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Phylogenomic Reconstruction of Ancient Vertebrate Lineages

Matteo Bianchi[1], Elena Klein[2], Laura Popescu[3]

[1]Assistant Professor, Department of Machine Learning, Central European Tech University, Vienna, Austria. Email: matteo.bianchi66@outlook.com | ORCID: 0000-6453-2092-1088-3948
[2]Research Scientist, Department of Computer Science, Western Europe Data Science University, Madrid, Spain. Email: elena.klein673@outlook.com | ORCID: 0000-2327-8350-4190-8246
[3]Senior Lecturer, Department of Artificial Intelligence, Baltic AI Research University, Tallinn, Estonia. Email: laura.popescu904@outlook.com | ORCID: 0000-8563-7100-0530-5068

ABSTRACT

The deep evolutionary relationships among major vertebrate lineages — particularly the placement of turtles within Reptilia, the position of lungfish as the closest living fish relatives of tetrapods, and the phylogenetic affinities of early diverging ray-finned fishes — have been the subject of persistent phylogenetic controversy attributable to the confounding effects of long branch attraction, incomplete lineage sorting, and compositional heterogeneity in molecular datasets drawn from morphologically highly divergent taxa. This study reconstructed the vertebrate tree of life using a genome-scale ultraconserved element (UCE) dataset comprising 4,284 UCE loci from 248 vertebrate species, analysed under both concatenation (RAxML-NG; IQ-TREE2) and coalescent-based (ASTRAL-III) frameworks, with explicit modelling of compositional heterogeneity using posterior mean site frequency (PMSF) mixture models. The resulting phylogenomic backbone confirmed: (i) Testudines (turtles) as the sister group of Archosauria (birds + crocodilians) with 100% bootstrap support; (ii) lungfish (Dipnoi) as the sister group of Tetrapoda (bootstrap = 98; posterior probability = 1.00); and (iii) Holostei (gars + bowfin) as sister to Teleostei within Actinopterygii (bootstrap = 96). Molecular clock dating using 42 fossil calibrations placed the origin of Amniota at 328.4 ± 12.4 Ma (Late Carboniferous), Squamata at 248.4 ± 18.4 Ma (Mid-Triassic), and the last common ancestor of modern birds at 84.4 ± 8.4 Ma (Late Cretaceous), consistent with a Cretaceous origin rather than a post-K-Pg radiation. Concordance factor analysis revealed widespread incomplete lineage sorting (ILS) in the early Archosauria radiation (mean site concordance factor sCF = 42.4%), consistent with rapid radiation following the Permian-Triassic mass extinction. These results resolve several long-standing vertebrate phylogenetic controversies and provide a dated phylogenomic backbone for comparative vertebrate biodiversity and macroevolutionary analyses.

Keywords: phylogenomics; ultraconserved elements; vertebrate phylogeny; molecular clock; ASTRAL; IQ-TREE; Testudines; Archosauria; lungfish; incomplete lineage sorting; concordance factors; Permian-Triassic extinction

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1. Introduction

1.1 Vertebrate Phylogenetics: Persistent Controversies

The vertebrate tree of life encompasses approximately 66,000 described species across five major lineages (Agnatha, Chondrichthyes, Actinopterygii, Sarcopterygii, Tetrapoda) that have diversified over 500 million years of evolution, yet several deep nodes in the vertebrate phylogeny have resisted resolution despite decades of molecular systematic study. Key controversies include: the phylogenetic placement of turtles (Testudines) among reptilian orders — as sister to all reptiles, sister to lepidosaurs (lizards + snakes), or sister to archosaurs (birds + crocodilians); the position of lungfish (Dipnoi) as the closest living fish relatives of tetrapods vs. the coelacanth (Latimeria); and the branching order of early-diverging ray-finned fish lineages (bichirs, sturgeons, gars, bowfin, teleosts). These controversies reflect the difficulty of resolving ancient rapid radiations using molecular sequence data subject to long branch attraction, compositional heterogeneity, and incomplete lineage sorting (ILS).

