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Archaeobatrachian Paraphyly and Pangaean Diversification of Crown-Group Frogs

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Abstract.—Current models for the early diversification of living frogs inferred from morphological, ontogenetic, or DNA sequence data invoke very different scenarios of character evolution and biogeography. To explore central controversies on the phylogeny of Anura, we analyzed nearly 4000 base pairs of mitochondrial and nuclear DNA for the major frog lineages. Likelihood-based analyses of this data set are congruent with morphological evidence in supporting a paraphyletic arrangement of archaeobatrachian frogs, with an (*Ascaphus* + *Leiopelma*) clade as the sister-group of all other living anurans. The stability of this outcome is reinforced by screening for phylogenetic bias resulting from site-specific rate variation, homoplasy, or the obligatory use of distantly related outgroups. Twenty-one alternative branching and rooting hypotheses were evaluated using a nonparametric multicomparison test and parametric bootstrapping. Relaxed molecular clock estimates situate the emergence of crown-group anurans in the Triassic, approximately 55 million years prior to their first appearance in the fossil record. The existence of at least four extant frog lineages on the supercontinent Pangaea before its breakup gains support from the estimation that three early splits between Laurasia- and Gondwana-associated families coincide with the initial rifting of these landmasses. This observation outlines the potential significance of this breakup event in the formation of separate Mesozoic faunal assemblages in both hemispheres. [Anura; archaeobatrachian frogs; Bayesian divergence age estimation; Pangaean breakup; Phylogenetic hypothesis testing.]

The sequence and timing of the early diversification of crown-group frogs (the last common ancestor of living frogs and all of its descendants) is highly elusive for a number of reasons. First, the Mesozoic fossil record of Anura (*sensu* Gao and Wang, 2001, i.e., the last common ancestor of †*Mesophryne*, †*Notobatrachus*, †*Prosalirus*, †*Vieraella*, and crown-group frogs, and all of its descendants) is particularly fragmentary and provides only limited information on the phylogenetic significance of potentially diagnostic traits or on the age of lineage divergences. For instance, revised interpretations of several character states in the Jurassic anurans †*Vieraella herbstii* and †*Notobatrachus degiustoi* challenged their previous status of oldest known crown-group fossils (Estes and Reig, 1973; Duellman and Trueb, 1986) and instead identified them as stem lineages (Baéz and Basso, 1996; Rocek, 2000; Gao and Wang, 2001), thereby causing a forward extension of the time interval in which the earliest divergence between extant lineages possibly took place. Second, phylogenetic analysis of any morphological data set, whether extant or fossil, is complicated by striking convergences in character evolution in one of the most conserved body plans in vertebrates (Shubin and Jenkins, 1995). Finally, DNA sequence studies that allow us to evade part of these drawbacks have repeatedly supported a phylogenetic hypothesis that seems largely inconsistent with morphological or paleontological evidence.

A major focus of research on early anuran evolution is the origin of archaeobatrachian frogs. Initially conceived to define a suborder combining representatives of Discoglossidae, Rhinophrynidae, and pelobatoid frogs (Reig, 1958), the term Archaeobatrachia (or its derived adjective) is frequently used to specify a broader set of anurans, including *Ascaphus*, *Leiopelma*, and Pipidae as well (Duellman, 1975; Ford and Cannatella, 1993; Hay et al., 1995; Pugener et al., 2003; Hoegg et al., 2004; Hertwig et al., 2004). Throughout this article, we adopt the latter specification. In contrast to the large radiation of Neobatrachia, archaeobatrachians represent only a fraction (∼4%) of the present-day anuran species diversity, but they stand out by an array of character states that seem less derived with respect to those in Neobatrachia. The gradient distribution of these traits has relatively early resulted in the concept of a paraphyletic assemblage of archaeobatrachian frogs at the base of the crown-group tree (Noble, 1931; Reig, 1958; Sokol, 1975; Fig. 1a). Separate studies have produced different variations on this scheme, with a basal divergence of either *Ascaphus* + *Leiopelma* + Discoglossidae *sensu lato* (i.e., including *Alytes*, *Barbourula*, *Bombina*, and *Discoglossus*; Laurent, 1979; Duellman and Trueb, 1986), *Ascaphus* + *Leiopelma* (Lynch, 1973), or *Ascaphus* alone (Ford and Cannatella, 1993). Pipoid and pelobatoid frogs were generally considered transitional lineages, either paraphyletic with respect to Neobatrachia (Lynch, 1973; Duellman and Trueb, 1986), or forming a clade (Mesobatrachia; Laurent, 1979; Ford and Cannatella, 1993). Recent studies, however, exploiting the anuran larval morphology as a fruitful source for extensive character sampling, have suggested a more basal origin of Pipoidea than generally perceived (Haas, 2003), or even supported the earlier idea (Orton, 1957; Hecht, 1963; Starrett, 1973) of a sister group relationship with all other living frogs (Maglia et al., 2001; Pugener et al., 2003).

