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# Notes and Comments

## Initial Diversification of Living Amphibians Predated the Breakup of Pangaea

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ABSTRACT: The origin and divergence of the three living orders of amphibians (Anura, Caudata, Gymnophiona) and their main lineages are one of the most hotly debated topics in vertebrate evolution. Here, we present a robust molecular phylogeny based on the nuclear RAG1 gene as well as results from a variety of alternative independent molecular clock calibrations. Our analyses suggest that the origin and early divergence of the three living amphibian orders dates back to the Palaeozoic or early Mesozoic, before the breakup of Pangaea, and soon after the divergence from lobe-finned fishes. The resulting new biogeographic scenario, age estimate, and the inferred rapid divergence of the three lissamphibian orders may account for the lack of fossils that represent plausible ancestors or immediate sister taxa of all three orders and the heretofore paradoxical distribution of some amphibian fossil taxa. Furthermore, the ancient and rapid radiation of the three lissamphibian orders likely explains why branch lengths connecting their early nodes are particularly short, thus rendering phylogenetic inference of implicated relationships especially difficult.

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Living amphibians (Lissamphibia) are a successful and highly diversified group of vertebrates that includes thousands of forms (5,770 species; AmphibiaWeb, January 26, 2005; http://www.amphibiaweb.org/) distributed throughout most habitats in all continents except Antarctica (Duellman and Trueb 1994). They experienced a long evolutionary history dating back at least to the early Triassic, the earliest known fossils being *Triadobatrachus* from Madagascar (Rage and Rocek 1989) and *Czatkobatrachus* from Poland (Evans and Borsuk-Bialynicka 1998). The Lissamphibia are widely thought to be a monophyletic group, constituted by three monophyletic orders (Anura, Caudata, and Gymnophiona) whose origin and interrelationships remain hotly debated (see Meyer and Zardoya 2003 for a recent review). The poor fossil record of some major lissamphibian groups and the fact that the three living amphibian orders possibly acquired their specialized morphology very early in their evolutionary histories (Zardoya and Meyer 2001) have left many questions unresolved regarding the origins, relationships, and historical distribution of the Lissamphibia.

A recent molecular phylogeny of lissamphibians based on mitochondrial rRNA genes grouped salamanders and caecilians to the exclusion of frogs and suggested that the early evolutionary history of living amphibians was associated with the Mesozoic continental fragmentation of the supercontinent Pangaea (Feller and Hedges 1998). Paradoxically, some distributional patterns and some data from the fossil record (Estes and Wake 1972; Estes and Reig 1973; Rage and Rocek 1989; Jenkins and Walsh 1993; Duellman and Trueb 1994; Evans et al. 1996; Evans and Borsuk-Bialynicka 1998; Rocek 2000) point at an initial divergence of living amphibians much earlier than the Mesozoic continental fragmentation of the Pangaea supercontinent. Moreover, alternative molecular phylogenies based on complete mitochondrial genomes (Zardoya and

Meyer 2001; San Mauro et al. 2004) support the "Batrachia" hypothesis (Anura + Caudata).

In order to test whether lissamphibian splits were triggered by Mesozoic continental breakup events, and to distinguish among competing hypotheses, we reconstructed a robust molecular phylogeny based on the RAG1 gene, encompassing for the first time a wide taxon sampling of major lissamphibian lineages. We applied a multiple-calibration Bayesian approach to estimate divergence times. This method was developed to avoid biases that were detected in traditional global molecular clock dating methods (Rodríguez-Trelles et al. 2002; Benton and Ayala 2003). It does not require the assumption of a constant rate of evolution, admits several independent calibrations, and allows the use of prior constraints on divergence time instead of fixed time points (Douzery et al. 2004). To confirm the reliability of the estimates based on the Bayesian relaxed molecular clock dating method, we further provide an empirical comparison of age estimates of basal nodes in the Lissamphibia obtained with a variety of alternative independent molecular clock calibrations (both single and multiple).

## Material and Methods

### *Taxon Sampling and DNA Sequencing*

We analyzed 44 amphibian nucleotide sequences of the 3′ end part of the RAG1 gene. This is a nuclear single-copy protein-coding gene that outperforms mitochondrial genes in reconstructing ancient phylogenies (Groth and Barrowclough 1999). The relative rate of evolution of this gene at the nucleotide level is about 2.5 times slower than that of COI (cytochrome c oxidase subunit I) at the amino acid level (San Mauro et al. 2004). For 22 taxa, the sequences were determined for this study using the primers, conditions, and methods reported in San Mauro et al. (2004). Additionally, the following primers were designed to sequence the fragments in some species in which general primers did not amplify: RAG1.R (5′-GGT GYT TYA ACA CAT CTT CCA TYT CRT A-3′), Sal-RAG1.F (5′-CAC YGG GCG CCA GAT YTT CCA RCC-3′), and Sal-RAG1.R1 (5′-AGG TTC TCA GTG TGG CTC CTG GTG A-3′). All nucleotide sequences reported in this article have been deposited in the GenBank database under accession numbers AY583334–AY583355.

Another 22 amphibian RAG1 sequences were obtained from previous studies (Hoegg et al. 2004; San Mauro et al. 2004). The sequences of eight amniotes were used to root the tree; in addition, the coelacanth was used as outgroup for the molecular clock analysis. A complete list of taxa and their higher classification, voucher specimens, collection localities, and GenBank accession numbers can be found in appendix A in the online edition of the *American Naturalist*.

### *Phylogenetic Reconstruction and Molecular Clock Calibration*

Nucleotide sequences were aligned by hand and only one gapped codon was excluded from the analyses (see app. B in the online edition), yielding an alignment of 1,368 positions (only 891 bp were available for *Leiopelma hochstetteri*). RAG1 sequences showed no severe saturation effects, as judged by plots of pairwise differences (absolute, only transitions, and only transversions) versus corrected sequence divergence (measured as maximum likelihood distance, not shown). The RAG1 alignment was subjected to maximum likelihood (ML; Felsenstein 1981), Bayesian inference (BI; Huelsenbeck et al. 2001), minimum evolution (ME; Rzhetsky and Nei 1992), and maximum parsimony (MP; Fitch 1971). Maximum likelihood, ME, and MP analyses were carried out with PAUP* version 4.0b10 (Swofford 1998). Bayesian inference analysis was conducted with MrBayes version 3.0b4 (Huelsenbeck and Ronquist 2001). The best-fitting models of nucleotide substitution were selected using Modeltest version 3.6 (Posada and Crandall 1998), following the Akaike Information Criterion (AIC). Maximum likelihood and ME analyses assumed the parameter-rich GTR (Rodríguez et al. 1990) + $\Gamma$ + I model for all positions. Bayesian inference analyses were also performed using the GTR + $\Gamma$ + I substitution model, although in this case parameter estimations were independently assessed for each codon position ("unlink" option). Maximum likelihood, ME, and MP analyses were performed using heuristic searches with TBR branch swapping and 10 random stepwise additions of taxa. Nonparametric bootstrapping was used to test the reliabilities of the ML, ME, and MP trees (100 pseudoreplicates for ML, and 1,000 pseudoreplicates for ME and MP). Bayesian inference analyses were performed simulating four simultaneous chains, for a million generations, sampling every 100 generations. Generations sampled before the chain reached stationarity (100,000) were discarded ("burn-in").

