Mitochondrial genome evolution: the influence of partitioning, calibration, and gene heterogeneity on pleurodontan substitution rates

Running title: Pleurodontan mitogenome substitution rates

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Abstract

Substitution rate estimates are a key source of information in modern evolutionary biology, underpinning divergence time inference and other evolutionary analyses. Mitochondrial DNA nucleotide substitution rates, in particular, are commonly used for these purposes. However, these rates are typically derived from a small set of genes, closely related species, or from a limited number of model organisms. Such limitations become increasingly problematic at deeper phylogenetic levels, where errors in rate estimates and divergence times tend to accumulate with evolutionary distance. Here, we use nearly complete mitogenomes of 27 pleurodontan (Squamata: Pleurodonta) species to estimate substitution rates for the whole clade, paying special attention to the effect of data partitioning, calibrations and model choices on these estimations. The substitution rate estimates we obtained are consistent with previous findings for specific lineages within the group. Rates across individual genes ranged from approximately 0.004 to 0.02 substitutions/site/million years, with notable differences between coding and non-coding regions, and among codon positions. Calibrations had a less pronounced effect on the analyses than anticipated, although subtle differences were

observed. These findings underscore the challenges of estimating targeted nucleotide substitution rates, especially for lineages with limited genomic data, as is the case for several Squamata lineages. Moreover, the results provide valuable insights into the evolutionary dynamics of Pleurodonta and emphasize the importance of incorporating robust data and models to improve accuracy in substitution rates and divergence time estimations.

Key words: divergence time, mitogenome, molecular evolution, phylogenomics, Squamata.

Introduction

Genomic datasets are essential for addressing complex questions in modern evolutionary biology. In this context, substitution rate estimates are a cornerstone, providing critical insights into molecular evolution and serving as a foundation for various applications. For instance, in the absence of fossils or other secondary calibration points, substitution rates often represent the main available data for estimating divergence times (e.g., Ho, 2007; Arcones et al., 2021). Mitochondrial DNA (mtDNA), in particular, has long been used for this purpose, mainly due to its relatively stable coding function, high mutation rates, small effective population size, matrilineal inheritance, and relatively fast coalescent times (Avise et al., 1987; Ballard & Rand, 2005). Besides, mitochondrial proteins play a critical role in the oxidative phosphorylation pathway and exhibit functional conservation across different metazoan lineages (Gray et al., 1999; Broughton & Reneau, 2006). Consequently, the accuracy of mitochondrial substitution rate estimates is fundamental to advancing evolutionary biology.

Substitution rates vary considerably across the mitochondrial genome and among different taxonomic groups. Empirical studies have revealed substantial variation among different mitochondrial genes (Williams & Hurst, 2002; Sloan et al., 2009; Pons et al., 2010; Duchêne et al., 2011; Zhu et al., 2014; Yang et al., 2018) as well as across lineages (Parkinson et al., 2005; Bininda-Emonds, 2007; Mower et al., 2007; Nabholz et al., 2008; Welch et al., 2008; Eo & DeWoody, 2010; Yan et al., 2021). Importantly, many studies have historically relied on a limited fraction of the

mitogenome—primarily cytochrome b, cytochrome c oxidase I, II, and III, and the 12S and 16S ribosomal RNAs (Johns & Avise, 1998; Hebert et al., 2003; Roe & Sperling, 2007; Patwardhan et al., 2014)—and have been based on a few model organisms, typically at the intraspecific level or between closely related species (Avise et al., 1987; Ballard & Whitlock, 2004; Funk & Omland, 2005; Ballard & Rand, 2005; Rubinoff & Holland, 2005).

Consequently, despite their widespread use, molecular clock approaches based on mtDNA have important practical limitations. Overlooking those variations can introduce substantial biases in substitution rate estimates, posing challenges for accurate evolutionary inference. This is particularly concerning in deep-level phylogenies, where errors in phylogenetic inference tend to amplify with increasing branch length (Buckley, 2002; Lemmon et al., 2009). To mitigate this problem, some studies have attempted to calibrate molecular rates using complete (or nearly complete) mitogenomes, across different groups (Pons et al., 2010; Park et al., 2012; Plazzi et al., 2016; Mackiewicz et al., 2022). This specificity is crucial, as accurate divergence time estimates rely on the precision and accuracy of calibration points and the rates applied to each marker and lineage under investigation (Mello & Schrago, 2014; Zheng & Wiens, 2015; Ritchie et al., 2017; Smith et al., 2018). Also, effective calibrations help to counteract errors arising from clock model misspecification (Duchêne et al., 2014).

Squamates (lizards, snakes, and amphisbaenians; Order Squamata) form a globally distributed clade of reptiles comprising approximately 11,000 extant species (Simões & Pyron, 2021; Uetz et al., 2025), making them one of the most diverse vertebrate orders (Uetz et al., 2021). Despite recent advancements in next-generation sequencing, squamates remain underrepresented in genomic research compared to mammals and birds (Feng et al., 2020; Genereux et al., 2020; Gable et al., 2023). This limited genomic data availability hinders a comprehensive understanding of key evolutionary parameters within the group, including substitution rates. In particular, the Pleurodonta clade (the main focus of this study) encompasses a wide range of taxa predominantly distributed throughout the New World, with desert iguanas, horned, spiny, and collared lizards dominating many

modern squamate faunas in North and South America (Pianka and Vitt, 2003; Losos, 2011; Avila et al., 2013; Carvalho et al., 2013). Although Pleurodontan evolutionary history is marked by multiple adaptive radiations in response to varied ecological pressures (Blankers et al., 2013; Alencar et al., 2024), mitochondrial evolutionary parameters remain scarce for the group. Commonly used substitution rate values broadly range from 0.005 to 0.02 substitutions per site per lineage per million years (subs/site/MY), depending on the gene (e.g., Zarza et al., 2008; Chan et al., 2012; Fontanella et al., 2012; Olave et al., 2015; Werneck et al., 2015; Román-Palacios et al., 2018; Bernardo et al., 2019; Camurugi et al., 2022; Carvalho et al., 2024; Rogers et al., 2024). However, as in most vertebrate groups, these estimates are often based on a limited number of species, typically at shallow evolutionary scales, and frequently rely on a small set of mitochondrial genes.

To address this issue, we integrated recently sequenced mitochondrial data with existing mitogenomic data to conduct comprehensive phylogenetic analyses, assessing evolutionary rate variation among Pleurodonta mitochondrial genes. Specifically, we analyzed their mitochondrial genomes to estimate its mitochondrial substitution rates. Using fossil-calibrated Bayesian phylogenetic analyses, we inferred molecular evolutionary rates across several families and characterized new nearly complete mitogenomes for seven *Tropidurus* species: *T. guarani*, *T. melanopleurus*, *T. sp. nov.* (species currently under formal description), *T. spinulosus*, *T. tarara*, *T. teyumirim*, and *T. xanthochilus*. We expect that these newly estimated rates will improve the precision of molecular clock dating and evolutionary inferences in squamates, offering deeper insights into the evolutionary processes influencing biodiversity patterns in this group.