Analyses of large subunit rRNA (Hillis et al., 1993; Kjer, 1995) and protein coding sequences (Hoegg et al., 2004) have occasionally corroborated the paraphyly of archaeobatrachian frogs, but recovered low nodal support values to sustain this hypothesis. In contrast, all other molecular studies using mitochondrial rRNA have concurred on the reciprocal monophyly of Archaeobatrachia and Neobatrachia (Hedges and Maxson, 1993; Hay et al., 1995; Dutta et al., 2004; Hertwig et al., 2004; Fig 1b). This outcome has entailed the hypothesis that centers of diversification of Archaeobatrachia in Laurasian and of Neobatrachia in Gondwanan landmasses, originated from a single vicariant event at the initial north–south

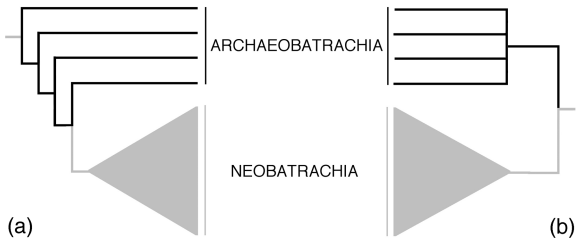

FIGURE 1. Simplified evolutionary schemes representing one of the major controversies on the early diversification of extant Anura. (a) Morphological evidence supports a paraphyletic assemblage of archaeobatrachian lineages, although different branching orders have been proposed; (b) the reciprocal monophyly of Archaeobatrachia and Neobatrachia has repeatedly received support by DNA sequence analyses.

break up of the Pangaean supercontinent (Hedges et al., 1996, Feller and Hedges, 1998). This scenario would imply an entirely post-Pangaean diversification of extant anurans, which could be consistent with the relative sudden Upper-Jurassic appearance of several crown-group lineages in the fossil record (Evans et al., 1990; Evans and Milner, 1993; Henrici, 1998; Gao and Wang, 2001). Moreover, although Neobatrachia currently have a cosmopolitan distribution, their early evolution seems unambiguously correlated with Gondwanan landmasses (Savage, 1973; Ruvinsky and Maxson, 1996; Biju and Bossuyt, 2003; Zhang et al., in press). However, the biogeographical history of archaeobatrachian frogs seems less obvious, and a Archaeobatrachia–Neobatrachia vicariance does not provide a simple explanation for the current occurrence of Pipidae and *Leiopelma* on remote landmasses of Gondwanan origin (Africa + South America, and New Zealand, respectively).

A close examination of previously proposed phylogenies reveals that, besides numerous differences in the *unrooted* arrangement of the major frog lineages, a primary point of conflict is the position of the anuran root. Obviously, correct rooting is of utmost importance for accurate polarization of evolutionary change and biogeographic pattern recognition. In order to resolve this issue, we sequenced approximately 4400 base pairs (bp) of nuclear DNA (nuDNA) and mitochondrial DNA (mtDNA) for representatives of the major frog lineages. Besides conventional and Bayesian phylogeny inference, we examine the potential impact of phylogenetic artifacts on the inferred root position and we use both nonparametric and parametric tests to evaluate competing rooting and branching hypotheses. The inferred phylogeny allows us to extract dating estimates for early branching events in the anuran crown-group. This provides a renewed perspective on the historical context in which early anuran diversification took place.

MATERIALS AND METHODS

Taxon Sampling and DNA Sequencing

Throughout this article, we mainly follow the taxonomic classification proposed by Frost (2004) for the use of family-group names. Our study includes representatives of the 10 archaeobatrachian frog families (Table 1) and eight neobatrachian species, representing major lineages of this group (Haas, 2003; Biju and Bossuyt, 2003). Three salamanders and two caecilians served as outgroups. Three nuclear protein-coding gene fragments were polymerase chain reaction (PCR)-amplified and cycle-sequenced on both strands: (1) a region of ~555 bp in the recombinase activating gene 1 (*Rag-1*); (2) a region of ~675 bp in exon two of the chemokine receptor 4 gene (*Cxcr-4*); and (3) a region of ~1280 bp in exon two of the sodium-calcium exchanger 1 gene (*Ncx-1*). A fourth fragment covers ~1940 bp of the mitochondrial genome, comprising ~745 bp of the 3′ end of the 16S rRNA, the complete sequences of $tRNA^{LEU}$, *ND-1*, $tRNA^{ILE}$, $tRNA^{GLN}$, and ~20 bp of $tRNA^{MET}$. Primers newly designed for this study are provided in Table 2; additional primers are mentioned elsewhere (Bossuyt and Milinkovitch, 2000; Biju and Bossuyt, 2003).

Sequence Alignment and Phylogeny Inference

Obtained sequences were first aligned with ClustalX 1.64 (Thomson et al., 1997). After removal of excessive "tail sequences" at the 5′- and 3′-ends for several taxa (e.g., resulting from the use of more externally located primers), we performed a second alignment using the probabilistic method implemented in the program ProAlign 0.5a0 (Löytynoja and Milinkovitch, 2003). We maintained ProAlign's estimated alignment probability of 90% as an arbitrary threshold value for the inclusion/exclusion of sites in our phylogenetic analyses, but made several minor adjustments in the alignment matrices using MacClade 4.0 (Maddison and Maddison, 2000). Positions forming pairs in stem regions of the 16S rRNA and tRNA segments were identified by comparison with the predicted secondary structures of *Xenopus laevis* (Gutell et al., 1994; www.rna.icmb.utexas.edu).