Divergence times were determined using a Bayesian approach that incorporates variation of rates of evolution among genes and among lineages (Thorne et al. 1998; Kishino et al. 2001; Thorne and Kishino 2002). We used the ML topology that was inferred based on the RAG1 data set as the starting phylogeny. Branch lengths of the inferred topology and divergence times were estimated using the programs Estbranches and Multidivtime, respectively (http://statgen.ncsu.edu/thorne/). The Bayesian method also requires the specification of prior distributions for parameters. The prior assumption for the mean

and standard deviation of the time of the ingroup root node (rttm) was set to 42 time units, where 1 time unit in this analysis represents 10 million years. This value was obtained based on the split of coelacanth and tetrapod lineages 420 million years ago (mya; Zhu et al. 2001). The standard deviation of the prior distribution was set to its maximum value (equal to the mean) to avoid violation of the definition of a prior. The divergence among diapsids and synapsids (Kumar and Hedges 1998) was used as the main calibration point. Considering the criticism of Graur and Martin (2004), we calibrated this split at 338–288 mya, as proposed by these authors, and, in addition, included multiple internal calibrations within the Lissamphibia as upper and lower time constraints. Four of these internal calibrations were based on fossil record: minimum age of the frogs-salamander split (node 36; see app. C in the online edition) at 230 mya (fossil record of frog ancestor *Triadobatrachus*; Rage and Rocek 1989); minimum age of the split among hynobiid and cryptobranchid salamanders (node 33) at 161 mya (cryptobranchid fossil record; Gao and Shubin 2003); minimum age of the split of pipid frogs from their sister group (node 24) at 140 mya (records of Mesozoic fossil pipids; Rocek 2000); minimum age of the split between *Caudiverbera* and *Lechriodus* (node 5) at 53 mya (fossil records of *Caudiverbera*; Baez 2000). The other four internal calibrations were based on biogeographical events: minimum age of the split among the caecilians *Gegeneophis* and *Geotrypetes* (node 37) at 130 mya (Gondwana fragmentation, separation of India-Seychelles-Madagascar from Africa; Rabinowitz et al. 1983); minimum age of the separation among South American and African pipid frogs (node 21) at 86 mya (separation of Africa and South America; Pitman et al. 1993); minimum age of the split between *Agalychnis* and *Litoria* (node 1) at 42 mya (last connection between Australia and South America; Seddon et al. 1998); maximum age of the split between *Mantidactylus wittei* and *Mantidactylus* sp. from the Comoro islands (node 7) at 15 mya (volcanic origin of the oldest Comoro island Mayotte; Vences et al. 2003). These calibrations exhibited a significant fit between time and divergence (see app. D in the online edition).

Divergence times were also independently reestimated using the following single and multiple calibrations (see table 1 for details): (1) the single calibration proposed by Kumar and Hedges (1998), (2) the correction to 1 proposed by Graur and Martin (2004), (3) the single calibration proposed by Reisz and Müller (2004), (4) the single calibration used by Vences et al. (2003), and (5) our multiple calibration plus the single calibration proposed by Reisz and Müller (2004).

The Markov chain Monte Carlo (MCMC) method was employed to approximate both prior and posterior distributions (Kishino et al. 2001). Initial parameter values were randomly selected to initialize the Markov chain, and then a burn-in period of 100,000 cycles was completed before parameters were sampled from the MCMC chain. Afterward, the state of the Markov chain was sampled every 100 cycles until a total of 10,000 generations.

## Results and Discussion

### *Early Separation of the Three Lissamphibian Orders during the Paleozoic*

According to our results, the ancestral lineage of caecilians separated from the common ancestor of batrachians approximately 367 (417–328; 95% confidence interval [CI]) mya (fig. 1). The divergence of salamanders and frogs occurred shortly thereafter, 357 (405–317) mya (fig. 1). Although the "Batrachia" hypothesis is not strongly supported by our results, it can be considered as the best explanation given the available data on the basis that all phylogenetic methods yielded phylograms with this topology (no method recovered alternative arrangements) and ME and MP found substantial (>70%) statistical support for the clade Batrachia (see also Zardoya and Meyer 2001).

Analyses of our data set with single and alternative calibrations (e.g., those of Kumar and Hedges [1998] and Reisz and Müller [2004]) produced concordant results (table 1). In all cases, a Paleozoic age of separation between the three amphibian orders was estimated (367–297 mya). In addition, all estimates agreed that the initial splittings within living salamanders and frogs occurred during the Permian–Triassic (273–204 mya), whereas the basal splits among living caecilians were estimated to be slightly younger in some of the analyses (214–150 mya).

These results may indicate that the separation of the three orders of modern amphibians in the Paleozoic occurred almost immediately (in evolutionary time) after the "jump to land" of sarcopterygian fishes (360 mya), as had been postulated by Benton (1990), Milner (1993), and Carroll et al. (2004), and in parallel with the diversification of extinct lineages of amphibians (e.g., *Acanthostega* or *Ichthyostega*). Such a rapid radiation event may be the cause for the lack of fossils that represent plausible ancestors or morphological immediate sister taxa of all three lissamphibian orders and the particularly short branch lengths connecting the nodes among them, thereby rendering phylogenetic inference more difficult.

These results disagree with the hypothesis that salamanders (Laurasia) and caecilians (Gondwana) arose in the Mesozoic from a common ancestor by vicariance directly linked to the breakup of supercontinent Pangaea, with frogs separating from the amphibian stem lineage much earlier during the Paleozoic (Feller and Hedges

**Table 1:** Comparison of age estimates of basal nodes in the Lissamphibia, their standard deviation (SD), and 95% confidence intervals (CI) obtained with different calibrations (multiple and single)

| Node and calibration[a] | Node[b] | Age | SD | Upper CI | Lower CI |
|---|---|---|---|---|---|
| Gymnophiona-Batrachia: | | | | | |
| Multiple | 43 | 367 | 23 | 328 | 417 |
| Kumar and Hedges 1998 | 43 | 342 | 16 | 315 | 376 |
| Graur and Martin 2004 | 43 | 344 | 22 | 305 | 392 |
| Reisz and Müller 2004 | 43 | 359 | 39 | 299 | 453 |
| Vences et al. 2003 | 43 | 309 | 101 | 144 | 534 |
| Multiple + RM | 43 | 366 | 23 | 325 | 416 |
| Caudata-Anura: | | | | | |
| Multiple | 36 | 357 | 22 | 317 | 405 |
| Kumar and Hedges 1998 | 36 | 329 | 17 | 297 | 365 |
| Graur and Martin 2004 | 36 | 331 | 23 | 289 | 379 |
| Reisz and Müller 2004 | 36 | 346 | 38 | 285 | 436 |
| Vences et al. 2003 | 36 | 297 | 98 | 137 | 514 |
| Multiple + RM | 36 | 356 | 22 | 315 | 405 |
| Gymnophiona: | | | | | |
| Multiple | 42 | 214 | 20 | 177 | 256 |
| Kumar and Hedges 1998 | 42 | 168 | 27 | 115 | 221 |
| Graur and Martin 2004 | 42 | 169 | 28 | 115 | 224 |
| Reisz and Müller 2004 | 42 | 177 | 30 | 121 | 239 |
| Vences et al. 2003 | 42 | 150 | 58 | 61 | 281 |
| Multiple + RM | 42 | 213 | 20 | 177 | 254 |
| Caudata: | | | | | |
| Multiple | 35 | 273 | 19 | 238 | 312 |
| Kumar and Hedges 1998 | 35 | 229 | 23 | 182 | 273 |
| Graur and Martin 2004 | 35 | 231 | 26 | 180 | 280 |
| Reisz and Müller 2004 | 35 | 241 | 32 | 184 | 313 |
| Vences et al. 2003 | 35 | 206 | 72 | 90 | 365 |
| Multiple + RM | 35 | 271 | 19 | 237 | 312 |
| Anura: | | | | | |
| Multiple | 24 | 262 | 21 | 223 | 305 |
| Kumar and Hedges 1998 | 24 | 227 | 22 | 184 | 268 |
| Graur and Martin 2004 | 24 | 228 | 24 | 180 | 276 |
| Reisz and Müller 2004 | 24 | 238 | 31 | 183 | 307 |
| Vences et al. 2003 | 24 | 204 | 70 | 91 | 359 |
| Multiple + RM | 24 | 262 | 21 | 222 | 305 |
| Hyloidea: | | | | | |
| Multiple | 4 | 65 | 8 | 52 | 84 |
| Kumar and Hedges 1998 | 4 | 42 | 10 | 26 | 63 |
| Graur and Martin 2004 | 4 | 42 | 10 | 25 | 64 |
| Reisz and Müller 2004 | 4 | 44 | 11 | 26 | 68 |
| Vences et al. 2003 | 4 | 37 | 15 | 15 | 72 |
| Multiple + RM | 4 | 65 | 8 | 52 | 84 |
| Ranoidea: | | | | | |
| Multiple | 9 | 99 | 16 | 70 | 132 |
| Kumar and Hedges 1998 | 9 | 78 | 16 | 50 | 111 |
| Graur and Martin 2004 | 9 | 78 | 16 | 50 | 113 |
| Reisz and Müller 2004 | 9 | 82 | 18 | 52 | 121 |
| Vences et al. 2003 | 9 | 69 | 25 | 28 | 127 |
| Multiple + RM | 9 | 99 | 16 | 71 | 132 |

