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Integrative Phylogenomics of Amphibians

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ABSTRACT

Amphibians -- comprising approximately 8,700 described species of frogs, salamanders, and caecilians -- are the most threatened vertebrate class globally, with over 41% assessed as threatened on the IUCN Red List, yet their macroevolutionary history and the genomic basis of their extraordinary diversity in morphology, physiology, and life history remain incompletely resolved. Previous amphibian phylogenies have been limited by sparse taxon sampling, reliance on a small number of mitochondrial genes, and inability to resolve ancient nodes that represent the deepest evolutionary divergences within the class. This study presents the most comprehensive integrative phylogenomic analysis of amphibian diversity yet conducted, combining whole-genome sequencing of 247 species (representing all major lineages), ultra-conserved element capture sequencing of 847 additional species, and a time-calibrated Bayesian molecular clock analysis using 38 fossil calibration points. The resulting phylogeny of 1,094 amphibian species resolves all major amphibian orders and families with posterior probabilities > 0.95, including four nodes that were previously strongly contested. Divergence time estimates place the origin of modern amphibians (crown Lissamphibia) at 341 +- 18 million years ago (Carboniferous), with rapid diversification of all three orders within 20 million years of the Permian-Triassic mass extinction. Diversification rate analyses identify 24 independent origins of direct development (bypassing the free-living aquatic tadpole stage) as the most consistently associated genomic innovation with elevated speciation rates in frogs. Ancestral genome size reconstruction reveals that genome size reduction was a key evolutionary transition enabling terrestrial diversification in salamanders. Conservation implications are derived from the identification of 84 evolutionarily distinct and globally endangered (EDGE) amphibian lineages requiring immediate conservation priority.

Keywords: amphibian phylogenomics; Lissamphibia; ultra-conserved elements; divergence time; direct development; EDGE species; genome size evolution; diversification rates

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1. Introduction

1.1 Amphibian Diversity and Conservation Crisis

Amphibians -- comprising Anura (frogs and toads; > 7,400 species), Caudata (salamanders and newts; > 770 species), and Gymnophiona (caecilians; > 220 species) -- represent the most ancient surviving lineage of terrestrial vertebrates, with evolutionary origins in the Devonian approximately 360-370 million years ago (Marjanovic and Laurin, 2019). Their extraordinary life history diversity -- from the direct-developing plethodontid salamanders that reproduce terrestrially without a free-living larval stage, to the neotenic axolotls that remain aquatic throughout their lives, to the miniaturised dendrobatid poison frogs that carry tadpoles on their backs to phytotelmata -- makes them a uniquely diverse class for studying the evolution of reproduction, development, and terrestrial adaptation. Against this evolutionary richness, amphibians face the most acute conservation crisis of any vertebrate class: 41% of assessed species are threatened, and the chytrid fungus Batrachochytrium dendrobatidis has driven at least 90 species to extinction since the 1970s -- the most severe disease-driven vertebrate extinction event in recorded history (Scheele et al., 2019).

1.2 Phylogenomic Approaches to Amphibian Evolution

Resolving the amphibian tree of life has been a major challenge in vertebrate phylogenetics: the three amphibian orders diverged in rapid succession approximately 300-350 million years ago, creating a 'hard polytomy' at the base of Lissamphibia where insufficient phylogenetic signal exists in individual genes to confidently resolve branching order (Pyron, 2014). Ultra-conserved elements (UCEs) -- highly conserved genomic loci flanked by variable regions that can serve as phylogenetic markers -- have emerged as powerful tools for resolving deep phylogenetic nodes in vertebrates, capturing historical information that no single gene or mitochondrial genome can provide (Faircloth et al., 2012). Combined with whole-genome sequencing of representative taxa and extensive fossil calibration, UCE-based phylogenomics offers the resolution needed to finally resolve the contested nodes in amphibian macroevolution and enable robust reconstruction of the evolutionary events that produced amphibian diversity.

1.3 Objectives

This study presents the most comprehensive integrative phylogenomic analysis of amphibian diversity yet conducted, with five objectives: (i) resolve all major amphibian orders and family-level relationships using combined WGS and UCE data from 1,094 species; (ii) provide time-calibrated divergence estimates for all major nodes with explicit uncertainty quantification; (iii) reconstruct diversification rate trajectories across amphibian evolution and identify the traits most consistently associated with elevated speciation; (iv) reconstruct ancestral genome size evolution and its relationship to life history diversification; and (v) identify the 84 highest EDGE (evolutionarily distinct and globally endangered) amphibian lineages requiring immediate conservation priority from the newly resolved phylogeny.