Phylogeny estimations were obtained under the maximum parsimony (MP) and the maximum likelihood (ML) criteria and in a Bayesian framework. Because distant outgroups can influence inferred relationships among ingroup taxa, independent analyses were conducted on a taxon set composed of the ingroup alone and on a set including both ingroup and outgroup taxa. Heuristic MP searches were executed with PAUP* 4.0b10 (Swofford, 1998) in 2000 replicates of random taxon addition, equal weighting of characters and tree bisection reconnection (TBR) branch-swapping. Nonparametric bootstrap analyses under MP were conducted with 2000 bootstrap replicates, each with simple taxon addition. For likelihood-based analyses, a model was selected among 56 models, either stationary or incorporating among-site rate variation (ASRV models) using the hierarchical likelihood ratio tests and Akaike information criterion (AIC; Akaike, 1973), implemented in Modeltest 3.0.6 (Posada and Crandall, 1998). Heuristic ML searches with the selected model were executed in PAUP* with 10 0replicates of random taxon addition, TBR branch-swapping, and fixed-model parameter values obtained

TABLE 1. Taxa included in this study, with corresponding specimen vouchers.

(Sub)order	(Sub)family	Species	Voucher
Ingroup taxa			
Archaeobatrachia	Ascaphidae	*Ascaphus montanus*	MVZ 187733
		Ascaphus truei	MVZ 187732
	Bombinatoridae	*Bombina orientalis*	VUB 0906
		Bombina variegata	VUB 0099
	Discoglossidae	*Alytes obstetricans*	VUB 0095
		Discoglossus pictus	VUB 0528
	Leiopelmatidae	*Leiopelma archeyi*	No voucher available
		Leiopelma hochstetteri	No voucher available
	Megophryidae	*Brachytarsophrys feae*	VUB 0708
		Leptobrachium montanum	VUB 0628
		Leptolalax arayai	No voucher available
	Pelobatidae	*Pelobates cultripes*	VUB 0510
	Pelodytidae	*Pelodytes punctatus*	VUB 0529
	Pipidae	*Hymenochirus boettgeri*	VUB 0092
		Pipa pipa	VUB 0539
		Silurana tropicalis	VUB 1060
		Xenopus sp.	VUB 0921
	Rhinophrynidae	*Rhinophrynus dorsalis*	MVZ 164755
	Scaphiopodidae	*Scaphiopus hurterii*	TNHC DCC3005
		Spea multiplicata	JAJ 428
Neobatrachia	Astylosternidae	*Trichobatrachus robustus*	ZFMK 66453
	Ceratophryinae	*Ceratophrys ornata*	VUB 1006
	Hylidae	*Hyla meridionalis*	VUB 0534
	Leptopelinae	*Leptopelis kivuensis*	CAS 201700
	Microhylidae	*Scaphiophryne marmorata*	VUB 0540
	Limnodynastinae	*Limnodynastes salmini*	TNHC DCC2898
	Ranidae	*Meristogenys kinabaluensis*	VUB 0627
	Rhinodermatidae	*Rhinoderma darwinii*	MVZ 164829
Outgroup taxa			
Caudata	Hynobiidae	*Hynobius formosanus*	MVZ 197237
	Plethodontidae	*Eurycea quadridigitata*	VUB 1058
	Salamandridae	*Pleurodeles waltl*	VUB 0508
Gymnophiona	Caeciliidae	*Gegeneophis sp.*	No voucher available
		Geotrypetes seraphini	FMNH 256782

Collection abbreviations: CAS, California Academy of Sciences; FMNH, Field Museum of Natural History; JAJ, University of Oklahoma; MVZ, Museum of Vertebrate Zoology; TNHC, Texas Natural History Collections; VUB, Vrije Universiteit Brussel; ZFMK, Zoologisches Forschungsinstitut und Museum A. Koenig.

TABLE 2. PCR primers designed for this study.

Fragment	Primer	Sequence (5′ → 3′)
NCX1	Naca-A	TTTGTTGCCATGGTTTACATGTT
	Naca-C	CGGTTCATGTCCTCTATTGAAGT
	Naca-E	TTCTCAGGGTGTTTTTGYTTNAG
	Naca-G	TTGTCAGGRTGYTTCTGCTTYAG
	Naca-H	TTCYTTRTCAGGGTGYTTCTG
	Naca-J	GCAGATATHGARATGGATGGGAA
	Naca-L	TCCAAAGCAGATATTGAAATGGA
	Naca-O	ATACCTGCATGATCATCATCAAA
	Naca-P	AAGATACCTGCATGRTCATCRTC
	Naca-Q	TCAACTCTCACACTGAAAAYTTC
	Naca-R	TGATCATCATCRAAGATGGTNAC
Mitochondrial	NDH-D	GGTATGGGCCCAAAAGCTT
	NDH-J	TTTACGACCTCGATGTTGGA
	NDH-K	ATTAAGGCRTATTTNGARTT
	NDH-L	AAACTATTTAYYAAAGARCC
	NDH-M	GGGTATGANGCTCGNACTCA
	NDH-N	GGCTTTAAYGTRGAATAYGC
	NDH-O	CCAATTAGGGCRTATTTNGAGTT
	NDH-P	CCAATTAGGGCRTATTTNGAATT
	NDH-Q	TAAAACTATTCATNAARGAACC
	NDH-R	TAAAACTATTCATNAARGAGCC
	NDH-S	GGGTATGANGCTCGNATCCA
	NDH-T	GGGTATGANGCTCGNACCCA
	NDH-U	GGCTTCAAYGTRGAGTAYGC
	NDH-W	GGGTATGANGCTCGNATTCA