[a] The nodes refer to the splits between caecilians and the salamander-frog clade (Batrachia), between salamanders and frogs, and to the initial splits of caecilians, salamanders, frogs, hyloids, and ranoids. The calibrations used were (1) the preferred multiple calibration as described in "Material and Methods" and shown in figure 1; (2) the single calibration proposed by Kumar and Hedges (1998), namely, a fixed synapsid-diapsid divergence at 310 mya; (3) the correction to the synapsid-diapsid calibration proposed by Graur and Martin (2004), 288–338 mya; (4) the single calibration proposed by Reisz and Müller (2004) for the crocodile-bird split, 227–242 mya; (5) the calibration used by Vences et al. (2003) based on endemic frogs of the oceanic island Mayotte (maximum age constraint 15 mya); (6) the preferred multiple calibration plus the single calibration proposed by Reisz and Müller (2004; "Multiple + RM").

[b] Node numbers are as in appendix C in the online edition.

1998). That hypothesis was based on a ribosomal molecular phylogeny and the geographical distribution of the amphibian fossil record, but it lacked molecular clock estimates. The RAG1-based hypothesis of a Paleozoic origin of all modern amphibian groups predating the breakup of Pangaea, as well as the tentative salamander + frog clade in our tree, therefore invalidate Feller and Hedges's (1998) hypothesis. Furthermore, the presence of the putative stem-group caecilian *Eocaecilia* in Laurasia (early Jurassic of North America; Jenkins and Walsh 1993) could not previously be reconciled with that hypothesis.

### *Initial Splittings within the Living Caecilians in the Early Mesozic*

The presence of living caeciliids in South America, Africa, Seychelles, and India, as well as the African affinities of a Paleocene caeciliid fossil (*Apodops*) found in South America (Estes and Wake 1972) suggest that the split of the major extant caecilian lineages occurred before the breakup of Gondwana. A successive dispersal from the Indian Plate subsequent to its collision with Asia has been proposed to explain the origin of ichthyophiid caecilians in Southeast Asia (Duellman and Trueb 1994; Wilkinson et al. 2002). Our results indicate that the time of initial splitting within the modern caecilians occurred about 214 (256–177) mya (fig. 1), when the rhinatrematid lineage separated from the ancestry of all other caecilians, and that the main basal divergences (including the time of initial splitting within the higher caecilians comprising scolecomorphids, caeciliids, and typhlonectids 177 [218–148] mya) took place in the early Mesozoic (fig. 1). Both the old origin, before the breakup of Gondwana, and the presently restricted geographical distribution of many caecilian lineages may indicate that the most ancient clades (rhinatrematids and the ichthyophiid + uraeotyphlid clade) are relicts of groups that may once have been widespread in Gondwana, whereas more recently derived clades such as scolecomorphids and typhlonectids may have evolved in situ and never achieved a wider distribution (Duellman and Trueb 1994).

### *Initial Splittings within the Living Salamanders in the Late Paleozoic*

Salamanders have a mostly Laurasian distribution, and it seems fairly clear that all salamander lineages arose in the Laurasian part of Pangaea (Duellman and Trueb 1994). However, Mesozoic sirenid fossils are known from both South America (*Noterpeton*) and Africa (*Kababisha*) (Evans et al. 1996) and may raise doubts about an exclusive Laurasian origin of salamanders. Our results indicate that the initial splitting within modern salamanders occurred during the late Paleozoic, 273 (312–238) mya, when the sirenids and the hynobiid + cryptobranchid clade separated from the ancestor of all other salamanders (fig. 1). Interestingly, cryptobranchids, hynobiids, and sirenids all have external fertilization and angular and prearticular bones of the lower jaw not fused, which are considered ancestral traits (Duellman and Trueb 1994). The estimated time of separation of the plethodontids from the ambystomatid + salamandrid clade later occurred about 253 (294–213) mya, and of the ambystomatids from salamandrids about 230 (274–188) mya (fig. 1). Hence, the main divergences of salamanders must have taken place before the breakup of Pangaea and also before the earliest fragmentation of Laurasian landmasses, which began with the opening of the North Atlantic Ocean in the early Jurassic (Smith et al. 1994).

### *Initial Splittings within the Living Frogs in the Late Paleozoic*

The discoveries of *Triadobatrachus* from the early Triassic of Madagascar (Rage and Rocek 1989) and *Czatkobatrachus* from the early Triassic of Poland (Evans and Borsuk-Bialynicka 1998) suggest that Salientia (the stem group of frogs) occurred in all Pangaea. Duellman and Trueb (1994) considered the leiopelmatids to be the sister group of all other frogs, widely distributed before the breakup of Pangaea (Jurassic fossils, *Vieraella* and *Notobatrachus*, are known from Argentina; Estes and Reig 1973), of which the living genera (*Ascaphus* in North America and *Leiopelma* in New Zealand) are merely relicts. Our results show that the estimated time of initial splitting within the living frogs occurred about 263 (305–223) mya, when the leiopelmatids separated from the ancestor of all other living frogs (fig. 1). The subsequent estimated dates of origin of pipids at about 245 (288–204) mya, discoglossids at 235 (277–195) mya, and pelobatoideans 216 (260–176) mya indicate that the divergences of all major archaeobatrachian groups occurred much earlier than the Pangaean fragmentation (fig. 1). These age estimates, together with the recovered paraphyly of archaeobatrachians, may indicate that they are likely remnants of an ancient and relatively fast radiation (Duellman and Trueb 1994; Hoegg et al. 2004) and would call into question the earlier proposal (Feller and Hedges 1998) of a Mesozoic vicariant origin of archaeobatrachians and neobatrachians being directly linked to the fragmentation of Pangaea. Furthermore, the present and Mesozoic fossil Gondwanan distribution of pipid frogs (Duellman and Trueb 1994; Rocek 2000) is geographically inconsistent with that proposal (Feller and Hedges 1998).

