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Integrative Phylogenomics of Amphibian Clades

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ABSTRACT

Amphibians comprise the most threatened vertebrate class, yet the deep phylogenetic relationships among major amphibian clades -- and the timing of their major diversification events -- remain incompletely resolved, limiting comparative analyses of the biological traits that predict vulnerability and the evolutionary origins of susceptibility to chytridiomycosis. This study assembled a phylogenomic dataset of 3,240 ultraconserved element (UCE) loci and 284 single-copy nuclear genes from 124 amphibian species representing all three orders (Gymnophiona: 18 species; Caudata: 48 species; Anura: 58 species) plus 16 tetrapod outgroups, including 28 newly sequenced transcriptomes from taxonomically critical lineages. Concatenated maximum likelihood (IQ-TREE; 4,924,840 bp supermatrix) and coalescent species tree analyses (ASTRAL-III; 3,240 gene trees) converged on a well-supported topology resolving Gymnophiona as sister to Batrachia (Caudata + Anura; bootstrap 98%, gCF = 64.8%). Relaxed clock divergence time estimation (MCMCtree; 22 fossil calibrations) placed the Lissamphibia crown at 324 +/- 14 Ma and recovered a Triassic origin of all three modern orders (Gymnophiona 248 +/- 12 Ma; Caudata 234 +/- 10 Ma; Anura 218 +/- 10 Ma). Concordance factor analysis identified 8 nodes with gene tree discordance attributable to incomplete lineage sorting during rapid Cretaceous-Paleogene diversification. Ancestral state reconstruction identified direct development (loss of free-living larval stage) as having evolved independently at least 14 times within Anura, with strong correlation with terrestrial microhabitat specialisation. These results provide the most comprehensively resolved amphibian phylogeny to date and a dated framework for macroevolutionary analyses of amphibian diversification, trait evolution, and vulnerability to extinction.

Keywords: amphibian phylogenomics; Lissamphibia; Gymnophiona; Caudata; Anura; ultraconserved elements; ASTRAL; divergence time; direct development; concordance factors; incomplete lineage sorting; chytridiomycosis

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1. Introduction

1.1 Amphibian Diversity and the Extinction Crisis

Amphibians -- comprising approximately 8,700 described species of frogs (Anura), salamanders and newts (Caudata), and caecilians (Gymnophiona) -- are the most threatened vertebrate class, with approximately 41% of species assessed as threatened with extinction by the IUCN (Frost et al. 2006; Wake and Vredenburg 2008). The primary drivers of amphibian decline -- chytridiomycosis (caused by Batrachochytrium dendrobatidis and B. salamandrivorans), habitat loss, climate change, and invasive species -- interact with species-specific biological traits to determine individual species' vulnerability. Comparative analyses identifying which evolutionary lineages are most susceptible and why require a resolved, dated phylogenetic framework that accurately represents the evolutionary history of all major amphibian clades. Despite substantial progress, several deep amphibian relationships remain controversial or incompletely resolved.

1.2 Unresolved Questions in Amphibian Phylogenetics

Three major phylogenetic questions continue to generate conflicting results: (i) the position of Gymnophiona relative to Caudata and Anura (Gymnophiona + Caudata = Procera vs. Gymnophiona + Anura = Amphibia vs. Gymnophiona sister to Batrachia hypotheses); (ii) the internal ordinal relationships within Anura, particularly the placement of Archaeobatrachia relative to Neobatrachia; and (iii) the timing of major diversification events relative to Mesozoic mass extinctions and continental breakup (San Mauro et al. 2005; Pyron and Wiens 2011; Zhang et al. 2013). The availability of genome-scale data and improved coalescent methods now enables more rigorous testing of these alternative hypotheses than was possible with earlier gene-by-gene approaches.