by recurrent ML estimation on neighbor-joining (NJ) trees. Bayesian analyses were performed using MrBayes 3.0b4 (Ronquist and Huelsenbeck, 2003) with flat dirichlet prior settings for base frequencies and substitution rate matrices and uniform prior settings for rate parameters. A first round of analyses consisted of individual runs on the total data set, a data set composed of all nuDNA and a data set composed of all mtDNA, and implemented a single model and uniform parameter optimization across the whole data set. In a second round, we analyzed the total combined data set using two different "partitioned data" approaches, with uncoupled parameter optimization across predefined data subsets. For the first partition, data subsets were defined in accordance with the four sequenced loci. The second partition had a similar design, but mitochondrial stem positions were distinguished as a fifth subset and analyzed under an adapted doublet model, integrating compensatory substitutions in paired sites (Ronquist and Huelsenbeck, 2003). The convergence and stationarity of model parameters for all Markov chain Monte Carlo (MCMC) runs was evaluated using time series plots, and based on these we selected the following sampling configuration, which seemed adequate for all analyses:

four chains (three heated, one cold, heating temperature = 0.2) of 5,000,000 generations each, a sampling interval of 500 generations, and a burn-in corresponding to the first 1,000,000 generations. All runs were repeated at least twice, starting from different topologies, to confirm consistent approximation of the posterior parameter distribution.

Evaluation of Phylogenetic Robustness

Covarion-like evolution.—The performance of likelihood-based tests and Bayesian analyses are highly dependent on the appropriateness of the substitution model applied on the collected data. For instance, in combination with a considerable level of evolutionary rate heterogeneity, the application of models lacking biological "realism" (e.g., by being too simplified) may produce tree-building artifacts or overcredibility of the significance of the outcome (Bollback, 2002; Buckley, 2002; Erixon et al., 2003; Lemmon and Moriarty, 2004). A particularly pervasive obstacle for stationary substitution models is the accumulation of mutational saturation through time. Models that incorporate ASRV reduce this problem, by allowing a distinction between fast-evolving sites and slowly evolving or invariable sites, rather than assuming a single averaged substitution rate. On the other hand, most of these models ignore the possibility of covarion-like evolution; i.e., *site-specific* rate variation (SSRV) *across lineages*. Studies on both simulated and empirical data have shown that part of the saturation may be overlooked by ASRV models when existing SSRV is not taken into account (Lockhart et al., 1998; Penny et al., 2001; Galtier, 2001; Huelsenbeck, 2002; Inagaki et al., 2004).

We used two alternative approaches to explore the possibility of model misspecification due to underlying (hidden) SSRV. First, we tested via MC simulation whether our data set contains more SSRV than expected under the null hypothesis of strict ASRV-like evolution (i.e., assuming site-specific rate constancy across lineages). Evolution under the selected ASRV model was simulated 100 times with the program Seq-Gen 1.2.6 (Rambaut and Grassly, 1997), along an NJ-inferred tree and using parameter values estimated by PAUP* on the real data. For every simulated data set, likelihood parameters were estimated on the same NJ tree under the unequal SSRV (USSRV) model, implemented in the software package NHML 3 (Galtier, 2001). This model approximates SSRV by a variation rate parameter ν and a parameter π, describing the proportion of sites under SSRV (Galtier, 2001). The same covarion parameters were estimated from the observed data and compared with the parameter distributions of the ASRV-simulated data.

As a second approach, we verified whether Bayesian analyses integrating covarion-like evolution produce posterior probabilities that deviate from those obtained under the ASRV model selected by Modeltest. Bayesian searches were repeated using the same settings as described above, but instead of assuming a constant proportion of invariable sites, two additional parameters were implemented, describing the rate at which sites switch from an invariable to a variable condition (s_{01}) and vice versa (s_{10}) (Huelsenbeck, 2002).

Substitution rate and homoplasy.—We evaluated the robustness of the inferred root position with respect to the degree of mutational saturation and homoplasy by recurrent analyses after the sequential exclusion of sites according to their evolutionary rates (Brinkmann and Philippe, 1999). Site-specific substitution rates were estimated as follows: First, the data set was partitioned into phylogenetically well-supported subclades, based on analyses of the total data set. Next, for each site the number of substitutions within each subclade was estimated under MP and then summed over all subclades. Sites were then divided into discrete categories, arranged from fast-evolving (a large number of substitutions) to invariable (zero substitutions). These categories are consecutively removed and the remaining data sets were analyzed by nonparametric bootstrapping under NJ and MP and by heuristic ML searches. This approach has the inherent advantage that identification of the fast-evolving sites is entirely independent of the relationships that are to be evaluated (i.e., the ingroup root position).