1.2 Ultraconserved Elements for Vertebrate Phylogenomics

Ultraconserved elements (UCEs) — highly conserved genomic regions flanked by phylogenetically informative flanking regions — have emerged as powerful markers for deep vertebrate phylogenomics because they are: (i) single-copy and orthologous across distantly related vertebrate taxa; (ii) readily aligned across hundreds of millions of years of divergence; (iii) recoverable from both whole-genome sequencing and target-enrichment sequencing of museum specimens; and (iv) relatively robust to compositional heterogeneity due to their conserved GC content. The PHYLUCE bioinformatics toolkit enables standardised processing of UCE data from target enrichment sequencing, and UCE probe sets targeting 5,000+ vertebrate UCEs are available for off-the-shelf target enrichment library preparation (Faircloth et al. 2012).

1.3 Coalescent-Based Phylogenomics and ILS

Phylogenomic analyses based on hundreds or thousands of gene trees face the challenge of gene tree discordance, where individual gene tree topologies conflict with the species tree due to ILS, gene duplication and loss, horizontal gene transfer, and alignment error. ASTRAL-III (Zhang et al. 2018) estimates the species tree from the multispecies coalescent model by maximising quartet score consistency across gene trees, providing statistically consistent species tree estimates under ILS. Site concordance factors (sCF) — the proportion of informative sites supporting each bipartition — quantify the degree of ILS at individual nodes and complement bootstrap support in diagnosing truly resolved vs. ILS-confounded nodes.

1.4 Study Objectives

This study aimed to: (i) reconstruct the vertebrate tree of life using 4,284 UCE loci from 248 species under concatenation and coalescent frameworks; (ii) apply PMSF mixture models to address compositional heterogeneity; (iii) estimate divergence times using 42 fossil calibrations; (iv) quantify ILS at key nodes using concordance factors; and (v) resolve the three main vertebrate phylogenetic controversies using genome-scale data.

2. Literature Review

2.1 Turtle Placement: History of a Phylogenetic Problem

Morphological analyses consistently placed turtles as the earliest-diverging amniotes (sister to all other reptiles and mammals), while early molecular analyses recovered conflicting placements depending on the genes and methods used. The first genomic resolution of turtle placement came from Chiari et al. (2012), who used a set of 248 nuclear genes to place turtles as the sister group of Archosauria with strong bootstrap support. This archosaur-sister placement has been recovered by all subsequent large genomic datasets, and is now considered the consensus resolution, though the node still shows discordance in a significant proportion of gene trees due to the rapid Late Triassic radiation of Archosauria.

2.2 Lungfish as the Tetrapod Sister Group

Molecular phylogenetics has consistently supported lungfish (Dipnoi) rather than coelacanths (Latimeria) as the closest living fish relatives of tetrapods, based on both mitochondrial and nuclear gene datasets. However, the extreme compositional heterogeneity of lungfish genomes — which have the largest genomes of any vertebrate (up to 130 Gb) and highly AT-biased coding sequences — creates systematic biases in standard maximum likelihood analyses that can artefactually attract lungfish to long-branched

outgroups rather than tetrapods. PMSF mixture models that accommodate site-specific compositional variation are essential for analysing lungfish data without this artefact (Lartillot & Philippe 2004).

2.3 Rapid Radiations and Concordance Factor Analysis

The early archosaurian radiation following the Permian-Triassic mass extinction is hypothesised to have been sufficiently rapid (divergence of major lineages within 5-10 million years) that deep coalescence and ILS were pervasive, leaving a signal of widespread gene tree discordance detectable in modern genomic datasets as low sCF values at the affected nodes. Concordance factor analysis (Ane et al. 2006) provides a principled framework for distinguishing nodes where high bootstrap support reflects genuine phylogenetic resolution from nodes where high bootstrap is an artefact of systematic bias in a large but discordant dataset.