Methods

We assembled a comprehensive dataset of Pleurodontan mitochondrial genomes available from GenBank by November 2024, including seven recently described sequences from different *Tropidurus* species (Salles et al., 2025). One Chamaleonidae species (*Calluma parsonii*) was included as an outgroup, resulting in a final dataset with 28 species (Table 1). Only coding regions (13 genes)

and the two mitochondrially encoded ribosomal RNAs (12 and 16s) were used. We excluded additional mtDNA markers because they represent regions that are either non-coding and hyper-variable (D-loop) or ultra-conserved (tRNAs), and therefore inadequate for molecular clock calibrations. We separately aligned each mitochondrial gene with MAFFT v7.471 (Katoh & Standley, 2013) using specific customized settings (-globalpair, --maxiterate 1000, --adjustdirection). Alignments were broadly examined by eye, and AMAS (Borowiec, 2016) was used to concatenate alignments and compute final summary statistics.

Table 1. Species used in all analyses in the present study. New mitochondrial genomes are in bold.

Species	Family	GenBank accession number
Calluma parsonii	Chamaeleonidae	AB474915
Basiliscus vittatus	Corytophanidae	AB218883
Amblyrhynchus cristatus	Iguanidae	NC_028031
Conolophus subcristatus		NC_028030
Cyclura pinguis		NC_027089
Iguana delicatissima		NC_044899
Iguana iguana		NC_002793
Leiocephalus personatus	Leiocephalidae	AB266739
Liolaemus darwinii	Liolaemidae	NC_057242
Liolaemus millcayac		NC_057243
Liolaemus parthenos		NC_057244
Chalarodon madagascariensis	Opluridae	AB266748
Oplurus grandidieri		AB218720
Holbrookia lacerata	Phyrnosomatidae	NC_041001
Phrynosoma blainvillii		NC_036492
Sceloporus occidentalis		AB079242
Urosaurus nigricaudus		NC_026308

Anolis punctatus		NC_044125
Anolis cybotes	Polychrotidae*	AB218960
Polychrus marmoratus		AB266749
Plica plica		AB218961
Tropidurus guarani		*will be submitted to genbank*
Tropidurus melanopleurus		*will be submitted to genbank*
Tropidurus sp. nov.	Tropiduridae	*will be submitted to genbank*
Tropidurus spinulosus		*will be submitted to genbank*
Tropidurus tarara		*will be submitted to genbank*
Tropidurus teyumirim		*will be submitted to genbank*
Tropidurus xanthochilus		*will be submitted to genbank*

* Traditionally, *Anolis* was classified within Polychrotidae. However, molecular phylogenetic studies have led to a major taxonomic reassessment. Recent evidence supports placing *Anolis* and related genera within Dactyloidae, rendering Polychrotidae paraphyletic or obsolete. While some taxonomic authorities now recognize Dactyloidae, references to Polychrotidae persist in the literature. Our option here was to consider *Anolis* and *Polychrus* to form a distinct phylogenetic group, despite of their taxonomical status. The group monophyly was not enforced and, hence, taxonomic arrangements had no influence in our analyses.

Effect of calibration points on substitution rate estimates

We implemented different calibration strategies to understand its possible effects on substitution rate estimates. Specifically, estimates were obtained separately through calibrated and non-calibrated analyses. Calibration points within the Pleurodonta clade were obtained consulting the specialized literature, prioritizing those that have been used in multiple evolutionary studies, and which are broadly supported by the fossil record (Table 2). Some possibly accurate calibrations, also commonly cited in the literature, but for groups whose monophyly is still under debate, were not used here, as monophyly was enforced for each calibrated node. We also note that estimating a fully resolved topology or divergence times for the entire group was not our primary objective, as the species included in this study represent only a limited sample of Pleurodontan diversity and exclude some of the group's most representative lineages.

Table 2. Values (million years, MY) of uniformly distributed calibration priors applied in dating analyses, based on both fossil and molecular data. Settings for calibration Bayesian prior mean, standard deviation and offset are provided. MRCA = most recent common ancestor.

Calibrated node (MRCA prior)	Species included	Lower value	Upper value	Offset	References
Pleurodonta	All except outgroup (*Calluma parsonii*)	65	85	0.5	Conrad & Norell (2007); Townsend et al. (2011); Prates et al. (2015); Scarpetta (2019)
Anolis	*Anolis cybotes, Anolis punctatus*	40	60	0.5	Sherratt et al. (2015); Zheng & Wiens (2016); Román-Palacios et al. (2018)
Phrynosomatidae	*Holbrookia lacerata, Phrysonoma blainvillii, Sceloporus occidentalis, Urosaurus nigricaudus*	35	55	0.5	Townsend et al. (2011); Leaché & Linkem (2015); Zheng & Wiens (2016)
Liolaemus 2	*Liolaemus darwinii, Liolaemus parthenos, Liolaemus millcayac*	30	45	0.5	Portelli et al. (2022)
Liolaemus 1	*Liolaemus darwinii, Liolaemus parthenos*	10	25	0.5	Fontanella et al. (2012); Portelli et al. (2022)

Bayesian estimation of mitochondrial nucleotide evolution rates

For each mitochondrial partition, mean nucleotide substitution rates were estimated using BEAST v2.7 (Drummond & Rambaut, 2007), applying a relaxed molecular clock with an uncorrelated log-normal distribution (*ucld*) and either a Yule or Calibrated Yule speciation model, depending on the test. The relaxed *ucld*-model assumes independent substitution rates across branches, as there is no assumed correlation between the rate of a given lineage and that of its ancestor. This model requires a prior for the mean clock rate. For coding sequences, we set the mean clock rate to 0.01 substitutions/site/MY, and for rRNAs, to 0.0055, based on prior estimates for various Pleurodonta species (Supporting Table S1). A normal distribution was used for the *ucld* mean rate prior, with the above values as the mean, a standard deviation (Sigma) of 0.005 for coding sequences and 0.0015 for rRNAs, with these same values used as the Offset. These hyperprior values

(Sigma and Offset) were determined based on preliminary analyses to ensure appropriate parameterization, and also computational and statistical demands.