Most of the neobatrachian families sampled in this study were clearly placed in either of two well-defined clades,

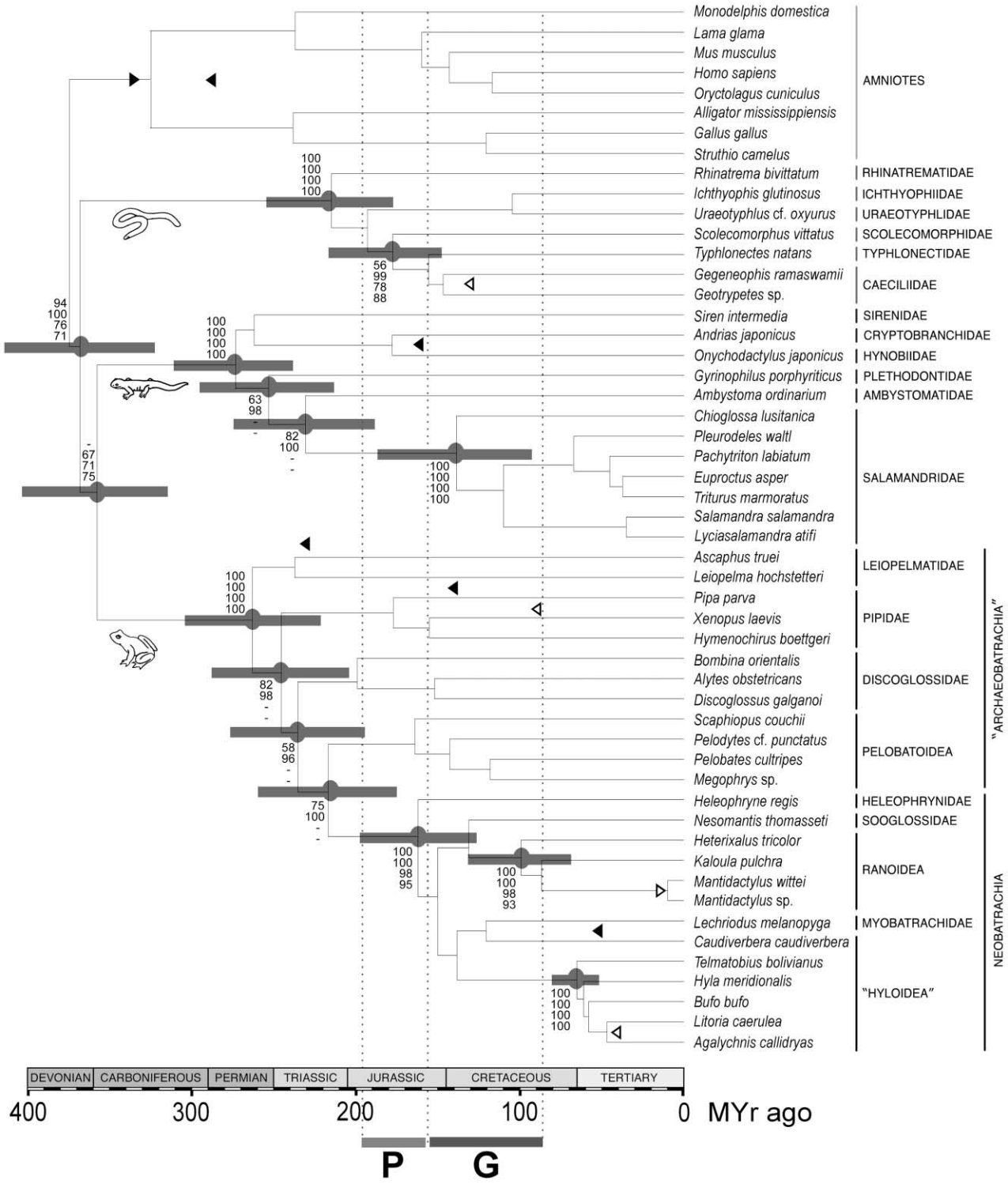


**Figure 1:** Maximum likelihood phylogeny and estimates of time divergence for the major lineages of living amphibians, estimated from 1,368 nucleotide positions of the RAG1 gene. Calibrations, as listed in "Material and Methods," are marked by triangles (upper and lower bounds). Filled triangles represent calibrations based on fossil record; open triangles represent calibrations based on biogeography. Gray bars mark confidence intervals of age estimates. Dotted vertical lines mark the periods of the initial breakup of Pangaea in Laurasia and Gondwana (*P*) and the breakup of Gondwana (*G*). Numbers adjacent to nodes indicate support for maximum likelihood (upper value of each quartet), Bayesian inference (middle-upper value), minimum evolution (middle-lower value), and maximum parsimony (lower value). Hyphens indicate support values of <50. Statistical support and confidence intervals are shown only for nodes relevant to the "Discussion." A detailed table with support values and age estimates for all nodes can be found in appendix C in the online edition.

the Hyloidea and Ranoidea, which are species-rich radiations containing many additional families (Hoegg et al. 2004). These radiations, according to our molecular clock estimates, occurred at around 65 (83–52) mya and 99 (132–70) mya, respectively (fig. 1), which agrees well with the hypothesis (Feller and Hedges 1998) that they occurred in South America and Africa after the separation of these two continents at 110–86 mya (Pitman et al. 1993). Their young age is not an artifact caused by any of the internal calibrations because calculations based on the single synapsid-diapsid split (table 1) led to similar estimates. Interestingly, the leptodactylid *Caudiverbera* is strongly recovered outside the clade comprising all other Hyloidea (fig. 1; see also app. B), suggesting that its family attribution needs to be revised.

It is remarkable that several species-poor neobatrachian clades originated in much earlier periods than the radiations of hyloideans and ranoideans. This includes the South African heleophrynids, the Australian myobatrachids, the Seychellean sooglossids, and, according to our data, the Neotropical *Caudiverbera*, which is restricted to the southern tip of South America. Probably also the recently discovered Indian *Nasikabatrachus* is one of these early lineages of neobatrachians (Biju and Bossuyt 2003) that radiated, according to our new data, between about 162 (199–128 mya; split of *Heleophryne* from other neobatrachians) and 120 (154–91 mya; split of *Caudiverbera* from myobatrachids) mya. This initial diversification occurred before the breakup of Gondwana, as indicated by the wide, though localized, distribution of their extant representatives. Their current restriction to geographic refuges indicates that these early neobatrachians may have been more widespread but were outcompeted by the more modern hyloid and ranoid radiations in large parts of their original distribution area.

### *Reliability of Relaxed Clock Estimates*

Until recently, molecular datings were estimated under the assumption of a constant-rate evolution (Nei et al. 2001). To estimate divergence times, a linearized (ultrametric) tree was constructed, and a timescale for the tree was produced using one or several (through a linear regression fitting) calibration points. Molecular clocks estimated this way are highly controversial because they often conflict with paleontological evidence (Benton and Ayala 2003). The source of this discrepancy relies on constraints inherent to both kinds of data. Divergence times inferred by paleontologists can only be underestimates of the actual origin of a lineage (Benton and Ayala 2003), provided that chronological assignments of fossils are correct. Moreover, if the fossil record for a given lineage is particularly poor, these underestimations can become misleading (Reisz and Müller 2004). On the other hand, conventional molecular dating approaches suffer from several limitations that lead to overestimation biases (Rodríguez-Trelles et al. 2002; Benton and Ayala 2003). Limited taxon sampling or calibration points can seriously affect molecular dating estimates (Douzery et al. 2004). However, the most pervasive handicaps are the significant violations of the assumption of a constant rate of evolution that may be undetected due to the limited statistical power of relative-rate tests (Bromham et al. 2000) and the asymmetric distribution of molecular time estimates (with an unconstrained older end) that leads to a systematic overestimation bias (Rodríguez-Trelles et al. 2002). Well-known examples of this controversy (i.e., consistently older molecular estimates than known fossil evidence) have been reported at the origin of vascular land plants, modern birds, and placental mammals (Benton and Ayala 2003).