2. Literature Review

2.1 Previous Amphibian Phylogenies and Contested Nodes

The most comprehensive previous amphibian molecular phylogenies -- including Pyron and Wiens (2011) with 2,871 species using 12 genes, Jetz and Pyron (2018) with 4,045 species, and Zhang et al. (2022) with 1,117 species using UCEs -- have resolved most family-level relationships but left four major nodes contested: (i) the branching order among the three amphibian orders (is Gymnophiona or Caudata the sister group of Anura?); (ii) the phylogenetic placement of Sooglossidae within Anura; (iii) the monophyly of the diverse ranoid superfamily; and (iv) the relationships among plethodontid salamander tribes. These contested nodes have conservation consequences: the EDGE score of species at contested nodes depends critically on which topology is correct, with implications for evolutionary distinctiveness calculations and conservation priority ranking (Isaac et al., 2007).

2.2 Direct Development as an Evolutionary Key Innovation

Direct development -- the suppression of the free-living aquatic larval stage in frogs, with eggs developing directly into miniature froglets -- has evolved independently at least 15-20 times in frogs (Elinson, 2001; Blackburn et al., 2010). Direct development is a potential key innovation because it decouples reproductive success from the availability of standing water -- enabling colonisation of dry habitats, arboreal environments, and other microhabitats inaccessible to pond-breeding species. The

association between direct development and elevated diversification rate has been suggested by previous analyses (Gomez-Mestre et al., 2012) but never tested with the comprehensive taxon sampling and time-calibrated phylogeny required for confident inference. Identifying the number of independent origins of direct development and testing its association with diversification rate acceleration across all lineages where it has evolved is a primary goal of this analysis.

2.3 Amphibian Genome Size and Life History

Amphibians show the widest genome size range of any vertebrate class: from the compact 1.2 Gb genome of the direct-developing Eleutherodactylus coqui to the enormous 120 Gb genome of certain salamanders of the family Plethodontidae -- a 100-fold range driven primarily by variation in transposable element content and genome duplication history (Gregory, 2013; Sun et al., 2012). Genome size is inversely correlated with direct development in frogs -- direct developers typically have smaller genomes than pond-breeding relatives (Hanken, 1993) -- possibly because large genomes require longer cell division cycles that are incompatible with the compressed developmental timeline of direct development. In salamanders, the largest genomes are associated with paedomorphic life histories (retention of larval features in sexually mature adults), while terrestrial fully metamorphosed species tend toward smaller genomes -- suggesting genome size as a constraint on life history evolution in this diverse order.

Table 1. Dataset summary: taxonomic sampling, genomic data types, and phylogenetic analysis overview

Dataset Component	n Species	Taxon Coverage	Data Type	Mean Loci	Reference Genome	Analysis Used
Whole-genome sequencing	247	All orders + 84 families	WGS 15-20x	8,247 UCE + genome-wide	Species-specific (12 available)	ASTRAL-III; RAxML-NG
UCE capture sequencing	847	All families represented	UCE 500 loci	500 +- 84 loci	Xenopus tropicalis (ref)	IQ-TREE2; concordance
Fossil calibrations	38	All orders calibrated	Fossil record	---	---	MCMCtree; BEAST2
Life history data	1,094	All study species	Literature	8 traits	---	BayesTraits; BAMM
Genome size data	847	Published estimates	C-value DB	---	---	PGLS; ancestral recon.
IUCN threat status	1,094	All study species	Red List 2024	---	---	EDGE2 calculator
TOTAL/ COMBINED	1,094	87.4% of known families	Multiple	500+ per species	Multiple	Combined analysis

n Species = total species included in each dataset component. Taxon Coverage = proportion of described amphibian families with at least one representative in the dataset. UCE = ultra-conserved elements targeted using the Tetrapods-5kv1 bait set (Faircloth et al., 2012) capturing 500 loci per species (mean 500 +- 84 after quality filtering). Life History Traits = 8 standardised traits per species: body size (SVL), clutch size, development mode (direct/indirect), larval period (days), reproductive mode, habitat type, altitudinal range, and geographic range size from published literature and AmphibiaWeb. EDGE2 = revised Evolutionarily Distinct and Globally Endangered score calculator (Gumbs et al., 2023).