For each calibration scheme (whether or not it included calibration points), we estimated substitution rates for each mitochondrial gene and for each codon position within protein-coding sequences. Tree topologies were linked across partitions, while clock models were unlinked both between genes and among codon positions within genes. Site models were linked across codon positions within individual genes but unlinked between genes, with model selection performed using BEAST Model Test (bModelTest; Bouckaert and Drummond, 2017) under the 'namedExtended' model set. Uncalibrated analyses consisted of two independent MCMC runs of 500 million generations each, with parameters sampled every 25,000 generations. Calibrated analyses followed the same sampling scheme, but each run was extended to 850 million generations. Convergence of all parameters was verified using Tracer v1.4 (Rambaut et al., 2007), ensuring effective sample sizes (ESS) ≥ 200 whenever possible. In summary, we performed four BEAST analyses (two calibrated and two uncalibrated) and reported the final results as the combination of two runs per analysis using LogCombiner (Rambaut and Drummond 2014).

Results

Alignments and evolutionary models

The alignment of protein-coding sequences alone comprised 11,426 bp, while the inclusion of non-coding sequences increased the total length to 14,059 bp. All coding genes exhibited multiple substitution models within the 95% highest posterior density (HPD) interval estimated through the bModelTest. Only the two rRNAs had a single best-fitting model to explain site substitution, specifically the GTR model (Supporting Table S2).

Substitution rates

Analysis of substitution rates across codon positions revealed heterogeneity in evolutionary rates across the mitochondrial genome of Pleurodontans; 95% posterior distributions of substitution rates for each gene can be observed in Fig. 1. When considering only protein-coding sequences, substitution rates appear relatively homogeneous across genes, with substantial overlap of the HPD intervals (Fig. 1A). Conversely, coding and non-coding regions exhibit markedly different substitution rates, with non-coding regions evolving approximately ten times slower (Fig. 1B). Calibrated analysis (for all genes) has consistently shown similar rates to non-calibrated ones, but with slightly smaller estimates. Median substitution rate estimates for individual genes, based exclusively on third codon positions, are presented in Table 3 (estimates for all codon positions are available in the original BEAST output files archived on Zenodo). In the case of non-calibrated analysis, the fastest mean rate was observed for ND4 and the slowest for *12s* and *16s*. Regarding calibrated analysis, rRNAs also exhibited the lowest estimates, but in this case ND2 presented the higher value.

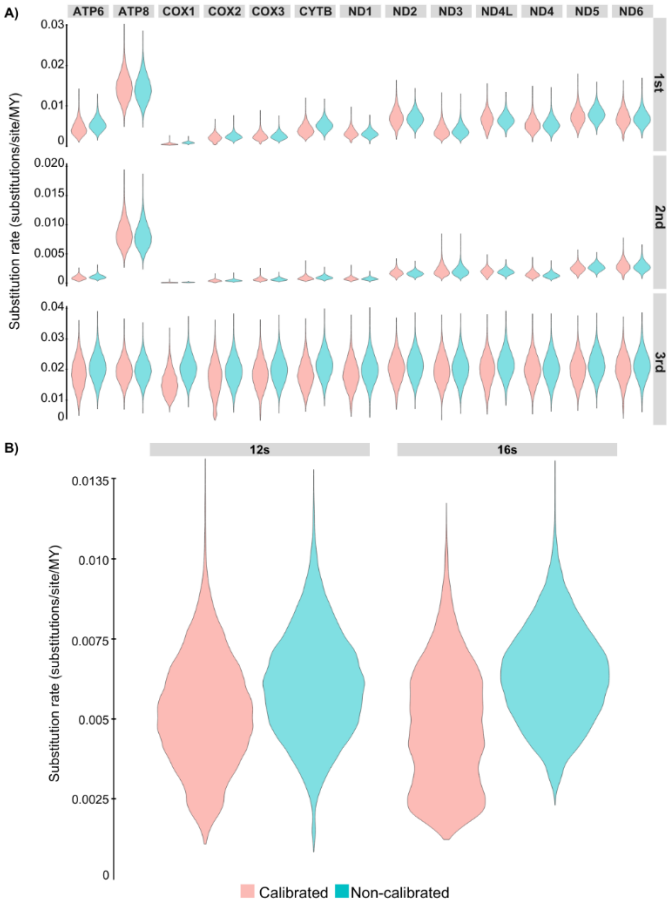

Fig. 1. Posterior distributions of mitochondrial substitution rates (substitutions/site/MY) from calibrated (pink) and non-calibrated (blue) analyses. **(A)** Violin plots for 13 protein-coding genes, illustrating the range and density of estimated rates for each codons position **(B)** Violin plots for two rRNA genes. In both cases, the width of each violin indicates the distribution density, and horizontal lines represent median values.

Table 3. Nucleotide substitution rates per site per million years estimated from 13 mitochondrial protein-coding genes (3rd codon position) and 2 rRNAs across 27 Pleurodontan species plus one outgroup. These rates were inferred using BEAST with a relaxed clock model assuming a lognormal distribution. The reported values represent the combined results from two independent runs.

Gene	Non-calibrated		Calibrated	
	ucld mean rate	Stdev	ucld mean rate	Stdev
12s	0.00598	0.0016623	0.00518	0.0017229
16s	0.00632	0.0015115	0.00468	0.0019285
ATP6	0.02059	0.0044696	0.01861	0.0049339
ATP8	0.01977	0.0038046	0.01958	0.0038725
COX1	0.02036	0.0039585	0.01578	0.0042038
COX2	0.01945	0.0055384	0.01706	0.0055097
COX3	0.01995	0.0043769	0.01836	0.0048264
CYTB	0.02136	0.0042930	0.01840	0.0045799
ND1	0.02011	0.0045590	0.01826	0.0047083
ND2	0.02114	0.0043318	0.02025	0.0044484
ND3	0.02049	0.0044802	0.01977	0.0046298
ND4	0.02151	0.0042928	0.01986	0.0048263
ND4L	0.02036	0.0045192	0.01996	0.0045306
ND5	0.02157	0.0042466	0.01991	0.0044204
ND6	0.02121	0.0044322	0.02006	0.0048847

Discussion

The influence of different partitioning schemes on the estimation of substitution rates

Our data support the widely accepted theory that nucleotides at the third codon position evolve at distinct rates and through different mechanisms compared to those at the first and second codon positions (Kimura, 1980). In practical terms, this also means that, within a gene, first codon positions are expected to evolve more similarly to other first codon than to second or third positions, with the same reasoning applying to each position (Bofkin & Goldman, 2007)—which is exactly what we detected here (Fig. 1). Thus, we emphasize that any studies drawing inferences from mitochondrial data must carefully account for the inherent heterogeneity in the composition of each gene, as ignoring this fact can introduce different phylogenetic artifacts (Hassanin, 2006).