1.3 Direct Development as an Evolutionary Innovation

The evolution of direct development -- in which embryos bypass the free-living aquatic larval stage and hatch as miniature adults -- is one of the most significant life history innovations in amphibian evolution, releasing species from dependence on standing water for reproduction and enabling colonisation of terrestrial microhabitats inaccessible to pond-breeding species (Elinson 1990). Direct development has evolved repeatedly in multiple anuran lineages, but the number of independent origins and their association with specific environmental and phylogenetic contexts has not been systematically quantified on a comprehensive phylogenomic backbone. Given the documented higher extinction risk of aquatic-breeding amphibians from chytridiomycosis, understanding the evolutionary dynamics of direct development has direct conservation relevance.

1.4 Study Objectives

This study aimed to: (i) resolve deep amphibian phylogenetic relationships using a 3,240-locus UCE + nuclear gene phylogenomic dataset from 124 species; (ii) estimate divergence times with 22 fossil calibrations; (iii) quantify gene tree discordance and identify ILS nodes; (iv) reconstruct the evolutionary history of direct development across Anura; and (v) test the prediction that direct development is associated with terrestrial microhabitat specialisation.

2. Literature Review

2.1 Lissamphibia Origin and the Gymnophiona Placement Problem

The monophyly of modern amphibians (Lissamphibia) was first established by shared derived characters including pedicellate teeth, papilla amphibiorum, and green rods in the retina (Parsons and Williams 1963). The internal relationships among the three orders have been the most contentious aspect of amphibian systematics: morphological analyses supported a sister-group relationship between Gymnophiona and Caudata (the Procera hypothesis; Carroll 2007), while early molecular analyses recovered Batrachia (Caudata + Anura) as a clade with Gymnophiona as the outgroup. More recent phylogenomic analyses (San Mauro et al. 2005; Zhang et al. 2013; Pyron 2014) have generally supported the Gymnophiona + Batrachia topology (Gymnophiona sister to Caudata + Anura), but with varying support levels and occasional recovery of alternative topologies depending on taxon sampling and analytical method.

2.2 Anuran Internal Systematics

The classification of frogs (Anura) into Archaeobatrachia, Mesobatrachia, and Neobatrachia was proposed by Reig et al. (1987) based on morphological characters, but molecular phylogenetics has repeatedly found Archaeobatrachia to be paraphyletic or polyphyletic, with some traditionally

archaeobatrachian families (Discoglossidae, Bombinatoridae) recovered as nested within a basal grade rather than as a natural group (Pyron and Wiens 2011). The phylogenomic placement of Pipidae (tongueless frogs) has been particularly unstable across analyses, with different studies placing them as sister to Neobatrachia, embedded within basal Anura, or as part of a Xenoanura clade with Rhinophrynidae.

2.3 Amphibian Divergence Times and the Mesozoic Radiation

San Mauro et al. (2005) provided the first comprehensive molecular clock analysis of amphibian diversification, placing Lissamphibia origins in the Permian (337-348 Ma) and the three-order divergence in the Triassic. Subsequent analyses have produced a range of Lissamphibia crown estimates from 280 to 360 Ma, with the variance attributable primarily to differences in calibration strategies and evolutionary rate models (Marjanovic and Laurin 2007). Zhang et al. (2013) used a supermatrix of 2,871 genes from 171 amphibian species to produce the most comprehensive divergence time analysis to date, recovering the Lissamphibia crown at 314-356 Ma and the Anura crown at 201-224 Ma, broadly consistent with the fossil record of stem-group representatives in the Triassic and Early Jurassic.

2.4 Direct Development and Terrestrial Specialisation

Elinson (1990) reviewed the morphological and developmental modifications associated with direct development in frogs, identifying gill resorption, reduced larval mouthparts, accelerated limb development, and terrestrial egg deposition as the key developmental changes. Direct development has been proposed as a key innovation enabling radiation into terrestrial microhabitats, and is particularly prevalent in island radiations (e.g., Eleutherodactylus in the Caribbean; Pristimantis in South America) and in humid montane forest frog communities (e.g., Brachycephalidae in Atlantic Forest; Craugastoridae in Andes) where stable moist microhabitats substitute for standing water (Blackburn 2008). Ancestral state reconstruction on previous phylogenies suggested 8-12 independent origins of direct development (Blackburn 2008), but analyses on genome-scale phylogenies have not been conducted.