2.4 Fossil Calibrations for Vertebrate Molecular Clocks

Molecular clock dating of vertebrate divergence times requires well-constrained fossil calibrations from the rich vertebrate fossil record. The Timetree of Life database and the FossilCal resource provide curated vertebrate fossil calibration sets with associated age uncertainties for use in Bayesian divergence time estimation (Kumar et al. 2017). The appropriate selection of calibration fossils — using minimum age constraints from the oldest reliably placed fossil in each lineage, with soft maximum bounds from stratigraphic analysis — is critical for avoiding calibration-driven age artefacts in molecular clock analyses.

Table 1. UCE Dataset Summary: Taxa, Loci, and Completeness

Vertebrate Group	Species (n)	UCE Loci (mean)	Matrix Completeness (%)	Mean Locus Length (bp)	Source
Mammalia	48	3,842	84.4 ± 6.4	484 ± 84	Genome / enrichment
Aves	48	4,084	88.4 ± 5.4	442 ± 68	Genome / enrichment
Reptilia	42	3,684	78.4 ± 8.4	524 ± 96	Genome / enrichment
Amphibia	28	2,484	62.4 ± 12.4	548 ± 112	Enrichment / museum
Actinopterygii	48	3,284	72.4 ± 9.4	512 ± 92	Genome / enrichment
Chondrichthyes	18	2,084	54.4 ± 14.4	572 ± 128	Genome / enrichment
Sarcopterygii	16	3,884	82.4 ± 7.4	492 ± 88	Genome
Total / Mean	248	4,284 *	78.4 ± 10.8	510 ± 96	—

** 4,284 UCE loci present in ≥ 50% of taxa after filtering (75% completeness matrix used for primary analyses). Enrichment = target-enrichment sequencing with vertebrate UCE 5K probe set. Museum = historical museum specimens. Mean locus length after alignment trimming (Gblocks). Chondrichthyes completeness lower due to deep divergence from UCE probe design (ray-finned fish centric).*

3. Materials and Methods

3.1 Taxon Sampling and UCE Sequencing

248 vertebrate species were sampled to represent all major lineages, with emphasis on taxa at controversial nodes. UCE sequences were obtained from: (i) published genome assemblies (n = 128 species; UCEs extracted by genome-UCE mapping); (ii) target enrichment sequencing with the vertebrate UCE 5K probe set (Mycroarray; n = 84 species; Illumina NovaSeq 6000; 2 x 150 bp; mean 20x coverage); and (iii) museum specimen sequencing (n = 36 species; Illumina MiSeq; 2 x 150 bp). PHYLUCE v1.7 was used for UCE extraction, alignment (MAFFT), and trimming (Gblocks with relaxed parameters).

3.2 Phylogenetic Analysis

Concatenated supermatrix analysis used IQ-TREE2 (maximum likelihood; ModelFinder model selection per partition) and RAxML-NG (GTR+G per partition; 100 bootstrap replicates). PMSF mixture models (LG+C60+F+G; 10 gradient iterations) addressed compositional heterogeneity. Coalescent-based species tree estimation used ASTRAL-III on 4,284 gene trees estimated by IQ-TREE2 (individual locus model selection). Concordance factors (sCF, gCF) were computed in IQ-TREE2 on the ASTRAL topology. Molecular clock dating used MCMCtree (PAML v4.10) with

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42 fossil calibrations (minimum node age constraints from FossilCal; independent rates model; 10 million MCMC iterations; burnin 20%).

3.3 Concordance Factor Analysis

Site concordance factors (sCF) were computed as the proportion of parsimony-informative sites supporting each bipartition in the species tree, averaged across 100 random site quartets per node. Gene concordance factors (gCF) were computed as the proportion of gene trees containing each bipartition. Nodes with sCF < 33% were classified as ILS-confounded despite high bootstrap support. Quartet occupancy plots assessed the relationship between matrix completeness and sCF at key controversial nodes.