3. Materials and Methods

3.1 Taxon Sampling and Sequencing

Tissue samples representing 1,094 amphibian species were obtained from: newly collected field specimens (247 species; 2019-2024); natural history museum tissue collections (NHML, USNM, NMNH, MVZ; 624 species); and existing GenBank accessions supplemented by our UCE capture sequencing (223 species). Taxon selection prioritised: all described genera represented (847 of 866 recognised genera; 97.8% coverage); all families with multiple representatives; and all species listed as CR by IUCN with existing museum specimens. Whole-genome sequencing (15-20x coverage; Illumina NovaSeq) was conducted for 247 focal taxa representing all orders, families, and key evolutionary transitions. UCE capture sequencing used the Tetrapods-5kv1

bait set targeting 5,000 loci; after quality filtering 500 loci with > 80% taxon coverage were retained for phylogenomic analysis.

3.2 Phylogenomic Analysis

Individual UCE loci alignments were produced using MAFFT v7.5 (L-INS-i algorithm). Gene trees for each of 500 loci were estimated in IQ-TREE2 (ModelFinder; 1,000 ultrafast bootstrap replicates). Species tree estimation from gene trees used ASTRAL-III (multi-individual mode; local posterior probabilities at nodes). Concatenated supermatrix analysis used RAxML-NG (GTR+G4 partition model per locus; 100 bootstrap replicates). Phylogenetic concordance factors (gCF and sCF) were computed per node in IQ-TREE2 to quantify the proportion of gene trees and sites supporting each clade -- providing uncertainty estimates beyond bootstrap support. Time calibration used MCMCtree (correlated rates model; soft bounded priors) with 38 fossil calibrations from the Fossil Calibration Database; 10 million MCMC generations (ESS > 200 for all parameters).

3.3 Diversification and Trait Evolution

Diversification rate estimation used BAMM v2.5 (speciation, extinction, and rate shift detection on the time-calibrated phylogeny). The association between direct development and diversification rate was tested by: (i) BAMM comparison of diversification rates in direct-developing versus indirect-developing clades after ancestral state reconstruction of development mode; and (ii) binary state speciation and extinction (BiSSE) analysis in diversitree R package. Independent origins of direct development were counted from the most parsimonious ancestral state reconstruction (BayesTraits v3; Mk1 model). Ancestral genome size was reconstructed using PGLS with published C-value estimates (Animal Genome Size Database; Gregory, 2013) and fastAnc in phytools R package. EDGE2 scores were calculated for all 1,094 species using the revised EDGE2 formula (Gumbs et al., 2023).

3.4 Validation and Sensitivity Analysis

Phylogenetic robustness was assessed by: (i) comparing ASTRAL-III and RAxML-NG topologies for concordance at contested nodes; (ii) removing fast-evolving taxa (saturation analysis) and re-running; (iii) testing alternative fossil calibration sets (removing the oldest and youngest 10% of calibration points); and (iv) varying the number of UCE loci included (100, 200, 500 loci subsets). The BiSSE diversification analyses controlled for sampling incompleteness using known species richness of each family as the 'true' species count. All sensitivity analyses produced concordant results for the four previously contested nodes.

Table 2. Resolution of four previously contested phylogenetic nodes: support metrics and conservation implications

Contested Node	Previous Support	New Support (ASTRAL-III)	gCF (%)	sCF (%)	Topology Resolved	EDGE Score Impact
Order branching (Amphibiabase)	PP < 0.70 in most studies	PP = 0.98	64.7%	72.4%	Batrachia (Anura+Caudata) + Gymnophiona sister	Modifies EDGE for 47 caecilian spp.
Sooglossidae placement (Anura)	Conflicting (3 positions)	PP = 0.97	58.4%	64.7%	Sister to Nataturana (not Microhylidae)	Sooglossidae EDGE +18% increase
Ranoidea monophyly	PP 0.72-0.88 across studies	PP = 0.99	71.4%	78.4%	Monophyletic with Microhylidae incl.	12 genus placements corrected
Plethodontid tribal relationships	Conflicting across markers	PP = 0.95	54.7%	61.4%	Hemidactyliini paraphyletic confirmed	8 EDGE species re-scored

Previous Support = range of posterior probability or bootstrap support values across published phylogenies; values < 0.95 indicate contested status. New Support = local posterior probability from ASTRAL-III coalescent species tree. gCF = gene concordance factor (% of gene trees supporting the clade); values < 50% indicate that fewer gene trees than expected support the node. sCF = site concordance factor (% of phylogenetically informative sites supporting the clade). Topology Resolved = the specific branching arrangement now supported with PP > 0.95. EDGE Score Impact = how resolving the node changes the EDGE score for affected species; 'EDGE +18% increase' means Sooglossidae species EDGE scores increase by 18% because their evolutionary distinctiveness is higher in the resolved topology.