In this study, we have tried to reduce the biases of conventional molecular dating by selecting a gene that has an appropriate rate of evolution for the question at hand, increasing taxon sampling, and applying the most recent Bayesian analytical techniques that relax molecular clock assumptions and allow the incorporation of multiple independent calibration constraints. A recent study (Douzery et al. 2004) showed that estimated molecular ages using the same Bayesian approach are less prone to overestimation than conventional molecular clock methods. Therefore, we believe that most of our molecular age estimates can be considered a reasonable approximation of the actual divergence times for the main lineages of living amphibians. Indeed, many molecular date estimates within the lissamphibian clade seem to agree very well with paleontological evidence. For instance, recent paleontological studies place the separation of the three orders of living amphibians back into the early Carboniferous (Carroll 2001; Carroll et al. 2004). The means of our estimated dates for these splits go back into the late Devonian, but CIs of these estimates also cover the early Carboniferous. Therefore, we cannot rule out a slight overestimation that is negligible when CIs are considered. Nonetheless, we are aware that some dates may be considerably overestimated, as is the case for example of the split between marsupials and placental mammals. This divergence is thought to have occurred sometime in the late Jurassic (Kumar and Hedges 1998) or early Cretaceous (Benton 1990). However, our analyses place this divergence between the late Permian and early Jurassic. Although the source of this discrepancy is unclear, it may be related to the limited taxon sampling within the outgroup.

Although we believe that most of our time estimates are most likely quite accurate, we are aware that they need to be interpreted with caution. In any case, overall the estimated dates for the initial splits within the living am-

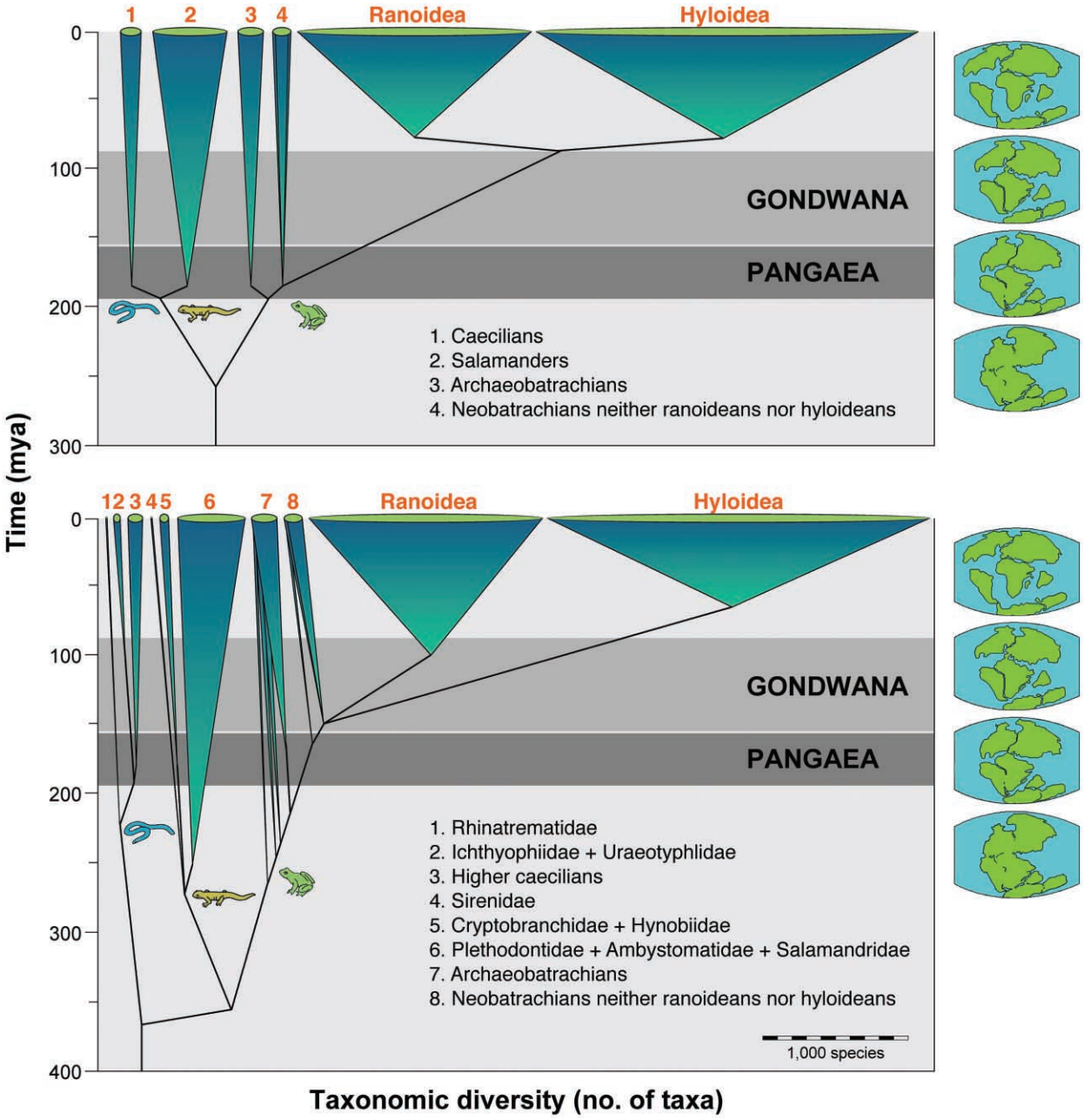


**Figure 2:** Comparative schematic graph of the radiations of living amphibians according to results in this study (*bottom*) and Feller and Hedges's (1998) hypothesis (*top*). The cross sections of the cones indicate roughly the number of extant species within a group. Shaded bands mark the periods of the breakups of Pangaea and Gondwana.

phibians are so old in comparison with the breakup of Pangaea that it is rather improbable that these splits were actually linked to the continental fragmentation of this supercontinent. Additional information from other genes (Nei et al. 2001; Thorne and Kishino 2002) and fossils (Reisz and Müller 2004) as well as finer calibrations would be desirable to obtain more accurate time estimates and would help reconcile molecular and fossil evidence.

## Conclusions

This study presents a comprehensive sampling of most major amphibian lineages for a nuclear protein-coding

gene, and it is the first that makes use of multiple and independent calibrations across the different lissamphibian groups to date major cladogenetic events within extant amphibians. Our results reject the hypothesis that early lissamphibian diversification was triggered by the continental breakup of Pangaea. A few phylogenetic patterns and datings recovered herein agree with scenarios of vicariance in the context of continental breakup, such as the hyloid-ranoid split and the initial diversification of neobatrachians (fig. 2). However, the origin as well as the initial diversification of salamanders, frogs, and caecilians predated the fragmentation of Pangaea (fig. 2). Antiquity of lissamphibian branches likely accounts for the long independent evolution of many convergent patterns in morphology and life history (Duellman and Trueb 1994). Our data provide old age estimates for many extant lissamphibian groups, but they also suggest that the most diverse clades (hyloid and ranoid neobatrachians, which together contain more species than all other amphibians combined; fig. 2) are younger than commonly thought. Ecological displacement by such young species-rich radiations might therefore have caused the extinction and current geographical restrictions of most older taxa, thereby masking the initial biogeographic patterns. Our study thereby provides a useful evolutionary framework that will be important in future studies on amphibian biology. The hypothesis presented here of a probable old origin of many of the major lineages of living amphibians, some of which are geographically restricted and now species poor, turns them into real "living fossils" among extant tetrapods, emphasizing the importance and urgency of the efforts that should be afforded for their conservation.