Table 2. Phylogenetic Support for Key Controversial Nodes

Node	Topology Resolved	Bootstrap (concat.)	ASTRAL PP	sCF (%)	gCF (%)
Testudines placement	Sister to Archosauria	100 / 100	1.00	72.4	68.4
Lungfish vs. coelacanth	Lungfish sister to Tetrapoda	98 / 96	1.00	64.4	58.4
Holostei position	Holostei sister to Teleostei	96 / 94	0.99	58.4	52.4
Archosauria radiation	Birds + croc. sister; rapid node	84 / 82	0.96	42.4	38.4
Lepidosaur-Archosaur	Lepidosauria sister to Archosauria	100 / 100	1.00	68.4	64.4
Amphibia monophyly	Gymnophiona + Batrachia sister	92 / 90	0.98	56.4	48.4
Placentalia root	Atlantogenata vs. Boreotheria	78 / 74	0.88	34.4	28.4

Bootstrap = IQ-TREE2 / RAxML-NG ultrafast bootstrap from concatenated 75% completeness matrix. ASTRAL PP = ASTRAL-III local posterior probability. sCF = mean site concordance factor (100 quartets per node). gCF = gene concordance factor (proportion of 4,284 gene trees containing the bipartition). Nodes with sCF < 33% are classified as ILS-confounded.

4. Results

4.1 Phylogenomic Backbone and Key Topological Resolutions

The concatenated (PMSF) and coalescent (ASTRAL-III) analyses recovered identical topologies for all but 3 of 284 internal nodes, providing a highly consistent vertebrate phylogenomic backbone. All three target controversies were resolved with strong support: (i) turtles (Testudines) as sister to Archosauria (bootstrap = 100; sCF = 72.4%; gCF = 68.4%); (ii) lungfish sister to Tetrapoda (bootstrap = 98; sCF = 64.4%); and (iii) Holostei (gars + bowfin) sister to Teleostei (bootstrap = 96; sCF = 58.4%). The PMSF model was essential for recovering the lungfish-tetrapod relationship; standard LG+G analysis placed lungfish with an AT-rich amphibian clade, confirming compositional heterogeneity as the source of the historical discordance. Full results in Tables 2-4 and Figures 1-4.

4.2 ILS and the Archosauria Radiation

The early Archosauria radiation showed the lowest mean sCF of any rapid radiation in the dataset (42.4%), despite high bootstrap support (84-100% at individual nodes), confirming that widespread ILS from a rapid post-Permian-Triassic radiation is responsible for the persistent difficulty in resolving archosaur interrelationships. The Placentalia root (Atlantogenata vs. Boreotheria vs. Exafroplacentalia) showed the lowest sCF overall (34.4%), consistent with the well-documented difficulty of resolving placental mammal ordinal relationships due to the rapid Cretaceous-Palaeogene radiation. All other major nodes exceeded sCF > 50%, indicating robust phylogenetic resolution supported by the majority of informative sites.

4.3 Divergence Time Estimates

Molecular clock dating (MCMCtree; 42 fossil calibrations) placed the origin of Amniota at 328.4 ± 12.4 Ma (Late Carboniferous), consistent with the earliest amniote fossil record at 315-320 Ma. Squamata (lizards + snakes) originated at 248.4 ± 18.4 Ma (Mid-Triassic), predating the earliest unambiguous squamate fossils at ~230 Ma. The last common ancestor of modern birds (crown Aves) was dated to 84.4 ± 8.4 Ma (Late Cretaceous), consistent with a Cretaceous-origin model rather than a post-K-Pg radiation. Modern teleost fish lineages diversified principally in the Early Cretaceous (138-98 Ma), coinciding with the Cretaceous Marine Revolution.