In this context, codon-position models offer a more robust framework for capturing the evolution of coding sequences in most multiple sequence alignments. By accounting for site heterogeneity and other evolutionary parameters, these models should provide greater accuracy and biological relevance compared to simpler alternatives. Consequently, substitution model testing can be a fundamental approach on this regard, and our results once again reveal notable patterns. We observed substantial differences in the evolutionary models best suited for each mitochondrial gene, including complex models that consider heterogeneity both in rates and nucleotide frequencies (Table S2), reflecting the inherent heterogeneity in substitution rates across the mitogenome. While the limitations of using overly simplistic evolutionary models may vary depending on the dataset, simpler models might invariably misestimate different evolutionary parameters by failing to account for the occurrence of multiple substitutions at the same site (Yang & Nielsen, 2000; Anisimova & Kosiol, 2009; Duchêne et al., 2014)—which can also lead to errors in phylogenetic inferences (Buckley et al., 2001; Su et al., 2014).

Although the mitogenome evolves as a single non-recombining unit, and typically exhibits a largely consistent phylogenetic signal across genes, our results also align with established evidence that evolutionary pressures act differentially on individual mitochondrial genes (Saccone et al., 1999;

Xu et al., 2006). While substitution rates showed broad similarity across the mitogenome, some degree of heterogeneity was observed among specific genes. In the calibrated analyses, genes such as ND2 and ND6 had substitution rates above 0.02 substitutions/site/MY, while others like COX1 and COX2 generally ranged between 0.015 and 0.017. These findings highlight the importance of using partitioned analyses that account for both site- and gene-specific rate variation, along with appropriately selected substitution models, given the observed heterogeneity across loci and codon positions (Table S2). Such approaches are critical for improving the precision of evolutionary inferences, including divergence time estimation and substitution rate calibration.

Substitution rates heterogeneity depending on the presence of calibration points

Our study also evaluated the influence of temporal calibrations on Pleurodontan mitochondrial substitution rate estimates. Analyses incorporating fossil calibrations yielded estimates slightly lower than those from uncalibrated analyses (Table 3), consistent with evidence that well-constrained calibrations reduce biases in molecular dating (Hipsley & Muller, 2014; Warnock et al., 2015). This underscores the importance of integrating multiple fossil calibrations, particularly at deep nodes, to improve the accuracy of divergence time inferences—a critical consideration for groups like Pleurodonta, that exhibit complex biogeographic histories and potential rate heterogeneity across subclades (Blankers et al., 2013; Alencar et al., 2024). Nonetheless, uncalibrated estimates did not depict large standard deviations, highlighting that, at least in the Bayesian framework we implemented, node calibrations were not as important as the used priors in the estimate's variation.

Difference between coding and non-coding regions

Mitogenomes are often established as superior to single genes-based approaches for divergence time estimation, as the latter typically overestimate node ages (e.g., Duchêne et al., 2011). In Pleurodontan squamates, our analyses revealed minimal substitution rate variation across mitochondrial coding regions, implying that, except for the rRNAs, practically any chosen gene subset may effectively capture their genome-wide evolutionary rate patterns. This finding offers

practical advantages for research on this squamate group, where targeted sequencing of subsets could reduce costs and labor while preserving phylogenetic signal. Selecting loci with intermediate substitution rates and robust phylogenetic resolution might be an important strategy for future research design. For instance, the use of genes such as 12S and 16S rRNAs should be critically pondered, as they present considerably lower substitution rates (Table 3), likely driven by functional constraints on ribosome assembly and saturation in conserved domains (Mueller, 2006; Duchêne et al., 2011). Additionally, loci exhibiting reduced phylogenetic informativeness (whether due to limited variability, homoplasy, or alignment ambiguity) require rigorous evaluation to avoid compromising analytical resolution (Zardoya and Meyer, 1996; Non et al., 2007). Either way, delineating such gene subsets demand taxon-specific substitution models and rigorous calibration to minimize biases, underscoring the need for tailored analytical frameworks, which can now be achieved by using our provided estimates.

Pleurodontan evolutionary dynamics and future perspectives

The substitution rate estimates from this study (nearly 0.01–0.02 substitutions/site/MY) align with prior estimates reported for Pleurodontan lineages (Supporting Table S1). However, we note that many of these earlier values were extrapolated from studies of distantly related taxa rather than empirically derived from lineage-specific calibrations. This reinforces the reliability of our methodological framework, which incorporated different partitioning schemes, appropriate substitution models, and string prior calibration strategies. Furthermore, our chosen priors, which were informed by values for different taxa within the Pleurodontan clade already reported on the literature (Table 2), proved to be robust. The close agreement of our substitution rate estimates with those previously reported for Pleurodonta also highlights the relative stability of mitochondrial evolutionary rates within the group. Prior research has demonstrated that mitochondrial substitution rates tend to cluster within narrow ranges among closely related taxa, often reflecting shared evolutionary constraints (Päckert et al., 2007; Pons et al., 2010). On the other hand, while mitochondrial protein-coding genes show conserved rate variation patterns across vertebrates—a

phenomenon stable for ~450 million years (Broughton & Reneau, 2006)—the drivers of this variation remain poorly understood. This gap highlights an opportunity to explore how structural, functional, and selective pressures differentially shape mitochondrial gene evolution.

Furthermore, by providing robust substitution-rate estimates for Pleurodontans as a whole, our study offers a valuable resource for future molecular dating analyses. In any case, lineage-specific rates for individual clades or species within the group might be warranted, depending on the study design. Such an approach could offer valuable insights into the evolutionary dynamics of particular species, particularly when ecological, physiological, or demographic factors influence mitochondrial evolution (e.g., Welch et al., 2008; Nabholz et al., 2016; Jing et al., 2024). Estimating lineage-specific rates could thus help identify these patterns and refine our understanding of the drivers of molecular evolution within Pleurodonta. However, the limited availability of complete mitochondrial genomes for several Pleurodontan lineages still hinders a full understanding of their evolutionary history from being achieved.

In this context, it is important to recognize that multiple methods exist for estimating substitution rates beyond the approach used here. For instance, germline-based estimates (e.g., Bergeron et al., 2023) are particularly relevant for assessing average nuclear genomic variation, a task that has only recently become feasible with advances in genomic sequencing and bioinformatics. However, obtaining such estimates is challenging, as it requires genomic data from multiple generations. Additionally, these methods remain taxonomically limited (Chintalapati & Moorjani, 2020; Bergeron et al., 2023), posing a major challenge in groups like Pleurodonta, where evolutionary parameters remain largely unknown for most species. Branch-specific substitution rate estimates, such as those generated using PAML (Yang, 2007), offer a robust alternative but are influenced by several factors that may affect their reliability for specific research objectives (e.g., sequence quality, alignment accuracy, and model assumptions) (Rasmussen & Kellis, 2007; Yan et al., 2023).