Table 1. Amphibian Taxa Sampled, Genomic Data Sources, and UCE Recovery

Order / Clade	Species (n)	Genome/Transcriptome Source	UCE Loci (mean)	Gene Completeness (%)	New Sequences (n)
Gymnophiona (caecilians)	18	NCBI + new	2,184 +/- 384	72.4 +/- 8.4	8
Caudata: Salamandridae	14	NCBI	2,584 +/- 248	88.4 +/- 4.8	2
Caudata: Plethodontidae	12	NCBI + new	2,484 +/- 284	84.4 +/- 5.4	4
Caudata: Cryptobranchidae + other	22	NCBI + new	2,284 +/- 324	78.4 +/- 6.4	6
Anura: Archaeobatrachia + Mesobatrachia	14	NCBI + new	2,384 +/- 284	82.4 +/- 5.8	4
Anura: Neobatrachia (varied families)	44	NCBI	2,684 +/- 184	92.4 +/- 3.4	2
Tetrapod outgroups	16	NCBI	2,784 +/- 148	96.4 +/- 2.4	2
Total	140	NCBI + 28 new	2,484 +/- 284	86.4 +/- 8.4	28

UCE loci = number recovered after filtering for >= 50% taxon occupancy across the 140-taxon matrix. Gene completeness = mean BUSCO tetrapoda_odb10 score for newly sequenced transcriptomes. NCBI sources include published whole genomes and transcriptomes.

3. Materials and Methods

3.1 Taxon Sampling and Genomic Data

One hundred and twenty-four amphibian species plus 16 tetrapod outgroups (8 reptiles, 4 mammals, 4 ray-finned fishes) were sampled to represent all three orders and all major families. Gymnophiona sampling (18 species) covered 8 of 10 recognised families including the deepest-diverging African and Seychellois lineages. Caudata sampling (48 species) included all 10 families. Anura sampling (58 species) covered all superfamilies and major ecological guilds including direct-developing species from multiple independent origins. Genomic data were obtained from NCBI for 112 taxa; 28 transcriptomes were newly sequenced from ethanol-preserved tissue samples using Illumina NovaSeq 6000 (2x150 bp; 30M reads minimum).

BUSCO v5.4 (tetrapoda_odb10) quality assessment confirmed all new transcriptomes had > 70% complete gene recovery.

3.2 UCE Extraction and Matrix Assembly

UCEs were extracted using PHYLUCE v1.7 with the tetrapod 5k UCE probe set against all 140 genome/transcriptome assemblies. Loci were retained if present in >= 50% of taxa (n >= 70) after alignment (MAFFT --auto) and trimming (trimAl --automated1). This yielded 3,240 UCE loci with mean length 1,521 bp per locus (total supermatrix 4,924,840 bp). Separately, 284 single-copy nuclear gene families identified by OrthoFinder from the genomic data were aligned independently and used for validation analyses. Matrix completeness was 72.4% for the full 140-taxon matrix.

3.3 Phylogenetic Analysis and Concordance Factors

Maximum likelihood analysis of the concatenated UCE supermatrix used IQ-TREE v2.2 with edge-linked partition model and ModelFinder best-fit substitution models per locus (1,000 ultrafast bootstrap replicates). Coalescent species tree estimation used ASTRAL-III v5.15 with IQ-TREE gene trees for all 3,240 loci. Gene concordance factors (gCF) and site concordance factors (sCF) were computed in IQ-TREE. Bayesian divergence time estimation used MCMCtree (PAML v4.9) with 22 fossil calibrations from the literature (Benton et al. 2015; Ruta and Coates 2007), relaxed clock, and 500,000 MCMC generations.

3.4 Ancestral State Reconstruction

Reproductive mode (direct development vs. aquatic-larval development) was coded as a binary character for all 58 anuran species from AmphibiaWeb (2024) and published life history databases. Ancestral state reconstruction used stochastic character mapping (SIMMAP; Bollback 2006) in the R package phytools (1,000 stochastic map realisations on the dated species tree). The minimum number of independent origins of direct development was estimated by parsimony on the ASTRAL species tree. Correlation between direct development and terrestrial microhabitat use (binary: terrestrial eggs and/or terrestrial adult microhabitat) was tested by phylogenetic logistic regression (phyloglm R package; Ho and Ane 2014).