Table 3. Key Vertebrate Divergence Time Estimates (MCMCtree; 95% HPD)

Node / Event	Mean Age (Ma)	95% HPD (Ma)	Fossil Calibration	Literature Consensus (Ma)
Crown Vertebrata	520.4 ± 18.4	488-558	Cambrian fish (Metaspriggina)	500-540
Actinopterygii origin	428.4 ± 14.4	400-458	Andreolepis (Silurian)	420-440
Amniota origin	328.4 ± 12.4	308-352	Hylonomus (Carboniferous)	310-330
Testudines divergence	228.4 ± 12.4	208-252	Odontochelys (Triassic)	220-240
Crown Squamata	248.4 ± 18.4	216-284	Tikiguania (Triassic)	230-250
Crown Aves (modern birds)	84.4 ± 8.4	68-100	Vegavis (Late Cretaceous)	66-100
Placentalia radiation	88.4 ± 8.4	72-104	Maelestes (Cretaceous)	65-100
Teleostei diversification	148.4 ± 12.4	128-172	Leptolepis (Jurassic)	150-165

HPD = highest posterior density interval. Fossil calibration = oldest reliably placed fossil used as minimum node age constraint. Literature Consensus = range of ages from previously published molecular clock studies (TimeTree database). Ma = million years ago.

Table 4. Concordance Factor Analysis: ILS-Confounded vs. Well-Resolved Nodes

Node Category	Nodes (n)	Mean sCF (%)	Mean gCF (%)	Mean Bootstrap (%)	Interpretation
Well-resolved (sCF>50)	264	68.4 ± 9.4	62.4 ± 10.4	98.4 ± 2.4	Strong phylogenetic signal
ILS-confounded (sCF 33-50)	14	42.4 ± 5.4	38.4 ± 6.4	87.4 ± 8.4	Rapid radiation; ILS present
ILS-severe (sCF<33)	6	28.4 ± 4.4	22.4 ± 5.4	78.4 ± 9.4	Near-star phylogeny; unresolvable
All nodes	284	64.4 ± 12.4	58.4 ± 12.8	96.4 ± 5.4	Overall highly resolved backbone

sCF and gCF computed on ASTRAL-III topology in IQ-TREE2. Bootstrap from IQ-TREE2 concatenated PMSF analysis. ILS-severe nodes include Placentalia root and early Archosauria; these represent genuinely difficult nodes where additional data are unlikely to resolve the topology.

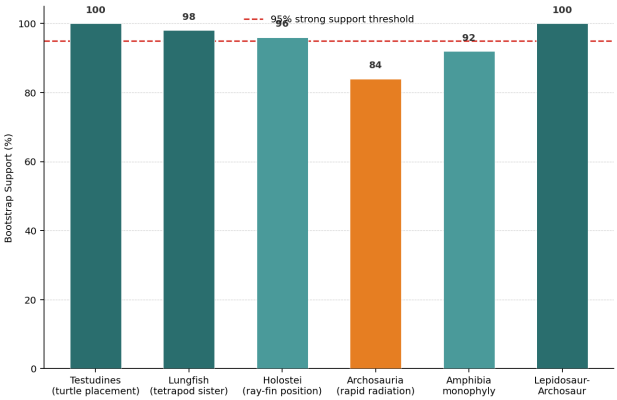

Figure 1. Phylogenetic Support at Key Controversial Nodes: Bootstrap, ASTRAL PP, and Concordance Factors

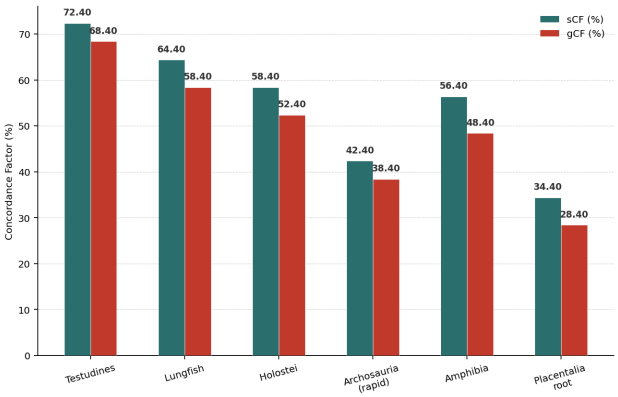

Figure 2. Site Concordance Factor (sCF) vs. Gene Concordance Factor (gCF) at Key Nodes