Lineage-specific rate estimation approaches can be computationally demanding, as it requires constructing tailored substitution models that account for codon position variation, partition-specific

evolutionary dynamics, and rate heterogeneity across the mitochondrial genome, as demonstrated here. Also, the accuracy of such specific estimates depends on the availability of high-quality sequence data and well-supported calibration points, both of which remain limited for many species, including those within Pleurodonta. This gains further importance as incomplete or biased sampling and poorly chosen calibrations can introduce substantial uncertainty into divergence time estimates (Zheng & Wiens, 2015; Schenk, 2016). Therefore, while lineage-specific rate estimation has the potential to refine our understanding of evolutionary rates, it must be applied cautiously, weighing the benefits of increased resolution against computational and methodological challenges.

Conclusion

In this study we examined the phylogenetic utility of nearly complete mitogenomes regarding the estimation of substitution rates, offering critical insights into the application of mitochondrial data in evolutionary studies. Despite the study's focus on a specific taxonomic scope (the Pleurodonta clade), the framework applied here may be broadly applicable across different taxa and divergence times.

Our findings reveal relatively homogeneity in substitution rates across Pleurodontan mitochondrial protein-coding genes, but heterogeneity between these and non-coding regions. Also, there is a considerable amount of difference in substitution rates when accounting for codon positions. Although this heterogeneity is relatively localized, employing rate estimates specific to the genes or genomic regions under study clearly enhance the accuracy of evolutionary inferences. Future research will be essential to determine whether this heterogeneity arises primarily from conserved replication mechanisms that drive variation in mutation rates across genomic regions, the effects of natural selection on individual genes, a combination of these factors, or other evolutionary processes.

Furthermore, evaluating the best modelling and partitioning schemes when conducting evolutionary analyses constitute a key factor and must not be overlooked when using mitochondrial markers. While subsets of informative genes might approximate these results, their effectiveness depends on robust methodological frameworks and careful taxon-specific selection. On this regard,

our results provide a valuable reference for future investigations into evolutionary dynamics specifically within the Pleurodonta clade and its closely related lineages, offering a foundation for comparative studies across Squamata. We then hope that our findings establish a foundation for optimizing mitochondrial phylogenetics in squamates, facilitating more accurate evolutionary reconstructions across diverse taxa and timescales.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data underlying this article, including phylogenetic datasets, corresponding trees, input and output files for all analyses, and any other relevant supplementary files are available in Zenodo, at https://doi.org/10.5281/zenodo.15952175.

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References

Alencar LR, Schwery O, Gade MR, Domínguez-Guerrero SF, Tarimo E, Bodensteiner BL, ... & Muñoz MM. 2024. Opportunity begets opportunity to drive macroevolutionary dynamics of a diverse lizard radiation. *Evolution Letters* 8: 623-637.

Anisimova M, Kosiol C. 2009. Investigating protein-coding sequence evolution with probabilistic codon substitution models. *Molecular Biology and Evolution* 26: 255-271.

Arcones A, Ponti R, Vieites DR. 2021. Mitochondrial substitution rates estimation for divergence

time analyses in modern birds based on full mitochondrial genomes. *Ibis* 163: 1463-1471.

Avila LJ, Martínez LE, Morando M. 2013. Checklist of lizards and amphisbaenians of Argentina: an update.

Avise JC, Arnold J, Ball RM, Bermingham E, Lamb T, et al. 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annu Rev Ecol Syst* 18: 489–522.

Ballard JWO, Whitlock MC. 2004. The incomplete history of mitochondria. *Molecular Ecology* 13: 729–744.

Ballard JWO, Rand DM. 2005. The population biology of mitochondrial DNA and its phylogenetic implications. *Annu. Rev. Ecol. Evol. Syst.* 36: 621-642.

Bergeron LA, Besenbacher S, Zheng J, Li P, Bertelsen MF, Quintard B, ... & Zhang G. 2023. Evolution of the germline mutation rate across vertebrates. *Nature* 615: 285-291.

Bernardo PH, Sánchez-Ramírez S, Sánchez-Pacheco SJ, Álvarez-Castañeda ST, Aguilera-Miller EF, Mendez de la Cruz FR, Murphy RW. 2019. Extreme mito-nuclear discordance in a peninsular lizard: the role of drift, selection, and climate. *Heredity* 123: 359-370.

Bininda-Emonds ORP. 2007. Fast genes and slow clades: comparative rates of molecular evolution in mammals. *Evolutionary Bioinformatics* 3: 59-85.

Blankers T, Townsend TM, Pepe K, Reeder TW, Wiens JJ. 2013. Contrasting global-scale evolutionary radiations: phylogeny, diversification, and morphological evolution in the major clades of iguanian lizards. *Biological Journal of the Linnean Society* 108: 127-143.

Bofkin L, Goldman N. 2007. Variation in evolutionary processes at different codon positions. *Molecular Biology and Evolution* 24: 513-521.

Borowiec ML. 2016. AMAS: a fast tool for alignment manipulation and computing of summary statistics. *PeerJ* 4: e1660.

Bouckaert RR, Drummond AJ. 2017. bModelTest: Bayesian phylogenetic site model averaging and model comparison. *BMC evolutionary biology* 17: 1-11.

Broughton RE, Reneau PC. 2006. Spatial covariation of mutation and nonsynonymous substitution rates in vertebrate mitochondrial genomes. *Molecular Biology and Evolution* 23: 1516-1524.

Buckley TR, Simon C, Chambers GK. 2001. Exploring among-site rate variation models in a maximum likelihood framework using empirical data: effects of model assumptions on estimates of topology, branch lengths, and bootstrap support. *Systematic Biology* 50: 67-86.

Buckley TR. 2002. Model misspecification and probabilistic tests of topology: evidence from empirical data sets. *Systematic Biology* 51: 509-523.

Camurugi F, Oliveira EF, Lima GS, Marques R, Magalhães FM, Colli GR, Mesquita DO, Garda AA. 2022. Isolation by distance and past climate resistance shaped the distribution of genealogical lineages of a neotropical lizard. *Systematics and Biodiversity* 20. https://doi.org/10.1080/14772000.2022.2084470

Carvalho ALG, de Britto MR, Fernandes DS. 2013. Biogeography of the Lizard Genus *Tropidurus* Wied-Neuwied, 1825 (Squamata: Tropiduridae): Distribution, Endemism, and Area Relationships in South America. PLoS One: 8.

Carvalho ALG, Paredero RCB, Villalobos-Chaves D, Ferreira E, Rodrigues MT, Curcio FF. 2024. A highly polymorphic South American collared lizard (Tropiduridae: Tropidurus) reveals that open-dry refugia from South-western Amazonia staged allopatric speciation. *Zoological Journal of the Linnean Society* 201: 493–533. https://doi.org/10.1093/zoolinnean/zlad138

Chan LM, Choi D, Raselimanana AP, Rakotondravony HA, Yoder AD. 2012. Defining spatial and temporal patterns of phylogeographic structure in Madagascar's iguanid lizards (genus *Oplurus*). *Molecular Ecology* 21: 3839-3851.