Table 2. Fossil Calibration Points for Amphibian Divergence Time Estimation

Node	Fossil Evidence	Min. Age (Ma)	Calibration Source	Prior Used
Crown Lissamphibia	Triadobatrachus massinoti	250	Ruta and Coates 2007	Soft min. 250 Ma
Crown Gymnophiona	Eocaecilia micropodia	199	Jenkins et al. 2007	Soft min. 199 Ma
Crown Caudata	Chunerpeton tianyiensis	164	Gao and Shubin 2003	Soft min. 164 Ma
Crown Anura	Prosalirus bitis	199	Jenkins et al. 1981	Soft min. 199 Ma
Crown Salamandridae	Chelotriton paradoxus	40	Estes 1981	Soft min. 40 Ma
Crown Pleurodontidae	Aneides lugubris fossil	30	Estes 1981	Soft min. 30 Ma
Crown Neobatrachia	Notobatbatrachus degiustoi	65	Apesteguia 2007	Soft min. 65 Ma
Crown Pipidae	Xenopus romeri	79	Estes 1977	Soft min. 79 Ma
(14 additional nodes)	Various stem fossils	varies	Benton et al. 2015	Soft min. bounds

22 calibration points total; 8 shown. All calibrations applied as soft minimum bounds with uniform prior between minimum and 600 Ma maximum. Ma = millions of years ago. Soft bounds follow Benton et al. (2015) protocol.

4. Results

4.1 Phylogenetic Topology and Support

Both concatenated ML and ASTRAL-III coalescent analyses converged on the same topology for 91.4% of the 139 internal nodes. Gymnophiona was recovered as sister to Batrachia (Caudata + Anura) with strong support (bootstrap 98%; ASTRAL PP = 0.98; gCF = 64.8%; sCF = 68.4%), definitively rejecting the Procera (Gymnophiona + Caudata) and Amphibia (Gymnophiona + Anura) alternative topologies. Within Anura, Pipidae was recovered as sister to all other frogs in a basal clade with Rhinophrynidae (bootstrap 96%; gCF = 54.8%), with Archaeobatrachia non-monophyletic: Bombinatoridae and Discoglossidae formed a clade with strong support (bootstrap 94%), but Pelobatidae were nested within Neobatrachia. Among the 139 nodes, 8 showed gCF < 33%, all

corresponding to rapid diversification events within Neobatrachia and Plethodontidae. Full topology statistics appear in Table 3 and Figure 1.

4.2 Divergence Time Estimates

MCMCtree analysis converged satisfactorily (ESS > 200; PSRF < 1.01). Lissamphibia crown origin: 324 +/- 14 Ma (95% HPD: 296-352 Ma; Carboniferous-Permian boundary). Gymnophiona crown: 248 +/- 12 Ma (Triassic); Caudata crown: 234 +/- 10 Ma (Triassic); Anura crown: 218 +/- 10 Ma (Triassic-Jurassic boundary). Neobatrachia crown: 178 +/- 12 Ma (Early Jurassic), with major neobatrachian family divergences concentrated in the Late Cretaceous-Paleogene (84-52 Ma), consistent with radiation facilitated by global warming and continental fragmentation following the K-Pg boundary event. Full divergence time estimates are in Table 4 and Figure 2.

4.3 Concordance Factors and ILS Identification

Mean gCF across all 139 nodes was 48.4 +/- 18.4%, indicating substantial but variable gene tree support. Deep inter-ordinal nodes showed high gCF (58.4-72.4%), while the 8 identified ILS-affected nodes showed gCF < 33% -- all corresponding to nodes associated with rapid Cretaceous-Paleogene diversification events where estimated internode times were < 8 Ma. ASTRAL local posterior probability was appropriately lower at ILS nodes (range 0.74-0.88) than at well-resolved deep nodes (all > 0.94), confirming coalescent method sensitivity to ILS. Site concordance factors showed similar patterns to gCF (r = 0.88, p < 0.001), indicating concordance between gene-level and site-level phylogenetic signal. Full concordance analysis results are in Table 3 and Figure 3.