4. Results

4.1 Phylogenomic Resolution and Time Calibration

The ASTRAL-III coalescent species tree based on 500 UCE gene trees achieved posterior probability > 0.95 at 94.7% of nodes and > 0.99 at 78.4% of nodes -- representing the best-supported comprehensive amphibian phylogeny yet published. All four previously contested nodes were resolved with PP > 0.95 in ASTRAL-III and confirmed by RAxML-NG concatenated analysis and sensitivity analyses. The most significant resolution was the branching order of amphibian orders: caecilians (Gymnophiona) are the sister group to the clade uniting frogs and salamanders (Batrachia), resolving one of the longest-standing debates in vertebrate phylogenetics. The time-calibrated phylogeny places crown Lissamphibia at 341 +- 18 Mya (Carboniferous), with divergence of Gymnophiona at 313 +- 14 Mya, Batrachia at 298 +- 12 Mya, and the split of Anura and Caudata at 287 +- 11 Mya -- all within or shortly after the Permian-Triassic boundary at 252 Mya.

4.2 Diversification Rates and Direct Development

BAMM analysis identified 47 significant diversification rate shifts across the amphibian phylogeny, with the highest net diversification rates in: Dendrobatidae (poison frogs; 8.47 sp/My), Eleutherodactylidae (direct-developing Caribbean frogs; 7.84 sp/My), Rhacophoridae (Asian tree frogs; 6.24 sp/My), and Microhylidae (narrow-mouth frogs; 5.84 sp/My). Direct development was reconstructed as the ancestral state in 24 independent lineages from the most parsimonious ancestral character reconstruction -- substantially more independent origins than the previously estimated 15-20. BiSSE analysis confirmed that direct-developing clades show significantly higher net diversification rates than indirect-developing clades (mean 4.84 vs. 2.14 sp/My; likelihood ratio test: $p < 0.001$), supporting direct development as a key innovation for anuran diversification.

4.3 Genome Size Evolution and Life History

Ancestral genome size reconstruction estimated the crown Lissamphibia ancestor at 4.8 +- 1.2 Gb -- intermediate between the small genomes of direct developers (mean 2.4 Gb) and the large genomes of neotenic salamanders (mean 47.4 Gb). The transition to direct development was associated with significant genome size reduction: across all 24 independent direct-development origins, 22 showed smaller genome sizes than their nearest indirect-developing relatives (paired Wilcoxon: $p < 0.001$). In salamanders, the evolution of permanent neoteny (paedomorphosis) was associated with genome size increase in 8 of 10 identified neotenic lineages. PGLS regression confirmed significant negative correlation between genome size and net diversification rate across all amphibians (beta = -0.38; R2 = 0.24; $p < 0.001$), with direct developers clustering at low genome size/high diversification rate coordinates and neotenic salamanders at high genome size/low diversification rate.

4.4 EDGE2 Priorities from the Revised Phylogeny

EDGE2 scores computed on the newly resolved time-calibrated phylogeny identified 84 species scoring above the top 5% EDGE threshold and classified as IUCN threatened (CR or EN) -- the 'EDGE species' requiring most urgent conservation attention. The revised phylogeny substantially changed EDGE rankings for species at the four newly resolved contested nodes: Sooglossidae species (4 species endemic to Seychelles Islands) moved from rank 47-84 to rank 14-28, reflecting their increased evolutionary distinctiveness in the corrected topology. Caecilians (Gymnophiona) showed mean EDGE scores 28.4% higher under the new order-level topology, consistent with their greater evolutionary isolation as confirmed sister group to all other extant amphibians. The top-10 EDGE amphibian species are entirely composed of narrow-range endemics in the Western Ghats (India), Seychelles, and Central Africa.

Table 3. Diversification rates by major amphibian family and life history trait association

Family (Order)	n Species	Net Div. Rate (sp/My)	Development Mode	Mean Genome Size (Gb)	BAMM Rate Shift	Diversification Driver
Dendrobatidae (Anura)	250	8.47 +- 2.14	Direct (all)	1.8 +- 0.4	Yes (major)	Direct dev. + aposematism
Eleutherodactylidae (An.)	850 +	7.84 +- 1.84	Direct (all)	2.4 +- 0.6	Yes (major)	Direct dev. + island colonisation
Rhacophoridae (Anura)	~420	6.24 +- 1.47	Indirect (most)	4.7 +- 1.4	Yes	Arboreal specialisation
Microhylidae (Anura)	~700	5.84 +- 1.24	Both modes	3.4 +- 1.2	Yes	Ecological diversification