Distantly related outgroups.—The lack of genealogical similarity with the ingroup may cause a distantly related outgroup to randomly bind on the ingroup topology. In empirical cases, this randomizing factor gains in importance with an increased evolutionary distance between outgroup taxa and the ingroup. This eventually results in rooting artifacts (e.g., random rooting or long branch attraction; Qiu et al., 2001; Huelsenbeck et al., 2002; Graham et al., 2002), or at least may cause overestimation of the Bayesian-inferred credibility for a certain root position. We applied a Bayesian approach adopted from Huelsenbeck et al. (2002) to evaluate whether the inferred root position and its observed posterior probability (*PP*) were predisposed due to the large evolutionary distance between the outgroup taxa (salamanders and caecilians) and the anuran ingroup. Such bias may be assessed by comparing the *PP* of this root position with an experimentally derived random rooting probability (*RRP*), which is the posterior probability of a random outgroup attaching to that particular ingroup branch in the absence of any phylogenetic signal. We experimentally assessed branch-specific *RRP*s, using 100 artificial outgroups generated in SeqGen, with the number of taxa and base composition equal to the empirical outgroup. The lack of phylogenetic signal between these outgroups and our ingroup data mimic an infinite evolutionary distance. The binding behavior of these artificial outgroups across the 53 branches of the ingroup topology was examined with MrBayes. Each MCMC run consisted of four chains of 1,000,000 generations, with a sampling interval of 100 generations. Convergence of the log-likelihoods was reached in all cases after 100,000 generations, but, for safety, trees sampled from the first 200,000 generations were discarded as burn-in.

Evaluation of Alternative Phylogenetic Hypotheses

Alternative rooting and branching scenarios for Anura were evaluated by a nonparametric approximately unbiased (AU) test (Shimodaira, 2002) and by parametric bootstrapping (PB) (Swofford et al., 1996; Huelsenbeck et al., 1996). In both cases, alternative hypotheses were represented by candidate trees estimated under ML using conventional, backbone, or reversed constraints in PAUP*.

Similar to the Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa, 1999), the AU test aims to provide better control of type-1 errors (the rejection of potentially true hypotheses) by simultaneous comparison of multiple hypotheses. Site-wise log-likelihoods estimated by PAUP* for all candidate trees were used as input for the software package CONSEL 0.1 g (Shimodaira and Hasegawa, 2001). Multiscale bootstrap resampling was conducted in ten sets of 10,000 replicates each, with scale parameters ranging from 0.5 to 1.4.

For the PB analyses, the log-likelihood difference between the ML tree and each candidate tree served as test statistic ($\delta \ln L$) for the individual evaluation of alternative hypotheses. Each candidate tree, with branch lengths and parameter values estimated from the real data, was entered into Seq-Gen to simulate a series of 500 replicate data sets under the GTR+Γ+I model. For every replicate data set, the ML tree and the log-likelihood of the candidate topology were estimated. This was done with the program PHYML 2.3 (Guindon and Gascuel, 2003), which implements a highly efficient hill-climbing algorithm that drastically reduces the computation time of ML searching and provides a phylogenetic accuracy similar to PAUP*. The resulting log-likelihood differences provide a null distribution, allowing valuation of the *P*-value of the observed $\delta \ln L$ under the alternative hypothesis. A Bonferroni correction was applied to adjust the significance level for rejection of alternative hypotheses in a case of multiple tests.

Estimation of Divergence Times

We estimated 95% credibility intervals for divergence times, using the Bayesian relaxed molecular clock method implemented in the MultiDivtime software package (Thorne and Kishino, 2002). Sequences of each of the four loci were realigned with the addition of homologous sequences of the rainbow trout (outgroup), chicken, mouse, and human retrieved from GenBank. Because the relationships among the three lissamphibian orders are still largely controversial, we excluded the two caecilian species from the analyses to avoid biased estimates caused by erroneous phylogenetic assumptions.

Bayesian estimations of the 95% credibility intervals were conducted with a prior of 338 million years ago (Mya) for the ingroup root (the split between lissamphibians and amniotes, corresponding to the age of the basal aïstopod †*Lethiscus stocki*; Ruta et al., 2003), and a standard deviation of 50 Mya, which represents a fairly conservative interval for this split. Markov chains were run for 1,000,000 generations, with sampling intervals of 100 generations, and burn-ins corresponding to the first 100,000 generations. We calibrated our estimates with time constraints on seven internal nodes, based on fossil and tectonic evidence. Because the selection of some of these constraints is based on phylogenetic results of this study, we discuss them in Results. Additional analyses were performed using only two well-supported, non-amphibian calibration points, to expose biased estimates caused by potential errors in the temporal and/or phylogenetic placement of amphibian fossils. Finally, all analyses were repeated to confirm successful convergence towards the proper distributions for divergence ages.

RESULTS

Sequence Alignment and Phylogeny Inference

Obtained sequences were deposited in GenBank under accession numbers AY523683 through AY523786. The exclusion of ambiguously aligned nucleotide sites resulted in a combined matrix of 3963 aligned positions. Of these, 2022 are variable and 1788 are parsimony informative (Table 3). Within the 16S rRNA and the tRNA regions, 314 sites were identified as paired stem positions. The hierarchical likelihood ratio tests performed by Modeltest proposed TrN+Γ+I as the most appropriate substitution model for likelihood-based analyses. However,

TABLE 3. Summary of sequence data for the four sampled loci and the combined data set.