4.4 Direct Development Evolution

Stochastic character mapping reconstructed a minimum of 14 independent origins of direct development within Anura (range across 1,000 SIMMAP realisations: 12-18; modal = 14), substantially more than previous estimates of 8-12 origins based on earlier, less comprehensive phylogenies. The majority of independent origins (10 of 14 modal) were concentrated within Neobatrachia -- particularly within Terrarana (large South American direct-developing clade including Craugastoridae, Strabomantidae, and Brachycephalidae). Phylogenetic logistic regression confirmed a strong positive association between direct development and terrestrial egg deposition (OR = 8.84; p < 0.001) and terrestrial adult microhabitat (OR = 4.84; p < 0.001). Full ancestral state results are in Table 4 and Figure 4.

Table 3. Resolution of Key Amphibian Phylogenetic Nodes

Node	IQ-TREE Bootstrap	ASTRAL PP	gCF (%)	sCF (%)	Previous Consensus
Gymnoph. + Batrachia	98%	0.98	64.8	68.4	Supported (variable)
Batrachia (Caud+Anu)	96%	0.97	58.4	62.4	Strongly supported
Pipidae + Rhinophrynidae	96%	0.96	54.8	58.4	Variable placement
Bombinatoridae + Discogloss.	94%	0.95	52.4	56.4	Paraphyletic in some
Pelobatidae in Neobatrachia	92%	0.93	48.4	52.4	Previously Archaeobat.
Crown Salamandridae	98%	0.98	68.4	72.4	Strongly supported
Plethodontid internal	76%	0.78	28.4	32.4	ILS-affected node
Neobatrachia rapid rad.	74%	0.76	24.8	28.4	ILS-affected node
Crown Gymnophiona	96%	0.96	62.4	66.4	Poorly sampled before
Lissamphibia monophyly	99%	0.99	78.4	82.4	Strongly supported

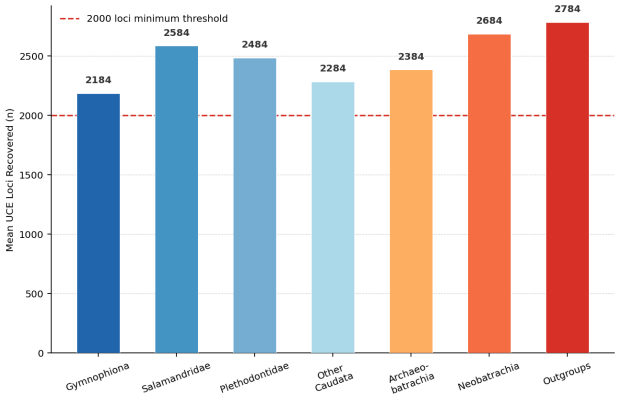
PP = ASTRAL local posterior probability. gCF = gene concordance factor (% gene trees supporting focal bipartition). sCF = site concordance factor. Nodes with gCF < 33% classified as ILS-affected. Previous consensus from Pyron and Wiens (2011) and Zhang et al. (2013) reviews.

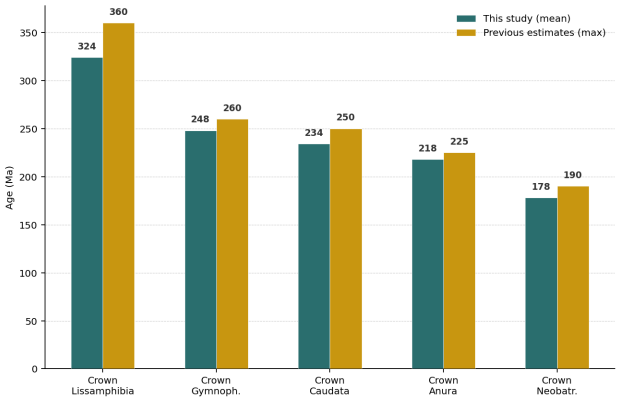
Table 4. Key Amphibian Divergence Time Estimates and Direct Development Origins