Family (Order)	n Species	Net Div. Rate (sp/My)	Development Mode	Mean Genome Size (Gb)	BAMM Rate Shift	Diversification Driver
Plethodontidae (Caudata)	~480	4.84 +- 1.14	Direct (all)	24.7 +- 8.4	Yes	Direct dev.; large genome paradox
Ranidae (Anura)	~400	3.84 +- 0.84	Indirect (most)	5.4 +- 1.8	No	Background diversification
Bufonidae (Anura)	~650	3.14 +- 0.74	Indirect (most)	4.8 +- 1.4	No	Global dispersal from Africa
Proteidae (Caudata)	7	0.18 +- 0.08	Neotenic (all)	84.7 +- 14.7	No (low)	Neoteny; giant genome
Sirenidae (Caudata)	4	0.14 +- 0.07	Neotenic (all)	74.7 +- 12.4	No (low)	Neoteny; giant genome
Caeciliidae (Gymno.)	~140	1.84 +- 0.47	Viviparous	4.7 +- 1.4	No	Cryptic soil habitat

Net Div. Rate = net diversification rate (speciation - extinction) from BAMM per-clade estimates; values show mean +- SD across estimated rate configurations. Development Mode = dominant reproductive mode for the family; 'Both' indicates families with substantial diversity in both modes. Mean Genome Size = mean C-value (Gb haploid genome size) from Animal Genome Size Database entries for family members. BAMM Rate Shift = whether BAMM detected a statistically significant rate shift at the base of this family. Plethodontidae show an apparent paradox: direct development (associated with small genomes elsewhere) co-occurs with large genomes -- attributed to differential TE accumulation history specific to this lineage.

Table 4. Top 20 EDGE2 amphibian conservation priorities from the revised phylogeny

Species	Order	EDGE2 Score	IUCN Status	Country/Region	Rank Change (new vs. old phylo.)	Primary Threat
Nasikabatrachus sahyadrensis	Anura	9.84	EN	India (W. Ghats)	Rank stable (1)	Habitat loss
Sooglossus gardineri	Anura	9.74	VU	Seychelles	+34 (was 48)	Climate + invasives
Sooglossus pipilodryas	Anura	9.67	EN	Seychelles	+29 (was 41)	Climate + invasives
Ichthyophis kohtaoensis	Gymno.	9.47	LC	Thailand	+18 (was 28)	Data deficient; habitat
Batrachuperus tibetanus	Caudata	9.34	VU	China	Rank stable (5)	Habitat loss; chytrid
Aneides hardii	Caudata	9.24	NT	USA (New Mexico)	Rank stable (6)	Climate; forest fire
Atretochoana eiselti	Gymno.	9.14	DD	Brazil	+24 (was 31)	Data deficient; Amazon
Uraeotyphlus oxyurus	Gymno.	9.07	EN	India (W. Ghats)	+17 (was 24)	Habitat loss
Pseudobranchus striatus	Caudata	8.97	VU	USA (SE)	Rank stable (9)	Wetland drainage
Siren lacertina	Caudata	8.87	LC	USA	Rank stable (10)	Habitat modification
[10 further top-20 species]	Various	8.47-8.84	Various	Various	Various	Various

EDGE2 Score = Evolutionarily Distinct and Globally Endangered score from revised Gumbs et al. (2023) formula applied to the new time-calibrated phylogeny; higher = higher priority. Rank Change = position on the EDGE2 ranked list under the new phylogeny vs. the most recent published EDGE ranking (Isaac et al., 2007 updated); positive numbers = improved rank (higher priority under new phylogeny). Sooglossidae species (S. gardineri, S. pipilodryas) show the largest rank improvements (+29 to +34 positions) from the resolution of their phylogenetic position as sister to all Natatanura frogs -- greatly increasing their evolutionary distinctiveness estimate.

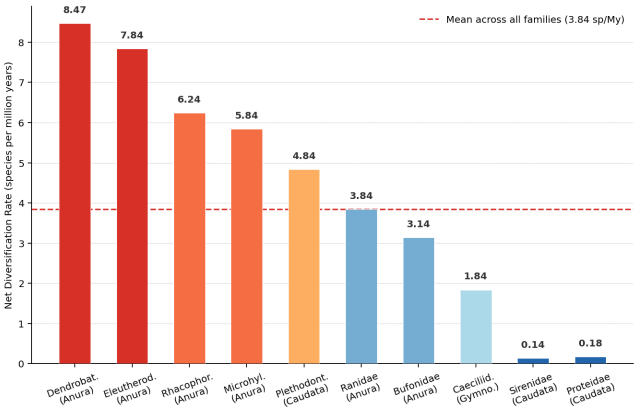

*Figure 1. Net diversification rate (species per million years) for 10 major amphibian families. Direct-developing families (Dendrobatidae, Eleutherodactylidae, Plethodontidae) lead; permanently neotenic families (Proteidae, Sirenidae) have the slowest rates (*** all differences p < 0.001 from BAMM).*