Chintalapati M, Moorjani P. 2020. Evolution of the mutation rate across primates. *Current opinion in genetics & development 62*: 58-64.

Conrad JL, & Norell MA. 2007. A complete Late Cretaceous iguanian (Squamata, Reptilia) from the Gobi and identification of a new iguanian clade. *American Museum Novitates*, *2007*(3584), 1-47.

Drummond AJ, Rambaut A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology 7*: 1-8.

Duchêne S, Archer FI, Vilstrup J, Caballero S, Morin PA. 2011. Mitogenome phylogenetics: The impact of using single regions and partitioning schemes on topology, substitution rate and divergence time estimation. *PLoS ONE* 6. https://doi.org/10.1371/journal.pone.0027138

Duchêne S, Lanfear R, Ho SY. 2014. The impact of calibration and clock-model choice on molecular estimates of divergence times. *Molecular Phylogenetics and Evolution* 78: 277-289.

Eo SH, DeWoody JA. 2010. Evolutionary rates of mitochondrial genomes correspond to diversification rates and to contemporary species richness in birds and reptiles. *Proceedings of the Royal Society B: Biological Sciences 277*: 3587-3592.

Feng S, Stiller J, Deng Y, Armstrong J, Fang Q, Reeve AH, Xie D, Chen G, Guo C, Faircloth BC, et al. 2020. Dense Sampling of Bird Diversity Increases Power of Comparative Genomics. *Nature* 587: 252–257.

Fontanella FM, Olave M, Avila LJ, Sites JW, Morando M. 2012. Molecular dating and diversification of the South American lizard genus *Liolaemus* (subgenus *Eulaemus*) based on nuclear and mitochondrial DNA sequences. *Zoological Journal of the Linnean Society* 164: 825-835.

Funk DJ, Omland KE. 2003. Species-Level Paraphyly and Polyphyly: Frequency, Causes, and Consequences, with Insights from Animal Mitochondrial DNA. *Annual Review of Ecology, Evolution, and Systematics* 34: 397–423. doi:10.1146/annurev.ecolsys.34.01

Gable SM, Mendez JM, Bushroe NA, Wilson A, Byars MI, Tollis M. 2023. The State of Squamate Genomics: Past, Present, and Future of Genome Research in the Most Speciose Terrestrial Vertebrate Order. In Genes (Vol. 14, Issue 7). Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/genes14071387

Genereux DP, Serres A, Armstrong J, Johnson J, Marinescu VD, Murén E, Juan D, Bejerano G, Casewell NR, Chemnick LG, et al. 2020. A Comparative Genomics Multitool for Scientific Discovery and Conservation. Nature 587: 240–245.

Gray MW, Burger G, Lang BF. 1999. Mitochondrial evolution. *Science* 283: 1476-1481.

Hassanin A. 2006. Phylogeny of Arthropoda inferred from mitochondrial sequences: strategies for limiting the misleading effects of multiple changes in pattern and rates of substitution. *Molecular Phylogenetics and Evolution* 38: 100-116.

Hebert PD, Ratnasingham S, De Waard JR. 2003. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London. Series B: Biological Sciences* 270: S96-S99.

Hipsley CA, Müller J. 2014. Beyond fossil calibrations: realities of molecular clock practices in evolutionary biology. *Frontiers in genetics 5*: 138.

Ho SY. 2007. Calibrating molecular estimates of substitution rates and divergence times in birds. *Journal of Avian Biology* 38: 409-414.

Jing Y, Long R, Meng J, Yang Y, Li X, Du B, ... & Luo Y. 2024. Influence of life-history traits on mitochondrial DNA substitution rates exceeds that of metabolic rates in teleost fishes. *Current Zoology*: zoae045.

Johns GC, Avise JC. 1998. A comparative summary of genetic distances in the vertebrates from the mitochondrial cytochrome b gene. *Molecular Biology and Evolution* 15: 1481-1490.

Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* 30: 772-780.

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of molecular evolution* 16: 111-120.

Leaché AD, & Linkem CW. 2015. Phylogenomics of horned lizards (Genus: Phrynosoma) using targeted sequence capture data. *Copeia* 103: 586-594.

Lemmon AR, Brown JM, Stanger-Hall K, Lemmon EM. 2009. The effect of ambiguous data on phylogenetic estimates obtained by maximum likelihood and Bayesian inference. *Systematic Biology* 58: 130-145.

Losos JB. 2011. Lizards in an evolutionary tree: ecology and adaptive radiation of anoles (Vol. 10).

Univ of California Press.

Mackiewicz P, Matosiuk M, Świsłocka M, Zachos FE, Hajji GM, Saveljev AP, ... & Ratkiewicz M. 2022. Phylogeny and evolution of the genus Cervus (Cervidae, Mammalia) as revealed by complete mitochondrial genomes. *Scientific Reports* 12: 16381.

Mello B, Schrago CG. 2014. Assignment of calibration information to deeper phylogenetic nodes is more effective in obtaining precise and accurate divergence time estimates. *Evolutionary Bioinformatics* 10: EBO-S13908.

Mueller RL. 2006. Evolutionary rates, divergence dates, and the performance of mitochondrial genes in Bayesian phylogenetic analysis. *Systematic biology* 55: 289-300.

Mower JP, Touzet P, Gummow JS, Delph LF, Palmer JD. 2007. Extensive variation in synonymous substitution rates in mitochondrial genes of seed plants. *BMC Evolutionary Biology* 7. https://doi.org/10.1186/1471-2148-7-135

Nabholz B, Glémin S, Galtier N. 2008. Strong variations of mitochondrial mutation rate across mammals-the longevity hypothesis. *Molecular Biology and Evolution* 25: 120-130. 10.1093/molbev/msm248.

Nabholz B, Lanfear R, Fuchs J. 2016. Body mass-corrected molecular rate for bird mitochondrial DNA. *Molecular ecology* 25: 4438-4449.

Non AL, Kitchen A, Mulligan CJ. 2007. Identification of the most informative regions of the mitochondrial genome for phylogenetic and coalescent analyses. *Molecular Phylogenetics and Evolution* 44: 1164-1171.

Olave M, Avila LJ, Sites Jr JW, Morando M. 2015. Model-based approach to test hard polytomies in the Eulaemus clade of the most diverse South American lizard genus *Liolaemus* (Liolaemini, Squamata). *Zoological Journal of the Linnean Society* 174: 169-184.

Päckert M, Martens J, Tietze DT, Dietzen C, Wink M, Kvist L. 2007. Calibration of a molecular clock in tits (Paridae)—Do nucleotide substitution rates of mitochondrial genes deviate from the 2% rule? *Molecular Phylogenetics and Evolution* 44: 1-14.