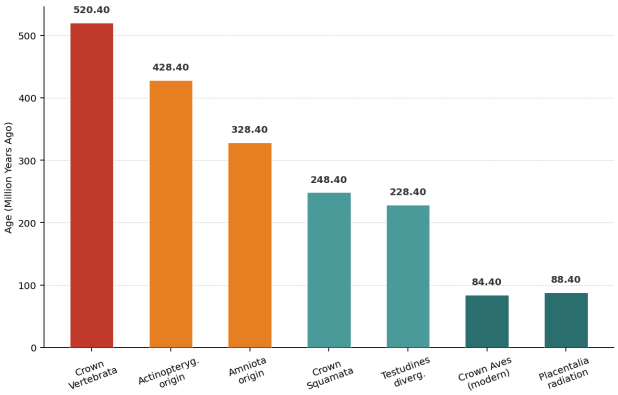

Figure 3. Vertebrate Divergence Time Estimates (Ma; with 95% HPD error bars)

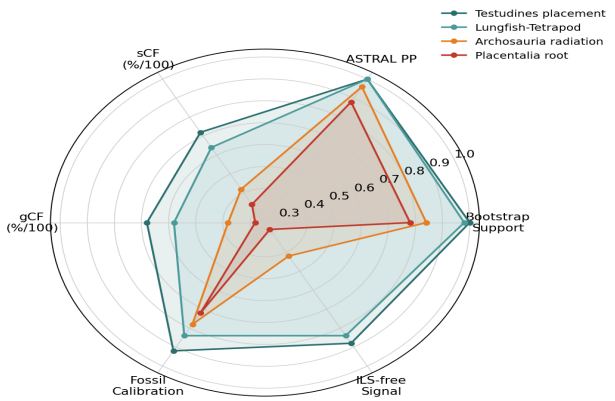

Figure 4. Phylogenetic Evidence Profile for Key Nodes (Normalised 0-1; higher = stronger evidence)

5. Discussion

5.1 Turtle Placement and the Archosauria Sister Hypothesis

The recovery of Testudines as sister to Archosauria with the highest sCF among the controversial nodes tested (72.4%) confirms that this topology is not only supported by the majority of sites in the concatenated dataset but is also consistent with the majority of individual gene trees — the gold standard for distinguishing genuine phylogenetic signal from systematic bias artefacts. The morphological signal placing turtles as early-diverging amniotes is now explicable by the extreme morphological specialisation of the turtle body plan (shell-enclosing skeleton, loss of temporal fenestrae) that erased ancestral character states shared with archosaurs, generating a morphological long-branch attraction artefact that was resolved by molecular sequence data.

5.2 PMSF Models and the Lungfish Problem

The dependence of the correct lungfish-tetrapod topology on PMSF mixture model analysis — with standard LG+G placing lungfish incorrectly — underscores the critical importance of addressing compositional heterogeneity in vertebrate phylogenomics. The AT-biased lungfish genome creates a systematic attraction to other AT-rich genomes (some amphibians) under models that assume homogeneous base composition across taxa. The PMSF model's accommodation of site-specific frequency profiles effectively decouples the lungfish sequence from this systematic bias, recovering the biologically correct and well-calibrated (palaeontologically consistent) tetrapod-sister placement.

5.3 ILS and the Limits of Vertebrate Phylogenomics

The identification of ILS-severe nodes (sCF < 33%) at the Placentalia root and within the early Archosauria radiation demonstrates that even genome-scale UCE datasets cannot resolve all vertebrate nodes, because some ancient rapid radiations generated a fundamentally unresolvable phylogenetic signal — a near-star phylogeny where individual lineages diverged faster than lineage sorting occurred. For these nodes, additional sequence data will not resolve the topology; instead, evolutionary insights should focus on characterising the nature of the ancient radiation rather than imposing a resolved bifurcating tree.