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# Appendix A from D. San Mauro et al., "Initial Diversification of Living Amphibians Predated the Breakup of Pangaea"

**(Am. Nat., vol. 165, no. 5, p. 590)**

## Voucher Specimens, Localities, and Classification of Taxa Studied

*Newly determined amphibian sequences (MNCN/ADN, Museo Nacional de Ciencias Naturales, Spain; SIH, University of Konstanz, Germany).* Anura: *Leiopelma hochstetteri* (Leiopelmatidae; New Zealand), *Hymenochirus boettgeri* (Pipidae; pet trade), *Alytes obstetricans* (Discoglossidae; MNCN/ADN 4313; Tielmes, Spain), *Bombina orientalis* (Discoglossidae; MNCN/ADN 4314; pet trade), *Discoglossus galganoi* (Discoglossidae; MNCN/ADN 4315; Reliegos, Spain), *Pelodytes* cf. *punctatus* (Pelodytidae; MNCN/ADN 8000; Portalegre, Portugal), *Lechriodus melanopyga* (Myobatrachidae; MNCN/ADN 8001; pet trade, Papua New Guinea), *Caudiverbera caudiverbera* (Leptodactylidae; MNCN/ADN 8002; pet trade, Chile), *Bufo bufo* (Bufonidae; MNCN/ADN 8003; Valdemanco, Spain), *Hyla meridionalis* (Hylidae; MNCN/ADN 8004; Logrosán, Spain), *Telmatobius bolivianus* (Leptodactylidae; MNCN/ADN 563; La Paz, Bolivia). Caudata: *Andrias japonicus* (Cryptobranchidae; MNCN/ADN 8005; pet trade), *Onychodactylus japonicus* (Hynobiidae; SIH-13), *Siren intermedia* (Sirenidae; Tallahassee, FL, USA), *Gyrinophilus porphyriticus* (Plethodontidae; MNCN/ADN 8006; North Carolina, USA), *Ambystoma ordinarium* (Ambystomatidae; MNCN/ADN 8007; Michoacán, Mexico), *Chioglossa lusitanica* (Salamandridae; MNCN/ADN 8008; Pobra do Caramiñal, Spain), *Salamandra salamandra* (Salamandridae; MNCN/ADN 8009; Miraflores de la Sierra, Spain), *Triturus marmoratus* (Salamandridae; MNCN/ADN 8010; Arrillor, Spain), *Euproctus asper* (Salamandridae; MNCN/ADN 8011; Zuriza, Spain), *Pachytriton labiatum* (Salamandridae; MNCN/ADN 8012; pet trade, China). Gymnophiona: *Geotrypetes* sp. (Caeciliidae; pet trade, Cameroon).

*Amphibian sequences from previous studies (with GenBank accession numbers).* Anura: *Ascaphus truei* (Leiopelmatidae; AY323754), *Pipa parva* (Pipidae; AY323761), *Xenopus laevis* (Pipidae; L19324), *Pelobates cultripes* (Pelobatidae; AY323758); *Scaphiopus couchii* (Pelobatidae; AY323759), *Megophrys* sp. (Megophryidae; AY323760), *Heleophryne regis* (Heleophrynidae; AY323764), *Nesomantis thomasseti* (Sooglossidae; AY323778), *Heterixalus tricolor* (Hyperoliidae; AY323768), *Mantidactylus* sp. (Mantellidae; AY323775), *Mantidactylus wittei* (Mantellidae; AY323774), *Kaloula pulchra* (Microhylidae; AY323772), *Litoria caerulea* (Hylidae; AY323767), *Agalychnis callidryas* (Hylidae; AY323765). Caudata: *Lyciasalamandra atifi* (Salamandridae; AY456261), *Pleurodeles waltl* (Salamandridae; AJ010258). Gymnophiona: *Rhinatrema bivittatum* (Rhinatrematidae; AY456257), *Ichthyophis glutinosus* (Ichthyophiidae; AY456256), *Uraeotyphlus* cf. *oxyurus* (Uraeotyphlidae; AY456259), *Scolecomorphus vittatus* (Scolecomorphidae; AY456258) *Gegeneophis ramaswamii* (Caeciliidae; AY456255), *Typhlonectes natans* (Typhlonectidae; AY456260).

*Outgroup sequences. Alligator mississippiensis* (Crocodylidae; AF143724), *Struthio camelus* (Struthionidae; AF143727), *Gallus gallus* (Phasianidae; M58530), *Monodelphis domestica* (Didelphidae; U51897), *Lama glama* (Camelidae; AF305953), *Mus musculus* (Muridae; M29475), *Oryctolagus cuniculus* (Leporidae; M77666), *Homo sapiens* (Hominidae; NM_000448), *Latimeria menadoensis* (Coelacanthidae; AY442925).

# Appendix B from D. San Mauro et al., "Initial Diversification of Living Amphibians Predated the Breakup of Pangaea"

**(Am. Nat., vol. 165, no. 5, p. 590)**

## Gapped Codon Excluded from the Alignment

A synapomorphic codon insertion was observed in the RAG1 nucleotide sequence of the representatives of our well-defined Hyloidea clade (*Telmatobius bolivianus*, *Litoria caerulea*, *Agalychnis callidryas*, *Hyla meridionalis*, and *Bufo bufo*) with respect to all other amphibians and amniotes. This indel corresponds to that reported by Venkatesh et al. (2001) between positions 637 and 638 of the human RAG1 amino acid sequence and is an amino acid deletion in tetrapods with respect to fishes. Our more comprehensive alignment allowed us to correctly relocate the indel at amino acid position 636–637, where lobe-finned fishes had a serine that is lost in tetrapods but secondarily reevolved in the above-mentioned anuran species, thereby providing further evidence for the monophyly of the group to the exclusion of *Caudiverbera* (which lacks this synapomorphic trait).

### Literature Cited in Appendix B

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# Appendix C from D. San Mauro et al., "Initial Diversification of Living Amphibians Predated the Breakup of Pangaea"