Parkinson CL, Mower JP, Qiu Y, Shirk AJ, Song K, Young ND, DePamphilis CW, Palmer JD. 2005. Multiple major increases and decreases in mitochondrial substitution rates in the plant family Geraniaceae. *BMC Evolutionary Biology* 5: 1–12. https://doi.org/10.1186/1471-2148-5-73

Patwardhan A, Ray S, Roy A. 2014. Molecular markers in phylogenetic studies-a review. *Journal of Phylogenetics & Evolutionary Biology* 2: 131.

Park E, Hwang DS, Lee JS, Song JI, Seo TK, Won YJ. 2012. Estimation of divergence times in cnidarian evolution based on mitochondrial protein-coding genes and the fossil record. *Molecular Phylogenetics and Evolution* 62: 329-345.

Pianka EP, Vitt LJ. 2003. Lizards: windows to the evolution of diversity (Vol. 5). Univ of California Press.

Plazzi F, Puccio G, Passamonti M. 2016. Comparative large-scale mitogenomics evidences clade specific evolutionary trends in mitochondrial DNAs of Bivalvia. *Genome Biology and Evolution* 8: 2544-2564.

Pons J, Ribera I, Bertranpetit J, Balke M. 2010. Nucleotide substitution rates for the full set of mitochondrial protein-coding genes in Coleoptera. *Molecular Phylogenetics and Evolution* 56: 796–807. https://doi.org/10.1016/j.ympev.2010.02.007.

Portelli SN, Abdala CS, Schulte II J, Gómez JMD, Fernández LD, Quinteros AS. 2022. Historical biogeographic reconstruction of the South American Liolaemus boulengeri group (Iguania: Liolaemidae). *South American Journal of Herpetology* 25: 41-56.

Prates I, Rodrigues MT, Melo-Sampaio PR, & Carnaval AC. 2015. Phylogenetic relationships of Amazonian anole lizards (Dactyloa): taxonomic implications, new insights about phenotypic evolution and the timing of diversification. *Molecular phylogenetics and evolution* 82: 258-268.

Rambaut A, Drummond AJ, Suchard M. 2007. Tracer v1. 6 http://beast.bio.ed.ac.uk.

Rambaut A, Drummond AJ. 2014. LogCombiner v2. 1.3. Institute of Evolutionary Biology, University of Edinburgh.

Rasmussen MD, Kellis M. 2007. Accurate gene-tree reconstruction by learning gene-and species specific substitution rates across multiple complete genomes. *Genome research* 17: 1932-1942.

Ritchie AM, Lo N, Ho SY. 2017. The impact of the tree prior on molecular dating of data sets containing a mixture of inter-and intraspecies sampling. *Systematic Biology* 66: 413-425.

Roe AD, Sperling FA. 2007. Patterns of evolution of mitochondrial cytochrome c oxidase I and II DNA and implications for DNA barcoding. *Molecular Phylogenetics and evolution* 44: 325-345.

Rogers TF, Stenhouse EH, Wilson H, Pendaries M, Bruford MW, Goetz M, Orozco-Wengel P. 2024. Using recent genetic history to inform conservation options of two Lesser Caymans iguana (*Cyclura nubila caymanensis*) populations. *Conservation Genetics* 25: 711-724.

Román-Palacios C, Tavera J, del Rosario Castañeda M. 2018. When did anoles diverge? An analysis of multiple dating strategies. *Molecular Phylogenetics and Evolution* 127: 655-668.

Rubinoff D, Holland B. 2005. Between Two Extremes: Mitochondrial DNA is neither the Panacea nor the Nemesis of Phylogenetic and Taxonomic Inference. *Systematic Biology* 54*:* 952–961. doi:10.1080/10635150500234674

Saccone C, De Giorgi C, Gissi C, Pesole G, Reyes A. 1999. Evolutionary genomics in Metazoa: the mitochondrial DNA as a model system. *Gene* 238: 195-209.

Salles, M. M. A., Carvalho, A. L. G., Leache, A. D., Martinez, N., Bauer, F., Motte, M., ... & Domingos, F. M. C. B. 2025. Ancient introgression explains mitochondrial genome capture and mitonuclear discordance among South American collared Tropidurus lizards. *bioRxiv*, 2025-04, https://doi.org/10.1101/2025.04.25.650633.

Scarpetta SG. 2019. The first known fossil Uma: ecological evolution and the origins of North American fringe-toed lizards. *BMC Evolutionary Biology* 19: 178.

Schenk JJ. 2016. Consequences of secondary calibrations on divergence time estimates. *PloS one* 11: e0148228.

Sherratt E, del Rosario Castañeda M, Garwood RJ, Mahler DL, Sanger TJ, Herrel A, ... & Losos JB. 2015. Amber fossils demonstrate deep-time stability of Caribbean lizard communities. *Proceedings of the National Academy of Sciences* 112: 9961-9966.

Simões TR, Pyron RA. 2021. The squamate tree of life. *Bulletin of the Museum of Comparative Zoology* 163: 47-95.

Sloan DB, Oxelman B, Rautenberg A, Taylor DR. 2009. Phylogenetic analysis of mitochondrial substitution rate variation in the angiosperm tribe Sileneae. *BMC Evolutionary Biology* 9. https://doi.org/10.1186/1471-2148-9-260

Smith SA, Brown JW, Walker JF. 2018. So many genes, so little time: a practical approach to divergence-time estimation in the genomic era. *PloS one* 13: e0197433.

Su Z, Wang Z, López-Giráldez F, Townsend JP. 2014. The impact of incorporating molecular evolutionary model into predictions of phylogenetic signal and noise. *Frontiers in Ecology and Evolution 2*: 11.

Townsend TM, Tolley KA, Glaw F, Böhme W, & Vences M. 2011. Eastward from Africa: palaeocurrent-mediated chameleon dispersal to the Seychelles islands. *Biology Letters* 7: 225-228.

Uetz P, Koo MS, Aguilar R, Brings E, Catenazzi A, Chang AT, Wake DB. 2021. A quarter century of reptile and amphibian databases. *Herpetol. Rev*. 52: 246-255.

Uetz P, Freed P, Aguilar R, Reyes F, Kudera J, Hošek J. (eds.). 2025. *The Reptile Database*, http://www.reptile-database.org, accessed January 31, 2025.

Xu W, Jameson D, Tang B, Higgs PG. 2006. The relationship between the rate of molecular evolution and the rate of genome rearrangement in animal mitochondrial genomes. *Journal of molecular evolution* 63: 375-392.

Warnock RC, Parham JF, Joyce WG, Lyson TR, Donoghue PC. 2015. Calibration uncertainty in molecular dating analyses: there is no substitute for the prior evaluation of time priors. *Proceedings of the Royal Society B: Biological Sciences* 282: 20141013.