	Cxcr-4	*Ncx-1*	*RAG-1*	mtDNA	Combined
No. of aligned positions	666	1276	558	2022	4524
No. of analyzed positions	597	1269	534	1563	3963
No. of varying positions	326	561	274	861	2022
No. of parsimony-informative positions	293	495	252	748	1788
Range of uncorrected pairwise distances (%)	0.2–30.4	0.2–22.3	1.1–28.3	5.0–27.5	2.3–25.7
π_A	0.22	0.31	0.28	0.29	0.28
π_C	0.25	0.19	0.23	0.25	0.23
π_G	0.22	0.23	0.24	0.18	0.21
π_T	0.31	0.27	0.25	0.28	0.28
Consistency index (CI)[a]	0.346	0.370	0.324	0.265	0.308
Retention index (RI)[a]	0.523	0.630	0.519	0.354	0.452
$r^{b,c}_{AC}$	1.769	1.734	1.214	3.629	2.305
$r^{b,c}_{AG}$	4.608	5.207	4.109	6.836	4.897
$r^{b,c}_{AT}$	1.129	1.639	1.252	3.446	1.955
$r^{b,c}_{CG}$	1.001	0.881	0.583	0.301	0.695
$r^{b,c}_{CT}$	5.189	6.250	5.044	21.299	8.489
Gamma shape parameter [b]	1.162	2.147	1.874	0.557	0.948
Proportion of invariable sites[b]	0.405	0.532	0.465	0.346	0.435

[a] Estimated on the MP tree of the combined data set.
[b] Estimated on the ML tree of the combined data set.
[c] Estimated relative to $r_{GT} = 1$.

additional chi-square tests comparing TrN with GTR, and TrN+Γ+I with GTR+Γ+I, significantly favored the latter models ($df = 3$, $P < 0.001$), indicating that Modeltest's outcome was caused by entrapment in a local optimum (Posada and Crandall, 1998). Because GTR+Γ+I also scored best under the AIC criterion, we used this model for all subsequent ML and Bayesian analyses.

The MP, ML, and Bayesian analyses of the ingroup alone produced congruent unrooted trees for Anura, and most branches are supported by fairly high MP bootstrap (BS) values and Bayesian posterior probabilities (*PP*s) (Fig. 2). The branch separating *Ascaphus*, *Leiopelma*, Bombinatoridae, and Discoglossidae from all other frogs forms a notable exception. Conflicts among the different criteria are principally limited to the arrangement of neobatrachian lineages, and to the pipid subtree, with a ((*Pipa* + *Hymenochirus*) + (*Xenopus* + *Silurana*)) topology supported by MP, and a (*Pipa* + (*Hymenochirus* + (*Xenopus* + *Silurana*))) topology supported by ML and Bayesian analyses. The unrooted arrangement of frog families shown in Figure 2 is very similar to those of Lynch (1973), Duellman and Trueb (1986), and Hay et al. (1995), and mainly differs from those of Maglia et al. (2001), Pugener et al. (2003), and Haas (2003) in the relative position of the (Pipidae + *Rhinophrynus*) subtree with respect to the (*Ascaphus* + *Leiopelma*) and (Discoglossidae + *Bombina*) subtrees.

All analyses with the outgroup included corroborate five major, well-supported frog clades (Fig. 3). Families of the Discoglossoidea *sensu lato* (Laurent, 1979; Duellman and Trueb, 1986) form a paraphyletic assemblage, and we henceforth restrict this name to the clade composed of Bombinatoridae and Discoglossidae, whereas the clade combining *Ascaphus* and *Leiopelma* is further referred to as Amphicoela (Noble, 1931; Reig, 1958). Heuristic MP searches produced a single most parsimonious tree (tree length [TL] = 11,646; consistency index [CI] = 0.3038; retention index [RI] = 0.4518), which, similar to previous molecular studies (Hedges and Maxson, 1993; Hay et al., 1995; Dutta et al., 2004; Hertwig et al., 2004), shows the reciprocal monophyly of Archaeobatrachia and Neobatrachia. However, the archaeobatrachian clade receives very low support from nonparametric bootstrap analyses (BS = 58.5). In contrast, heuristic ML searches recover a paraphyletic arrangement of archaeobatrachian lineages and identify Amphicoela as the sister group of all other living frogs (Fig. 3). Neobatrachia are placed in a deeply nested position within Anura, with Pelobatoidea as their closest relatives. Bayesian analyses largely corroborate the ML-inferred phylogeny (Fig. 3) and yield