**(Am. Nat., vol. 165, no. 5, p. 590)**

## Extended Result of the Phylogenetic and Molecular Clock Analyses

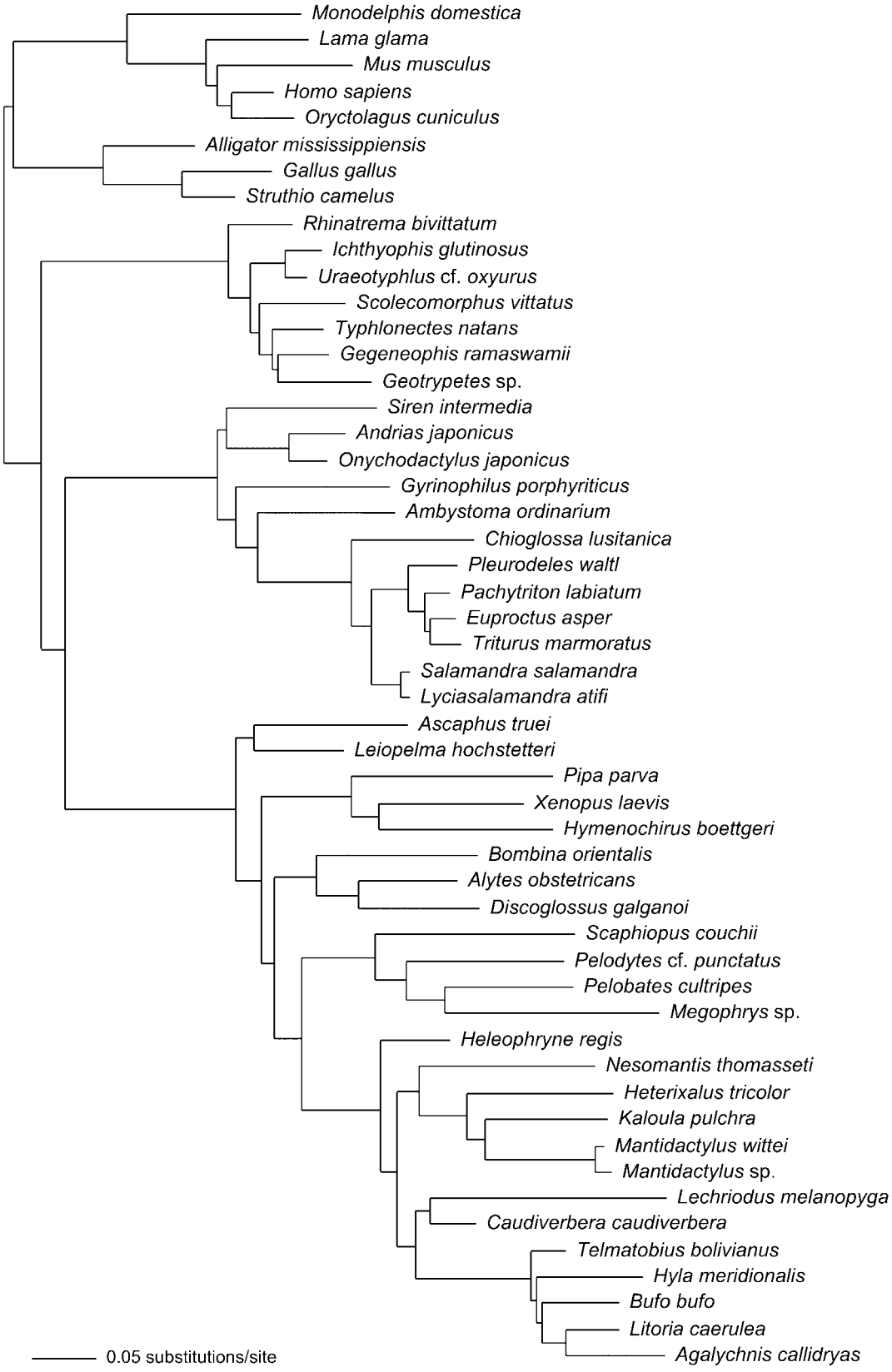


**Figure C1:** Unconstrained (nonultrametric) maximum likelihood phylogram showing the pattern of rate variability.

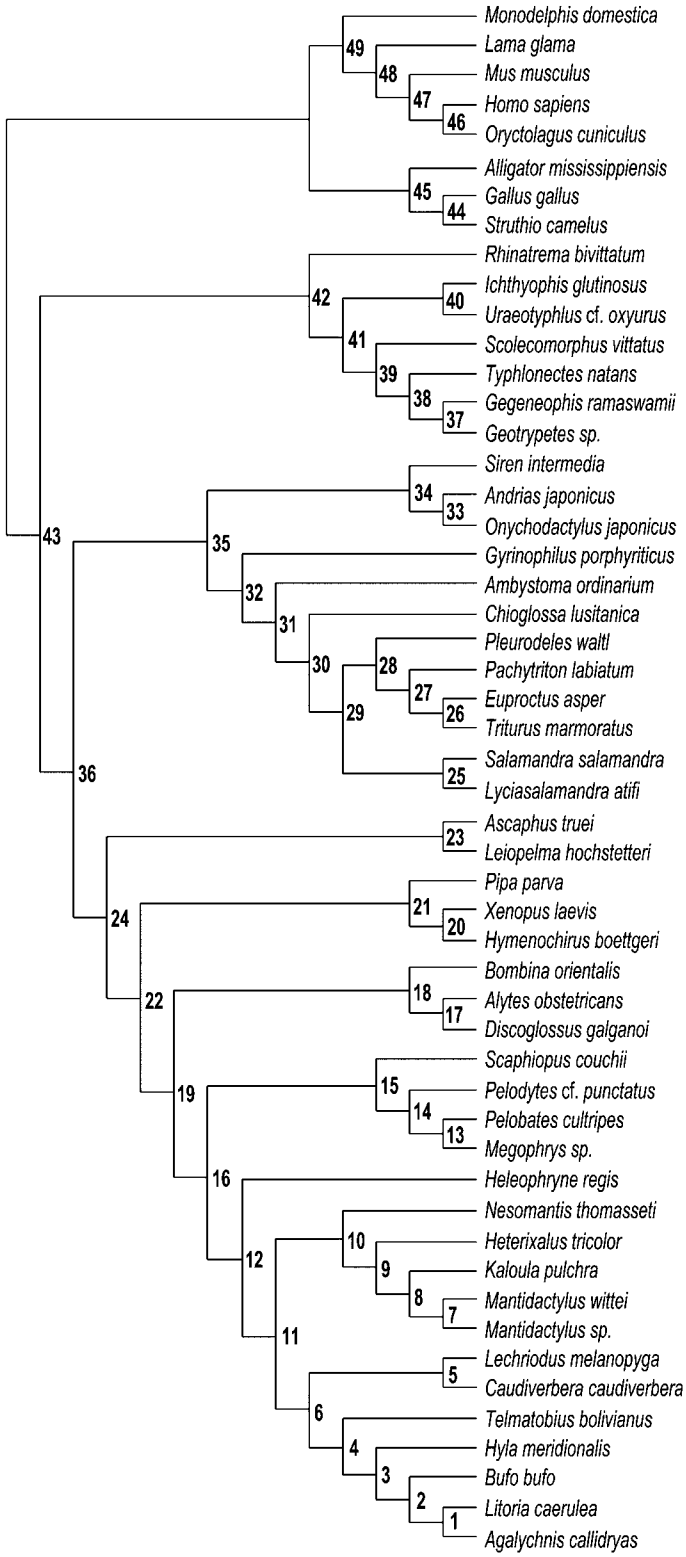


**Figure C2:** Topology of maximum likelihood tree with node numbers

**Table C1**
Statistical support and age estimates for each node

| | Statistical support | | | | Age estimate | | |
|---|---|---|---|---|---|---|---|
| Node | ML | BI | ME | MP | Mean | SD | CI |
| 1 | 99 | 100 | 62 | 78 | 46.786 | 4.507 | 42.136–58.676 |
| 2 | … | … | … | … | 57.932 | 6.894 | 46.703–73.632 |
| 3 | 62 | 80 | … | … | 60.911 | 7.313 | 48.793–77.276 |
| 4 | 100 | 100 | 100 | 100 | 65.054 | 7.976 | 51.916–82.813 |
| 5 | 59 | 96 | … | … | 120.302 | 16.153 | 90.608–154.232 |
| 6 | … | 66 | … | … | 138.029 | 16.259 | 108.041–171.613 |
| 7 | 100 | 100 | 100 | 100 | 9.783 | 2.835 | 4.455–14.604 |
| 8 | 77 | 85 | 54 | 75 | 86.527 | 14.961 | 58.430–117.456 |
| 9 | 100 | 100 | 98 | 93 | 99.044 | 15.831 | 69.940–132.137 |
| 10 | 51 | 69 | … | … | 130.791 | 17.253 | 99.065–166.582 |
| 11 | … | 99 | … | … | 149.698 | 17.569 | 117.136–186.236 |
| 12 | 100 | 100 | 98 | 95 | 161.706 | 18.087 | 127.892–198.934 |
| 13 | 77 | 100 | 57 | 51 | 117.860 | 21.874 | 77.179–161.823 |
| 14 | 55 | 97 | … | … | 142.478 | 22.350 | 100.511–186.596 |
| 15 | 100 | 100 | 100 | 97 | 163.582 | 22.470 | 121.398–208.485 |
| 16 | 75 | 100 | … | … | 216.353 | 21.210 | 176.105–259.721 |
| 17 | 100 | 100 | 96 | 95 | 151.529 | 24.047 | 104.840–199.422 |
| 18 | 100 | 100 | 100 | 97 | 198.574 | 22.812 | 155.298–243.508 |
| 19 | 58 | 96 | … | … | 234.862 | 21.336 | 194.878–277.135 |
| 20 | 54 | 93 | 75 | 60 | 154.815 | 25.044 | 107.122–204.529 |
| 21 | 100 | 100 | 100 | 98 | 176.697 | 24.524 | 130.300–226.269 |
| 22 | 82 | 98 | … | … | 244.773 | 21.597 | 204.091–288.157 |
| 23 | 86 | 91 | 100 | 94 | 236.661 | 22.575 | 192.567–281.017 |
| 24 | 100 | 100 | 100 | 100 | 262.470 | 20.798 | 223.183–304.551 |
| 25 | 100 | 100 | 100 | 100 | 35.001 | 15.688 | 11.878–72.953 |
| 26 | 61 | 90 | 66 | 66 | 37.216 | 12.877 | 17.492–67.106 |
| 27 | 100 | 100 | 96 | 99 | 45.100 | 14.773 | 22.066–78.803 |
| 28 | 100 | 100 | 97 | 100 | 66.955 | 18.681 | 36.038–108.754 |
| 29 | 89 | 98 | 100 | 91 | 109.771 | 22.505 | 68.634–155.305 |
| 30 | 100 | 100 | 100 | 100 | 138.300 | 24.024 | 92.913–186.583 |
| 31 | 82 | 100 | … | … | 230.107 | 22.234 | 187.516–274.276 |
| 32 | 63 | 98 | … | … | 252.585 | 20.505 | 213.251–293.835 |