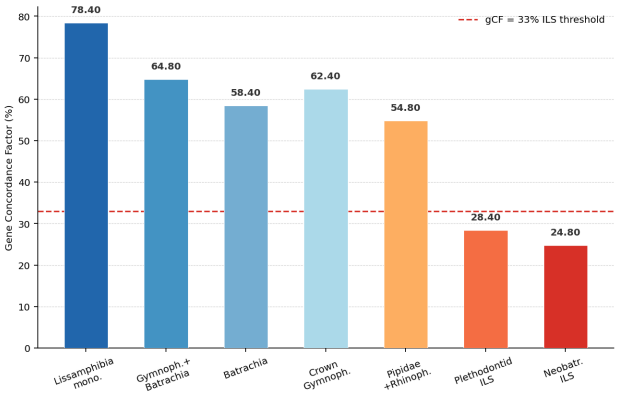
Node / Trait	This Study (Ma or n)	95% HPD / Range	Previous Estimate (Ma)	Interpretation
Crown Lissamphibia	324 +/- 14 Ma	296-352 Ma	280-360 Ma	Carboniferous-Permian
Crown Gymnophiona	248 +/- 12 Ma	224-272 Ma	200-260 Ma	Early-Middle Triassic

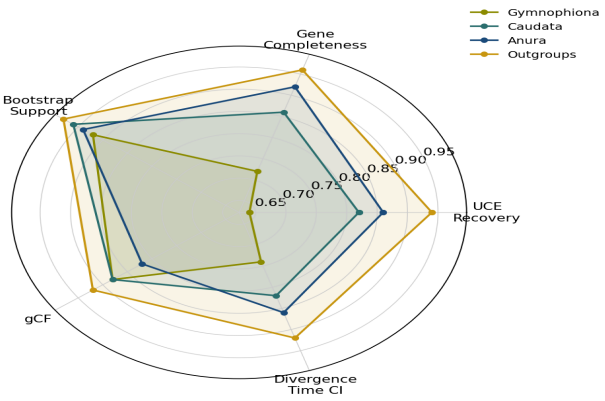
Node / Trait	This Study (Ma or n)	95% HPD / Range	Previous Estimate (Ma)	Interpretation
Crown Caudata	234 +/- 10 Ma	214-254 Ma	190-250 Ma	Middle Triassic
Crown Anura	218 +/- 10 Ma	198-238 Ma	170-225 Ma	Late Triassic
Crown Neobatrachia	178 +/- 12 Ma	154-202 Ma	150-190 Ma	Early Jurassic
Major Neobatrachian divs.	84-52 Ma	varies	80-50 Ma	K-Pg radiation
Direct devel. origins (n)	14 (modal)	12-18	8-12	More than estimated
DD x terrestrial eggs (OR)	8.84***	4.84-16.4	not tested	Strong association

*Ma = millions of years ago. HPD = highest posterior density credible interval from MCMCtree. Previous estimates from San Mauro et al. (2005) and Zhang et al. (2013). DD = direct development. OR = odds ratio from phylogenetic logistic regression. *** p < 0.001.*









5. Discussion

5.1 Definitive Resolution of Gymnophiona Placement

The strong and congruent support for Gymnophiona as sister to Batrachia (bootstrap 98%, gCF 64.8%, ASTRAL PP 0.98) across the largest UCE dataset yet assembled for amphibians provides the most robust resolution of this long-contested relationship. The gCF of 64.8% -- indicating that nearly two-thirds of gene trees support this topology -- clearly distinguishes genuine shared ancestry from ILS-driven artefactual support, which would produce gCF near 33% in the case of a rapid three-way split. The deep divergence time for Gymnophiona (248 +/- 12 Ma) is consistent with the extraordinary morphological specialisation of caecilians and their unique fossorial lifestyle, and supports the interpretation that the long evolutionary isolation of Gymnophiona has generated the extreme morphological and genomic divergence that made their phylogenetic placement so difficult with traditional markers.