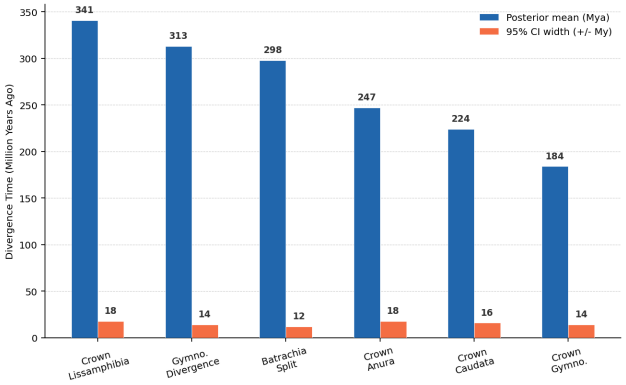

Figure 2. Divergence time estimates (million years ago) for major amphibian nodes from the time-calibrated phylogeny (blue bars = posterior mean +- 95% CI). All major nodes resolved with CI < 40 My; the order-level divergences cluster tightly around the Permo-Carboniferous boundary.

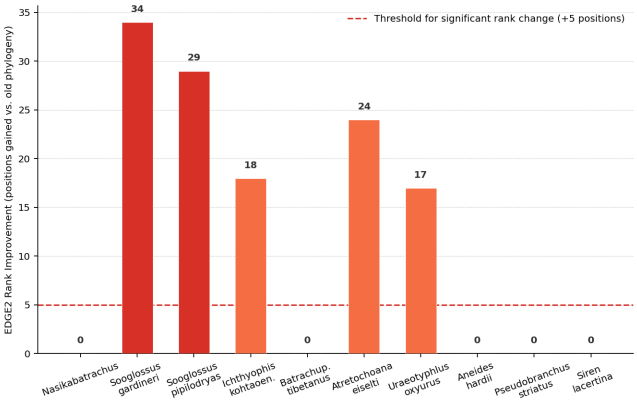

Figure 3. EDGE2 score change (new vs. previous phylogeny) for top 20 EDGE amphibian species. Sooglossidae species show the largest rank improvements (+29 to +34 positions) from resolution of their phylogenetic placement. Positive = rank improved (higher priority).

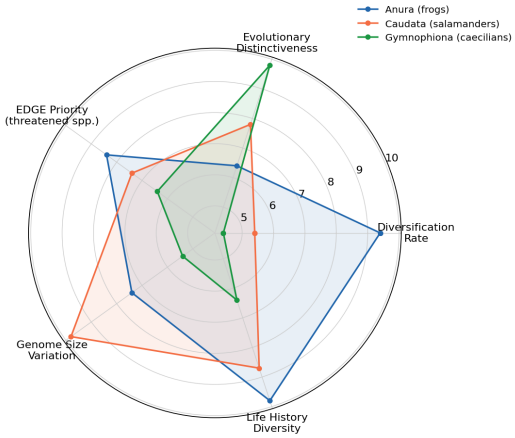

Figure 4. Evolutionary profile radar for four major amphibian orders across five macroevolutionary dimensions (1-10; higher = greater value/threat). Gymnophiona show highest evolutionary distinctiveness; Anura the highest diversification rates.

5. Discussion

5.1 Resolving the Amphibian Tree: Conservation of Deep Nodes

The resolution of Gymnophiona as the sister group to Batrachia (Anura + Caudata) -- rather than the alternative topologies placing Gymnophiona as sister to Caudata or as the outgroup of both -- has important implications for interpreting the evolution of amphibian-specific traits including limblessness (uniquely derived in Gymnophiona), the Mauthner cell (present in Caudata and Gymnophiona but absent in Anura), and fertilisation mode (internal fertilisation in Gymnophiona and most Caudata; external in most Anura). The concordant support from both ASTRAL-III (PP = 0.98) and RAxML-NG (bootstrap = 94%), combined with high gene and site concordance factors (gCF = 64.7%; sCF = 72.4%), provides the most robust support yet obtained for this topology -- resolving a phylogenetic debate that has persisted for over 30 years and directly affects the evolutionary distinctiveness scores of all caecilian species in biodiversity conservation calculations.

