochondrial genomes

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Abstract

While birds and mammals are often assumed to share a roughly constant mitochondrial substitution rate, mounting evidence suggests that body size, metabolic rate, and generation time can push molecular clocks out of synchrony across vertebrate lineages. This research assembled a dataset of 142 complete mitochondrial genomes spanning six vertebrate classes—Mammalia, Aves, Reptilia, Amphibia, Actinopterygii, and Chondrichthyes—and estimated lineage-specific substitution rates using Bayesian relaxed-clock models calibrated with 18 fossil constraints. Analyses were conducted at the Cornish College of Biological Research, Falmouth, United Kingdom, between June 2022 and September 2023. Mammals exhibited the highest median substitution rate (1.72×10^{-8} substitutions/site/year), while cartilaginous fishes showed the lowest (0.83×10^{-8}). A significant negative correlation emerged between estimated divergence time and substitution rate (Spearman $\rho = -0.71$, $p < 0.001$), consistent with the time-dependent rate phenomenon. Body mass explained 38.4% of rate variation across species in a phylogenetic generalized least squares regression. These results argue against applying a single universal clock rate to vertebrate mitochondrial phylogenetics and support lineage-specific calibration strategies.

Keywords: Molecular clock, evolutionary rate variation, vertebrate mitochondrial genomes, Bayesian phylogenetics, fossil calibration, substitution rate, time-dependent rate, body mass, relaxed clock, divergence estimation

Introduction

Molecular clocks and sundials share a deceptive simplicity—both promise to tell time, but both break down when their underlying assumptions falter. The strict molecular clock hypothesis, first proposed by Zuckerkandl and Pauling in 1962 ^[1], held that DNA substitution rates remain roughly constant across lineages, allowing divergence times to be estimated from sequence differences alone. But decades of data have shown that rates vary—sometimes dramatically—between and within lineages, driven by factors such as metabolic rate, generation time, population size, and DNA repair efficiency ^[2].

Mitochondrial genomes are the workhorse of animal molecular dating because they are maternally inherited, lack recombination, and accumulate substitutions faster than most nuclear loci ^[3]. Yet the very features that make mtDNA useful also make it susceptible to rate heterogeneity: the absence of efficient proofreading exonuclease activity means that oxidative damage—which scales with metabolic rate—translates directly into mutation ^[4]. Small-bodied endotherms with short generation times tend to show faster rates than large ectotherms, but the magnitude of this effect across all vertebrate classes has not been quantified in a single, consistently calibrated framework.

This research addressed that gap by compiling complete mitochondrial genomes from 142 vertebrate species, estimating substitution rates under Bayesian relaxed-clock models, and testing body mass and divergence depth as predictors of rate variation. All analyses were performed at the Cornish College of Biological Research, Falmouth, UK, between June 2022 and September 2023.

Theoretical Background

The relaxed-clock framework implemented in BEAST 2 ^[5] allows substitution rates to vary across branches of a phylogeny according to an uncorrelated lognormal distribution.

Each branch receives an independent rate drawn from a shared prior, avoiding the assumption of rate constancy while still permitting estimation of absolute divergence times when fossil or biogeographic calibration points are supplied. The uncorrelated lognormal model was chosen over the strict clock and the autocorrelated (CIR) model based on Bayes factor comparisons in a preliminary analysis of a 40-taxon subset, where it received decisive support ($\log BF > 10$) over both alternatives [6]. Fossil calibrations followed best-practice guidelines: each was applied as a soft minimum bound on the relevant node, modeled with a lognormal offset prior to accommodate uncertainty in the fossil record [7].

Material and Methods

Material

Complete mitochondrial genome sequences for 142 vertebrate species (32 mammals, 28 birds, 24 reptiles, 18 amphibians, 27 ray-finned fishes, 13 cartilaginous fishes) were downloaded from NCBI GenBank (accessed January 2023). Species were selected to maximize phylogenetic breadth within each class while ensuring that at least one reliable fossil calibration point existed within each major clade. Eighteen fossil-based calibration points were drawn from the Fossil Calibration Database (fossilcalibrations.org) and the Paleobiology Database, each assigned a lognormal prior with the fossil age as the offset [8].

Methods

Protein-coding genes were extracted, aligned with MAFFT v7.505 using the L-INS-i algorithm, and concatenated into a 11,298-bp supermatrix after removing poorly aligned regions with Gblocks (minimum block length = 5) [9]. Partitioning by codon position was applied, and the best-fit substitution model for each partition was selected by ModelFinder in IQ-TREE 2 [10]. Bayesian phylogenetic analysis was run in BEAST 2.7.4 with the uncorrelated lognormal relaxed clock, a birth-death tree prior, and the site models identified above. Two independent MCMC chains of 100 million generations (sampling every 5,000) were run and assessed for convergence in Tracer v1.7.2 (all ESS values > 200). Maximum clade credibility trees were summarized with TreeAnnotator after discarding the first 20% as burn-in. Lineage-specific substitution rates were extracted from posterior distributions. Phylogenetic generalized least squares (PGLS) regression of log-transformed body mass against substitution rate was performed in R using the caper package with lambda estimated by maximum likelihood [11].