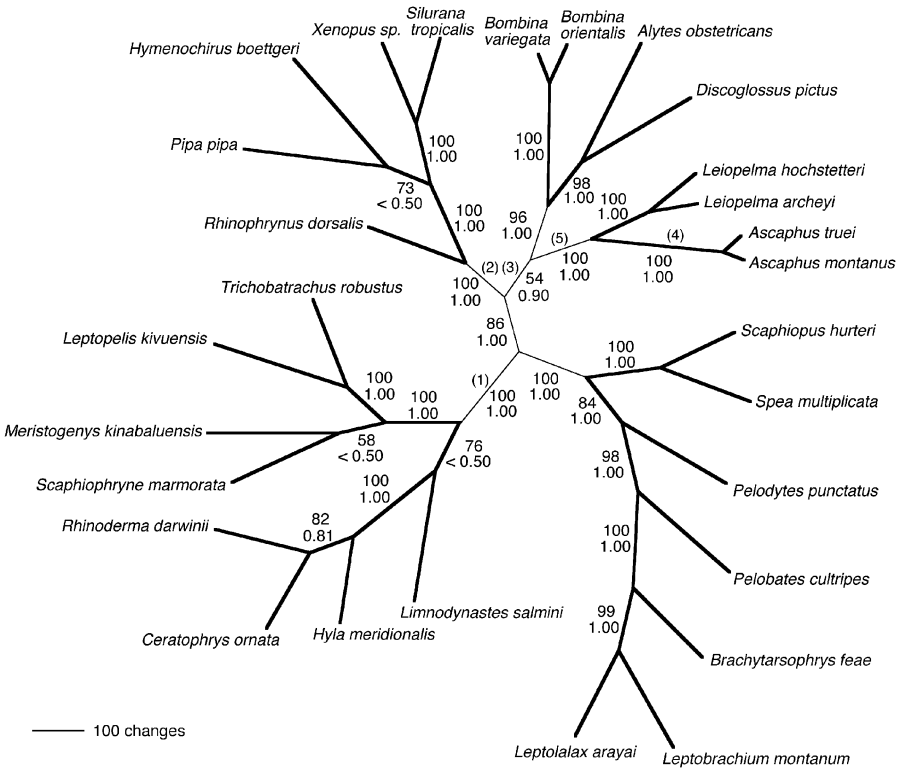

FIGURE 2. Unrooted MP phylogram (TL = 9257) for the major lineages of living Anura. Numbers on branches indicate MP bootstrap values (above) and Bayesian posterior probabilities (below), respectively. Numbers in parentheses denote competing ingroup root positions recovered by previously published analyses, or by the present study: (1) Hedges and Maxson (1993), Hay et al. (1995), Dutta et al. (2004), Hertwig et al. (2004), our MP analyses; (2) Maglia et al. (2001), Pugener et al. (2003); (3) Laurent (1979), Duellman and Trueb (1986); (4) Ford and Cannatella (1993); and (5) Lynch (1973), our ML and Bayesian analyses. Bold branches indicate the five well-supported clades that, in addition to the outgroup clade, were used for the estimation of site-specific substitution rates.

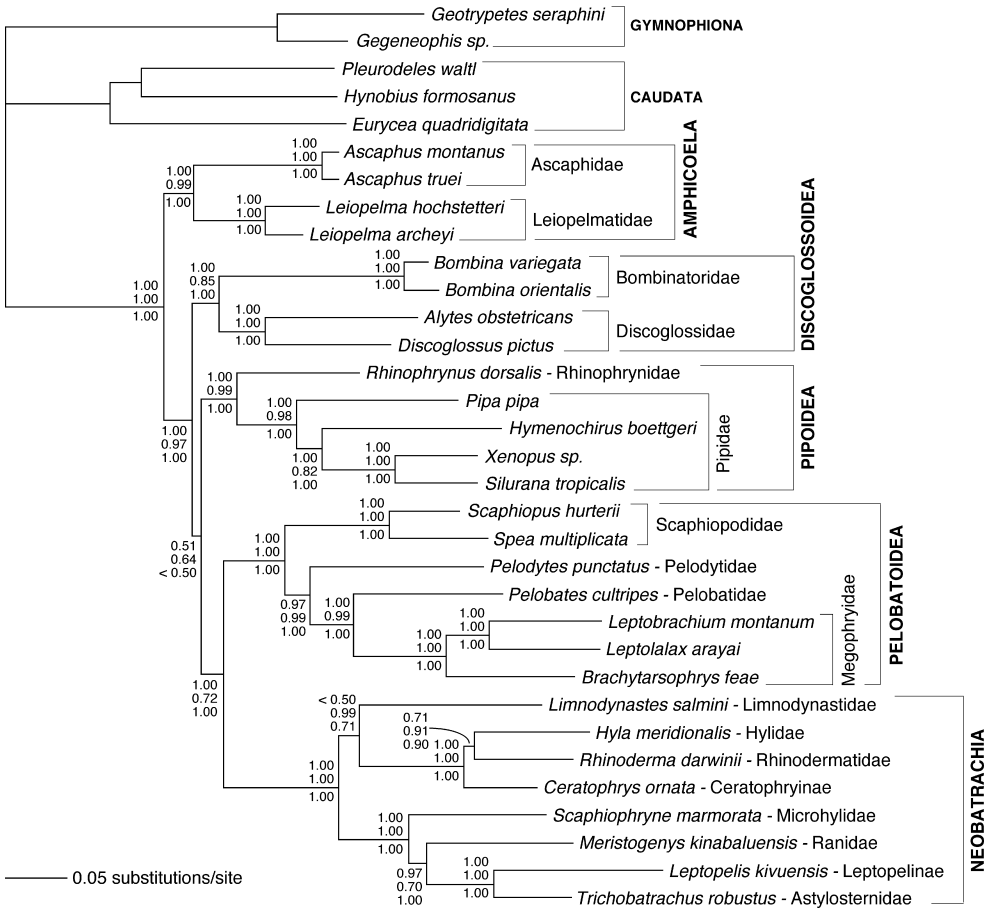

FIGURE 3. ML phylogram ($-\ln L = 52{,}100.00$) for the major lineages of living Anura, obtained from analysis of the total combined data set under a GTR+Γ+I model. Numbers on internal branches represent Bayesian posterior probabilities inferred from unpartitioned analyses of the nuDNA (top), the mtDNA (middle), and the total data set (bottom). Separate analyses on the total combined data set using locus-based data partitioning and a doublet model for stem regions yielded very similar posterior probabilities.

high *PP*s for both the basal divergence of Amphicoela and for the pairing of Neobatrachia with Pelobatoidea ($PP = 1.0$ in both cases). Additional runs using partitioning of model optimization across loci, either with or without a doublet model for stem regions, yielded nearly identical probability values. Furthermore, separate analyses of the nuDNA and the mtDNA yield similar posterior probabilities for a basal divergence of Amphicoela ($PP = 0.974$ and $PP = 1.0$, respectively).