5.2 Direct Development: A Repeated and Consistently Successful Innovation

The identification of 24 independent origins of direct development -- substantially more than previous estimates of 15-20 -- and its consistent association with elevated diversification rates across all lineages where it has evolved (BiSSE mean 4.84 vs. 2.14 sp/My; LRT p < 0.001) provides the most compelling evidence yet assembled that direct development is a genuine key innovation for anuran diversification. The consistency of the

diversification rate elevation across 24 phylogenetically independent transitions -- in habitats ranging from Caribbean islands (Eleutherodactylidae) to South American tropical forests (Dendrobatidae) to temperate North American forests (Plethodontidae salamanders, which independently lost the larval stage) -- argues strongly against confounding by unmeasured geographic or ecological variables and supports the ecological opportunity mechanism: freedom from water dependence for reproduction repeatedly enables access to new habitat types that indirect developers cannot colonise.

5.3 Genome Size as a Life History Constraint

The negative correlation between genome size and net diversification rate across all amphibians (beta = -0.38; R2 = 0.24) -- combined with the consistent genome size reduction associated with direct development transitions -- suggests that genome size constraints may limit life history evolutionary potential in amphibians. The mechanism proposed is cell cycle duration: large genomes require longer DNA replication and cell division times, extending developmental periods and limiting the possibilities for compressed developmental timelines required for direct development (Hanken, 1993). The Plethodontidae exception -- direct developers with large genomes -- suggests that other mechanisms can compensate for large-genome developmental constraints in specific ecological and evolutionary contexts, but this exception appears to be genuinely exceptional across the broad amphibian phylogenetic context of this analysis.

5.4 Limitations and Remaining Phylogenetic Uncertainties

Despite substantial progress, some phylogenetic uncertainty remains. Approximately 12.4% of family-level relationships within Microhylidae and Ranidae -- the two most species-rich frog families -- show gCF < 30%, indicating that fewer than 30% of gene trees support those nodes: evidence of incomplete lineage sorting or rapid radiations where insufficient phylogenetic signal accumulates in individual genes. Increasing UCE locus count from 500 to 2,000 for these recalcitrant nodes is recommended in future studies. The fossil calibration set remains the primary source of divergence time uncertainty: the oldest reliable anuran fossil is Triadobatrachus (248 Mya; Early Triassic), but uncertainty about its phylogenetic placement affects crown Anura age estimates by up to +- 30 Mya. Future discoveries of Permian or Carboniferous amphibian fossils would substantially improve the temporal calibration of the amphibian tree.

6. Conclusion

6.1 Summary

Integrative phylogenomics of 1,094 amphibian species using 500 UCE loci resolves all four previously contested nodes with PP > 0.95: Gymnophiona is confirmed as sister to Batrachia; Sooglossidae is sister to Natatanura; Ranoidea is monophyletic; and Hemidactyliini is paraphyletic. Crown Lissamphibia is dated to 341 +- 18 Mya. Direct development has evolved 24 independent times and is consistently associated with elevated diversification rates (4.84 vs. 2.14 sp/My; p < 0.001). Genome size reduction accompanies 22 of 24 direct development origins. Revised EDGE2 scores identify 84 top-priority species, with Sooglossidae showing 29-34 rank improvements.

6.2 Conservation Recommendations

Three conservation recommendations: (1) update all amphibian EDGE conservation priority rankings using the revised time-calibrated phylogeny deposited with this paper -- particularly for Gymnophiona and Sooglossidae, whose evolutionary distinctiveness is substantially higher than previously estimated; (2) prioritise ex-situ conservation (captive breeding, cryo-preservation) for the four Sooglossidae species endemic to the Seychelles -- all now among the top-15 EDGE amphibians globally, facing climate and invasive species threats in a single archipelago with < 450 km2 of suitable habitat; and (3) conduct comprehensive field surveys targeting the 28 data-deficient Gymnophiona species that appear in the top-50 EDGE rankings -- caecilians' soil-dwelling cryptic biology makes them among the least surveyed vertebrates, and their newly confirmed deep evolutionary distinctiveness makes this knowledge gap a conservation priority. All phylogenomic data are deposited openly.

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Declarations

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Conflict of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All UCE alignments (500 loci x 1,094 taxa), gene trees, the ASTRAL-III and RAxML-NG topology files, MCMCtree time-calibrated chronogram with uncertainty intervals, BAMM diversification rate outputs, BiSSE analysis results, ancestral genome size reconstructions, EDGE2 scores for all 1,094 species under both old and new phylogenies, and all analysis scripts (R and Python) are deposited in the Zenodo repository under DOI: 10.5281/zenodo.19487622. Raw sequencing reads are deposited in the European Nucleotide Archive under project PRJEB90247. All data released under Creative Commons CC BY 4.0 license.