Welch JJ, Bininda-Emonds OR, Bromham L. 2008. Correlates of substitution rate variation in mammalian protein-coding sequences. *BMC Evolutionary Biology* 8: 1-12.

Werneck FP, Leite RN, Geurgas SR, Rodrigues MT. 2015. Biogeographic history and cryptic diversity of saxicolous Tropiduridae lizards endemic to the semiarid Caatinga. *BMC Evolutionary Biology* 15: 1–24. https://doi.org/10.1186/s12862-015-0368-3

Williams EJB, Hurst LD. 2002. Is the synonymous substitution rate in mammals gene-specific? *Molecular Biology and Evolution* 19: 1395-1398.

Yan H, Hu Z, Thomas GW, Edwards SV, Sackton TB, Liu JS. 2023. PhyloAcc-GT: A Bayesian method for inferring patterns of substitution rate shifts on targeted lineages accounting for gene tree discordance. *Molecular Biology and Evolution* 40: msad195.

Yan L, Xu W, Zhang D, Li J. 2021. Comparative analysis of the mitochondrial genomes of flesh flies and their evolutionary implication. *International Journal of Biological Macromolecules* 174: 385–391. https://doi.org/10.1016/j.ijbiomac.2021.01.188

Yang Z, Nielsen, R. 2000. Estimating synonymous and nonsynonymous substitution rates under realistic evolutionary models. *Molecular Biology and Evolution* 17: 32-43.

Yang Z. 2007. PAML 4: phylogenetic analysis by maximum likelihood. *Molecular biology and evolution* 24: 1586-1591.

Yang H, Li T, Dang K, Bu W. 2018. Compositional and mutational rate heterogeneity in mitochondrial genomes and its effect on the phylogenetic inferences of Cimicomorpha (Hemiptera: Heteroptera). *BMC Genomics* 19. https://doi.org/10.1186/s12864-018-4650-9

Zardoya R, Meyer A. 1996. Phylogenetic performance of mitochondrial protein-coding genes in resolving relationships among vertebrates. *Molecular biology and evolution* 13: 933-942.

Zarza E, Reynoso VH, Emerson BC. 2008. Diversification in the northern neotropics: mitochondrial and nuclear DNA phylogeography of the iguana *Ctenosaura pectinata* and related species. *Molecular Ecology* 17*:* 3259-3275.

Zheng Y, Wiens JJ. 2015. Do missing data influence the accuracy of divergence-time estimation with

BEAST? *Molecular Phylogenetics and Evolution* 85: 41-49.

Zheng Y, Wiens JJ. 2016. Combining phylogenomic and supermatrix approaches, and a time-calibrated phylogeny for squamate reptiles (lizards and snakes) based on 52 genes and 4162 species. *Molecular phylogenetics and evolution* 94: 537-547.

Zhu A, Guo W, Jain K, Mower JP. 2014. Unprecedented heterogeneity in the synonymous substitution rate within a plant genome. *Molecular Biology and Evolution* 31: 1228–1236. https://doi.org/10.1093/molbev/msu079

Supporting information

Table S1. References regarding values for substitution rate priors adopted in the present study.

Gene	Values	References
COX1	0.01	Phrynosomatidae: Bernardo et al., 2019; *Tropidurus:* Camurugi et al., 2022
CYTB	0.019355 | 0.0113 | 0.0223	*Cyclura*: Rogers et al., 2024; Liolaemidae*:* Olave et al., 2015; *Liolaemus:* Fontanella et al., 2012; *Tropidurus:* Werneck et al., 2015;
ND1	0.013876	*Oplurus:* Chan et al., 2012
ND2	0.013	*Anolis:* Román-Palacios et al., 2018
ND4	0.0113 | 0.0078	*Cyclura*: Rogers et al., 2024 Iguaninae: Zarza et al., 2008
12s & 16s	0.006339 | 0.00576	Liolaemidae*:* Olave et al., 2015; *Liolaemus:* Fontanella et al., 2012; *Tropidurus:* Carvalho et al., 2024;

Table S2. Model selection results from BEAST, including only models within the 95% HPD interval for each gene. The four-digit model code represents how substitution rates are grouped, following the order of relative rates for A-C, A-G, A-T, C-G, C-T, and G-T. A complete list of model codes can be found here: https://taming-the-beast.org/tutorials/Substitution-model-averaging/.

Gene	Non-calibrated	Calibrated
	Models	
12s	GTR	GTR
16s	GTR	GTR
ATP6	HKY, GTR, $K81_{123324}$, TIM_{123345}, TVM_{123425}	GTR, K80, $K81_{123324}$, TIM_{123345}, $TN93_{121131}$, TIM_{123345}
ATP8	K80, $K81_{123321}$, $K81_{123324}$, TIM_{123341}, TIM_{123345}, $TN93_{121131}$	K80, $K81_{3321}$, $K81_{3324}$, TIM_{3341}, TIM_{3345}, $TN93_{1131}$
COX1	TIM_{3341}, TIM_{3345}, $TN93_{1131}$	TIM_{3341}, TIM_{3345}, $TN93_{1131}$
COX2	K80, $K81_{3321}$, $K81_{3324}$, TIM_{3341}, $TN93_{1131}$	K80, $K81_{3321}$, $K81_{3324}$, TIM_{3341}, $TN93_{1131}$
COX3	K80, $K81_{3321}$, $K81_{3324}$, TIM_{3345}, $TN93_{1131}$	K80, $K81_{3321}$, $K81_{3324}$, TIM_{3341}, $TN93_{1131}$
CYTB	GTR, TVM_{3425}	GTR, $K81_{3324}$, TVM_{3425}
ND1	GTR, $K81_{3324}$, TIM_{3345}, TVM_{3425}	GTR, $K81_{3324}$, TIM_{3345}, TVM_{3425}
ND2	$K81_{3324}$, TIM_{3345}, TVM_{3425}	$K81_{3324}$, TIM_{3345}, TIM_{3345}
ND3	K80, $K81_{3321}$, TIM_{3341}, TIM_{3345}	K80, $K81_{3321}$, $TN93_{1131}$, $K81_{3324}$, TIM_{3341}
ND4	GTR, TIM_{3345}, $TN93_{1131}$	GTR, TIM_{3345}
ND4L	GTR, K80, $K81_{3321}$, $K81_{3324}$, TIM_{3341}, TIM_{3345}	K80, $K81_{3321}$, $K81_{3324}$, TIM_{3341}, TIM_{3345}, TVM_{3425}
ND5	$K81_{3324}$, TIM_{3345}, TVM_{3425}	$K81_{3324}$, TIM_{3345}, TVM_{3425}
ND6	GTR, TVM_{3425}	GTR, TVM_{3425}