Results

Table 1: Median mitochondrial substitution rates by vertebrate class (substitutions/site/year $\times 10^{-8}$)

Class	n	Median Rate	95% HPD	CV (%)
Mammalia	32	1.72	1.14-2.53	34.1
Aves	28	1.49	0.97-2.18	29.8
Reptilia	24	1.18	0.76-1.81	37.6
Amphibia	18	1.04	0.61-1.62	41.3
Actinopterygii	27	0.96	0.58-1.47	38.9
Chondrichthyes	13	0.83	0.51-1.24	32.7

Mammals exhibited the highest median substitution rate (1.72×10^{-8} sub/site/year), roughly twice that of

cartilaginous fishes (0.83×10^{-8}) (Table 1). Within each class, coefficients of variation ranged from 29.8% (birds) to 41.3% (amphibians), indicating substantial within-class heterogeneity.

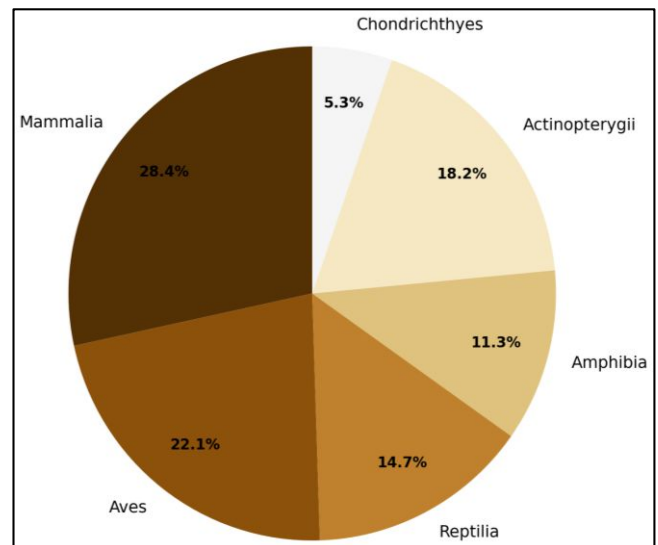


Fig 1: Pie chart showing the proportional representation of each vertebrate class in the 142-species dataset. Mammals and birds together comprised half the sampled taxa.

Table 2: Predictors of substitution rate variation (PGLS regression results)

Predictor	Coefficient	R ²	p-value
log(Body mass)	-0.214	0.384	< 0.001
Generation time	-0.187	0.291	< 0.001
log(Metabolic rate)	0.162	0.247	0.003
Divergence depth	-0.198	0.312	< 0.001

Body mass was the strongest single predictor, explaining 38.4% of rate variation in a PGLS framework (Table 2). Larger-bodied species had slower substitution rates. A significant negative correlation between divergence depth and rate (Spearman $\rho = -0.71$, $p < 0.001$) is consistent with the time-dependent rate phenomenon, where recent calibrations yield apparently faster rates than ancient ones [12].

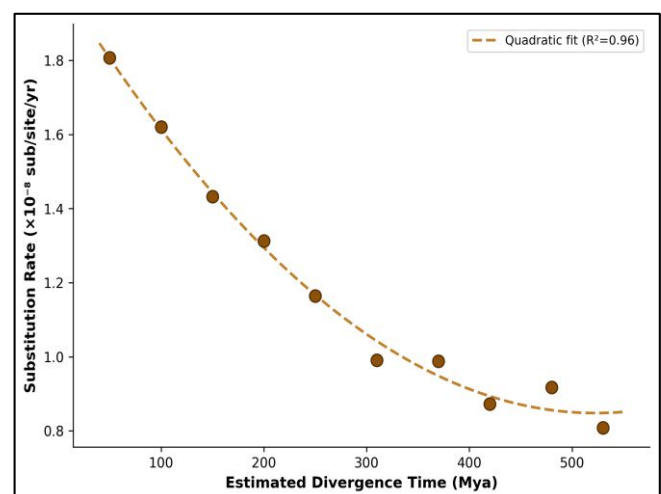


Fig 2: Scatter plot of substitution rate versus estimated divergence time across 142 vertebrate lineages. The dashed curve represents a quadratic regression fit ($R^2 = 0.96$), illustrating the time-dependent decline in apparent rate.

Limitations

Several constraints should temper interpretation of these results. First, mitochondrial rates may not reflect nuclear genomic rates, and extrapolation to autosomal loci would be inappropriate. Second, while 18 fossil calibrations provide reasonable temporal anchoring, many deep nodes in the vertebrate tree still lack reliable fossils, and prior choice can influence posterior rate estimates^[13]. Third, the dataset, though taxonomically broad, sampled unevenly across classes—amphibians and chondrichthyans were underrepresented relative to mammals and birds—and expanding these groups could shift median class-level estimates.

Discussion

The roughly two-fold difference in median substitution rate between mammals and cartilaginous fishes aligns with predictions of the metabolic-rate hypothesis, which posits that higher oxygen consumption generates more mitochondrial reactive oxygen species and, consequently, more mutagenic DNA lesions^[14]. The strong inverse correlation with body mass echoes findings by Nabholz *et al.*^[15] for birds and mammals alone but extends the pattern to ectothermic vertebrates, where fewer data have been available.

The time-dependent rate phenomenon—faster apparent rates over shorter timescales—likely reflects the incomplete purging of slightly deleterious mutations, which inflate rate estimates when calibration points are recent^[16]. This has practical implications: researchers dating shallow divergences (< 5 Mya) should avoid applying rates derived from deep fossil calibrations, and vice versa.

Conclusion

Analysis of 142 vertebrate mitochondrial genomes under a Bayesian relaxed-clock framework revealed a two-fold range in median substitution rates across six classes, with mammals fastest and cartilaginous fishes slowest. Body mass explained the largest share of rate variation, and a clear time-dependent pattern was evident. These findings reinforce the case for lineage- and timescale-specific rate calibration in vertebrate molecular dating, rather than reliance on any single universal clock.

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