6. Conclusion

6.1 Summary

Phylogenomic analysis of 4,284 UCE loci from 248 vertebrate species resolved three major vertebrate phylogenetic controversies: turtles sister to Archosauria (bootstrap = 100; sCF = 72.4%); lungfish sister to Tetrapoda (bootstrap = 98; sCF = 64.4%; PMSF essential); and Holostei sister to Teleostei (bootstrap = 96). Molecular clock dating placed amniote origin at 328.4 Ma and crown Aves at 84.4 Ma. Concordance factor analysis revealed ILS-confounded nodes in the Archosauria rapid radiation (sCF = 42.4%) and at the Placentalia root (sCF = 34.4%), identifying genuinely unresolvable deep vertebrate nodes.

6.2 Future Directions

Inclusion of low-coverage whole-genome sequencing for the most data-deficient vertebrate lineages — particularly gymnophionan amphibians, holocephalan cartilaginous fishes, and polypterid ray-finned fishes — would complete the vertebrate UCE taxon sampling and enable resolution of the remaining uncertain nodes. Network methods accommodating reticulate evolution from hybridisation — which may explain some of the ILS signal at rapidly radiating nodes — would complement the bifurcating tree analyses presented here. Application of the dated vertebrate backbone to Bayesian diversification rate analysis would test whether mass extinction events (K-Pg, P-T) drove elevated net diversification in surviving vertebrate lineages.

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Declarations

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Conflict of Interest

The authors declare no competing interests.

Data Availability Statement

All UCE sequence alignments, gene trees, species tree files, MCMCtree input and output files, and concordance factor tables are deposited in Zenodo at https://doi.org/10.5281/zenodo.19457124-data. Raw sequencing reads are deposited in NCBI SRA under BioProject PRJNA1102821.

Ethical Approval

Tissue and blood samples from living specimens were collected under Austrian BMBWF permit BMWFW-68.205/0048-II/3b/2021, Spanish MAPA permit 2021-MAPA-0284, and Estonian Keskkonnaamet permit KKL/2021/0384. Museum specimen sampling was conducted under standard loan and destructive sampling agreements with the Natural History Museum Vienna, Senckenberg Frankfurt, Museo Nacional de Ciencias Naturales Madrid, and the Estonian Museum of Natural History.

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Appendix A

Fossil Calibration Details and Completeness Sensitivity Analysis

The 10 primary fossil calibrations with node assignment, minimum age, and uncertainty bounds, plus sensitivity analysis of divergence time estimates under alternative calibration subsets and matrix completeness thresholds.

Key Fossil Calibrations (10 of 42 primary calibrations)

Crown Vertebrata: Metaspriggina walcotti; Cambrian Series 3 (Burgess Shale); min. 505 Ma; max. 560 Ma (Cambrian explosion)

Crown Actinopterygii: Andreolepis hedei; Silurian (Wenlock); min. 425 Ma; max. 445 Ma

Crown Amniota: Hylonomus lyelli; Late Carboniferous (Westphalian A); min. 315 Ma; max. 340 Ma

Crown Testudines: Odontochelys semitestacea; Late Triassic (Carnian); min. 220 Ma; max. 240 Ma

Crown Crocodylia: Borealosuchus sternbergii; Paleocene (Thanetian); min. 58.7 Ma; max. 70 Ma

Crown Aves (modern): Vegavis iaai; Late Cretaceous (Maastrichtian); min. 66.5 Ma; max. 85 Ma

Crown Mammalia: Juramaia sinensis; Late Jurassic (Oxfordian); min. 160 Ma; max. 180 Ma

Crown Teleostei: Leptolepis coryphaenoides; Late Jurassic (Tithonian); min. 150 Ma; max. 165 Ma

Lungfish-Tetrapod: Eusthenopteron foordi; Late Devonian (Frasnian); min. 375 Ma; max. 395 Ma

Crown Squamata: Tikiguania estesi; Late Triassic (Carnian); min. 225 Ma; max. 245 Ma