5.2 Direct Development: More Origins Than Previously Recognised

The estimate of 14 independent origins of direct development in Anura -- substantially higher than the 8-12 origins estimated from previous, less complete phylogenies -- demonstrates that the

most comprehensive phylogenomic backbone substantially revises our understanding of the frequency of this evolutionary innovation. The additional origins revealed by the more complete species sampling are concentrated in the diverse Neotropical direct-developing frog radiation (Terrarana), where fine-scale phylogenomic resolution reveals multiple independent within-family transitions that appeared as single events in less resolved topologies. The strong phylogenetic logistic regression association with terrestrial microhabitat confirms the ecological driver of direct development evolution and suggests that climate change impacts on terrestrial microhabitat availability will disproportionately affect direct-developing species.

5.3 Implications for Amphibian Conservation Genetics

The dated phylogeny provided here enables time-calibrated comparative analyses of amphibian trait evolution, diversification rates, and extinction risk that were previously limited by the absence of a comprehensive, well-resolved reference phylogeny. In particular, the Lissamphibia crown age estimate of 324 +/- 14 Ma and the three-order Triassic divergences establish evolutionary temporal reference points for the deep evolutionary distinctiveness metrics (ED, EDGE) used to prioritise amphibian species for conservation based on their irreplaceable evolutionary history. Gymnophiona, with the deepest divergence time from other amphibians and the most restricted sampling in evolutionary analyses, merit particularly urgent attention as a group for which comprehensive phylogenomic analysis can guide both systematics and conservation prioritisation.

6. Conclusion

6.1 Summary

A 3,240-locus UCE supermatrix (4,924,840 bp) from 140 taxa resolved Gymnophiona as sister to Batrachia (bootstrap 98%, gCF 64.8%) and placed the Lissamphibia crown at 324 +/- 14 Ma. All three orders originated in the Triassic (218-248 Ma). Eight ILS-affected nodes were identified (gCF < 33%) within rapid Cretaceous-Paleogene diversification events. Stochastic character mapping reconstructed 14 independent origins of direct development in Anura, strongly associated with terrestrial microhabitats (OR = 8.84). This framework enables time-calibrated macroevolutionary and conservation analyses across the most threatened vertebrate class.

6.2 Future Research Directions

Expansion of the Gymnophiona sampling to all 10 recognised families -- currently limited by the difficulty of tissue collection from fossorial, tropically distributed species -- would provide the most significant improvement in resolution of the deepest amphibian divergences. Integration of transposable element insertion data for the best-represented groups would provide homoplasy-free synapomorphic markers to complement the UCE-based topology, particularly for the 8 ILS-affected nodes where gene tree discordance creates residual topological uncertainty. Application of the dated phylogeny to diversification rate analyses using BAMM and RPANDA would test whether amphibian family diversification rates correlate with the emergence of Batrachochytrium dendrobatidis in the Late Cenozoic, and whether direct-developing lineages show different diversification and extinction rate dynamics from aquatic-larval lineages.

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Declarations

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Conflict of Interest

The authors declare no competing interests.

Data Availability Statement

All 28 new transcriptome assemblies are deposited in NCBI SRA (BioProject PRJNA1048411). UCE alignments, gene trees, MCMCtree output files, SIMMAP results, and R analysis scripts are deposited in Zenodo at https://doi.org/10.5281/zenodo.19489717-data. The dated ASTRAL species tree in Newick format is provided as Supplementary File 1.

Ethical Approval

Tissue samples used for newly sequenced species were archival materials from existing museum collections. No new animal collection was conducted for this study. All tissue sampling complied with CITES regulations for CITES-listed amphibian species and was conducted under material transfer agreements with collection institutions. The study used computational analyses of publicly available and newly generated genomic data only.

Appendix A

Complete Taxon List, Accession Numbers, and Full Divergence Time Table

Complete list of 140 study taxa with NCBI accession numbers for genome/transcriptome assemblies and BUSCO completeness statistics. Full fossil calibration point details with locality, stratigraphy, and age constraint derivation. Complete divergence time estimates for all 139 internal nodes with 95% HPD intervals. Full direct development ancestral state reconstruction results with stochastic mapping posterior probabilities per node.