| 33 | 100 | 100 | 100 | 100 | 177.404 | 13.696 | 161.513–211.731 |
| 34 | … | … | … | … | 261.358 | 18.722 | 226.966–300.312 |
| 35 | 100 | 100 | 100 | 100 | 272.544 | 19.036 | 238.099–311.837 |
| 36 | … | 67 | 71 | 75 | 356.959 | 22.261 | 317.256–405.261 |
| 37 | 65 | 95 | … | … | 146.417 | 14.070 | 130.529–182.564 |
| 38 | 58 | 99 | … | 89 | 155.237 | 15.275 | 134.243–193.482 |
| 39 | 56 | 99 | 78 | 88 | 177.064 | 17.788 | 147.982–217.600 |
| 40 | 100 | 100 | 98 | 100 | 104.305 | 22.436 | 64.645–151.029 |
| 41 | 56 | 94 | 83 | 94 | 192.414 | 18.786 | 160.280–232.994 |
| 42 | 100 | 100 | 100 | 100 | 214.285 | 20.306 | 177.412–255.705 |
| 43 | 94 | 100 | 76 | 71 | 367.378 | 22.699 | 327.517–417.364 |
| 44 | 100 | 100 | 100 | 100 | 120.214 | 28.004 | 70.487–177.954 |
| 45 | 100 | 100 | 100 | 100 | 237.672 | 24.284 | 185.812–279.520 |
| 46 | 71 | 83 | 78 | 91 | 116.492 | 26.867 | 66.555–170.896 |
| 47 | 80 | 85 | … | 65 | 142.652 | 28.232 | 87.831–196.801 |
| 48 | 100 | 100 | 100 | 100 | 159.483 | 28.201 | 103.183–213.141 |
| 49 | 100 | 100 | 100 | 100 | 236.372 | 24.267 | 183.620–278.913 |

**Note:** Statistical support given by bootstrap proportions for maximum likelihood (ML; 100 pseudoreplicates) and minimum evolution and maximum parsimony (ME and MP; 1,000 pseudoreplicates) and by Bayesian posterior probabilities (1,000,000 generations) for Bayesian inference (BI). Age estimates are in millions of years; also included are standard deviations and 95% confidence intervals (CI). Ellipses indicate support values of <50.

# Appendix D from D. San Mauro et al., "Initial Diversification of Living Amphibians Predated the Breakup of Pangaea"

**(Am. Nat., vol. 165, no. 5, p. 590)**

## Fit between Time and Divergence for the Employed Calibrations

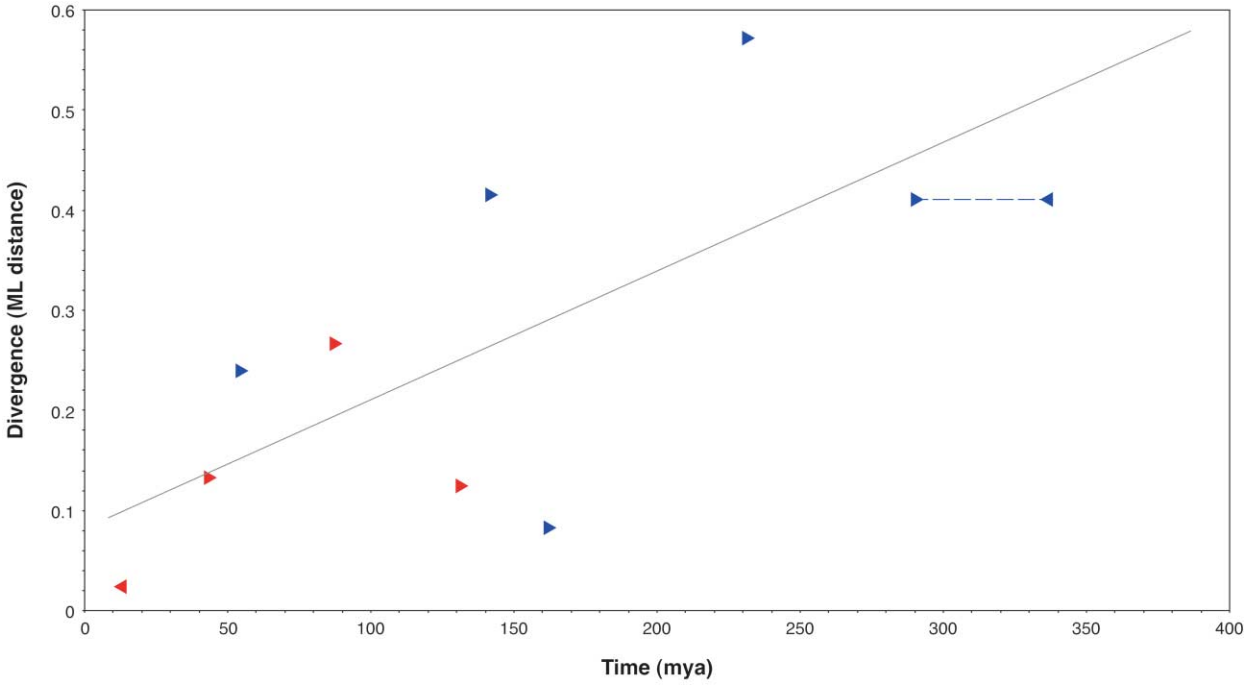


**Figure D1:** Scatterplot of divergence (measured as maximum likelihood [ML] distance) versus time (in millions of years) for the employed calibrations. These calibrations, as listed in "Material and Methods," are marked by triangles (upper and lower bounds). Red triangles represent calibrations based on biogeography; blue triangles represent calibrations based on fossil record. Dashed line indicates the interval for the synapsid-diapsid calibration. There is a significant correspondence between time and divergence even though the calibrations are not point calibrations but upper and lower time constraints (gray line represents linear regression fit; $R^2 = 0.458$; $F = 5.920$; $\mathrm{df} = 1, 7$; $P = .045$).