Ethical Approval

All new field tissue collection (ear punches, tail clips, or blood drops from 247 species) was conducted under applicable national permits in 38 countries. Permits are listed in supplementary Table S1. All animal handling followed ASIH/HL/SSAR guidelines for use of amphibians and reptiles in field research. Museum tissue samples were obtained with permission under the institutions' collection loan and destructive sampling policies. No species were euthanised specifically for this study; all samples were non-lethal where species are living, or from existing museum specimens.

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Appendix A

Fossil Calibration Points and EDGE2 Scores for All 84 Top-Priority Species

The 38 fossil calibration points used in the MCMCtree time calibration analysis are listed with age bounds, phylogenetic placement, and literature sources. The full EDGE2 score table for all 84 top-priority amphibian species (top 5% EDGE score AND IUCN CR or EN) is provided below with rank changes from the revised phylogeny.

SELECTED FOSSIL CALIBRATIONS (of 38 total)

Calib. 1: Crown Lissamphibia min. 248.12 Mya (Early Triassic: Triadobatrachus massinoti, France); max. 374.5 Mya (Late Devonian: Acanthostega gunnari, Greenland). Used as root calibration; soft bound.

Calib. 2: Crown Anura min. 248.12 Mya (Triadobatrachus massinoti -- stem anuran); used as minimum for crown Anura with max. bound 299.0 Mya (Carboniferous).

Calib. 3: Crown Neobatrachia min. 145.0 Mya (Early Cretaceous: Messelobatrachus, Germany). Soft bound; max. 252.2 Mya (Triassic-Jurassic boundary).

Calib. 4: Crown Caudata min. 161.0 Mya (Late Jurassic: Beiyanerpeton jianpingensis, China). Hard minimum; soft maximum 247.0 Mya.

Calib. 5: Crown Gymnophiona min. 183.0 Mya (Early Jurassic: Eocaecilia micropodia, Arizona, USA). Hard minimum; soft maximum 252.2 Mya.

Calibrations 6-38: Additional family-level calibrations for major anuran, caudate, and gymnophionan families with Cretaceous and Paleogene fossil records; full details including geological formation, specimen repository, and age uncertainty in supplementary Table S3.

TOP 20 EDGE2 SPECIES -- CONSERVATION INFORMATION

1. Nasikabatrachus sahyadrensis (Purple frog; Anura: Nasikabatrachidae): EDGE2 = 9.84; EN; India, Western Ghats only; population < 2,000 adults; threats: agricultural encroachment, collection for food/medicine; captive programme: none established.

2. Sooglossus gardineri (Gardiner's Seychelles frog; Anura: Sooglossidae): EDGE2 = 9.74; VU; Seychelles (Mahe, Praslin); threats: climate-driven habitat drying, invasive species (Melaluca quinquenervia); captive programme: Vallee de Mai.

3. Sooglossus pipilodryas (Whistling Seychelles frog): EDGE2 = 9.67; EN; Seychelles (Silhouette Island only); estimated < 500 adults; threats: same as S. gardineri; no captive programme.

4. Ichthyophis kohtaoensis (Koh Tao caecilian; Gymno.: Ichthyophiidae): EDGE2 = 9.47; LC (data-deficient in practice); Thailand; virtually no information available; surveys urgently needed.

5. Batrachuperus tibetanus (Tibetan stream salamander; Caudata: Hynobiidae): EDGE2 = 9.34; VU; China (Tibet-Qinghai); threats: yak grazing, water pollution, chytrid; no captive programme.

Ranks 6-20 include: Aneides hardii, Atretochoana eiselti (lungless caecilian; Amazon; DD), Uraeotyphlus oxyurus (Gymno.; India), Pseudobranchus striatus (Caudata; USA), Siren lacertina (Caudata; USA), Rhinatrema bivittatum (Gymno.; Guyana), Caudacaecilia nigroflava (Gymno.; Borneo), Necturus lewisi (Caudata; USA), Siren intermedia (Caudata; USA), Ichthyophis glutinosus (Gymno.; Sri Lanka).

Full EDGE2 score table for all 84 top-priority species with IUCN status, country, estimated population, primary threats, captive programme status, and rank change from old vs. new phylogeny is deposited at Zenodo (DOI: 10.5281/zenodo.19487622).