Evaluation of Phylogenetic Robustness

Covarion-like evolution.—ML analysis of our data set under the USSRV model recovered the value 0.0 for both covarion parameters ν and π, already indicating a negligible signal of SSRV. The same zero values are scored for 100 data sets simulated under the GTR+Γ+I model, indicating that the USSRV model does not detect more covarion-like evolution than expected under the site-specific rate constancy assumed by GTR+Γ+I (corresponding to $P \sim 1.0$). In addition, Bayesian analyses integrating the covarion-adapted (GTR+Γ+s_{01}+s_{10}) model supported the exact same relationships as found under GTR+Γ+I, with practically identical posterior support for the basal divergence of amphicoelous frogs ($PP = 0.997$) and the grouping of Neobatrachia with Pelobatoidea ($PP = 1.0$). This suggests that a covarion-like nature of the data, if present, did not affect the estimated posterior probability of these inferred relationships.

Substitution rate and homoplasy.—The incongruence between our MP and likelihood-inferred root position is explained when analyses are repeated after the sequential removal of fast-evolving site categories. We approximated site-specific mutation rates by summing the number of substitutions over the five well-supported anuran subclades (Fig. 2) and the outgroup clade. The recorded site-specific substitution numbers varied between 0 and 18 and were divided in 15 categories. MP bootstrap support for a root position on the branch between Archaeobatrachia and Neobatrachia (= reciprocal monophyly of both groups) remains constant until all positions with at least 11 recorded substitutions (193 sites excluded; 3770 remaining) are discarded, but declines rapidly after the exclusion of all positions with at least eight substitutions (= 461 sites excluded; 3502 remaining) (Fig. 4a). Conversely, a root position on the branch

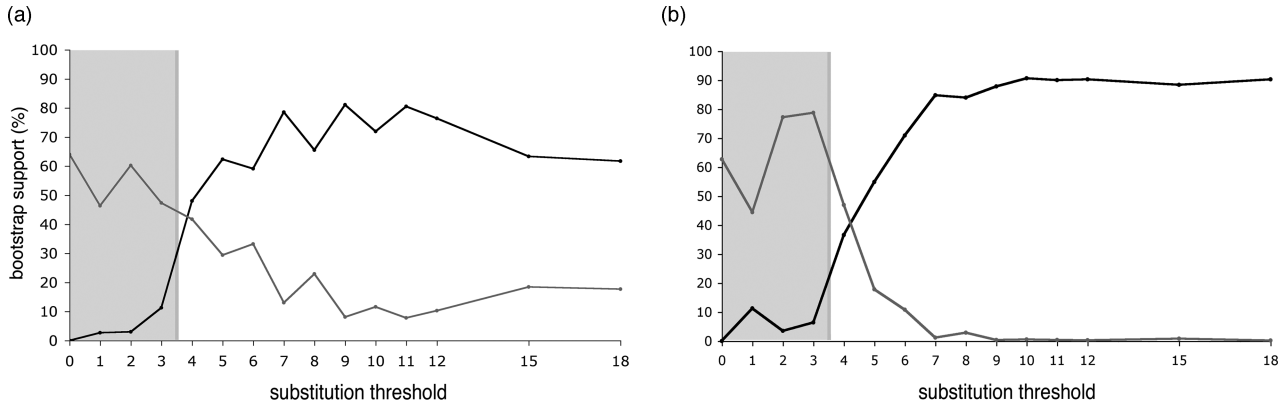

FIGURE 4. Bootstrap support (2000 replicates) for the monophyly (black) and paraphyly (grey) of archaeobatrachian frogs in function of the stepwise exclusion of fast-evolving sites: (a) under maximum parsimony; (b) via neighbor joining under the JC model. The grey zones in the plots delimit matrices for which MP and NJ trees corroborate the paraphyly of archaeobatrachians, with the basal divergence of Amphicoela.

between Amphicoela and all other frogs (= paraphyly of archaeobatrachians) is supported by increasing bootstrap values, and after exclusion of all sites with at least four recorded substitutions, MP trees switched from a basal Archaeobatrachia–Neobatrachia split to archaeobatrachian paraphyly with a basal divergence of Amphicoela. An identical trend is observed for NJ analyses under the Jukes-Cantor (JC) model (Fig. 4b), which corresponds to the tree reconstruction method applied in previous studies supporting monophyly of archaeobatrachians (Hedges and Maxson, 1993; Hay et al., 1995). In contrast, the root position obtained under ML remains constant when fast-evolving site categories are removed. These patterns suggest that: (1) NJ and MP, rather than ML, are liable to phylogenetic bias caused by multiple substitutions, and (2) that monophyly of the archaeobatrachian frogs under these methods is mainly supported by those sites that are most likely to bear homoplasy.

Distantly related outgroups.—Because the *PP* of rooting the ingroup on the branch separating Amphicoela from all other frogs approaches 1.0 when salamanders and caecilians are used as outgroups, the *PP* of any other root position is approximately 0. The credibility of this distinct rooting probability distribution is reinforced by examination of the rooting behavior of 100 artificial outgroups with respect to the ingroup topology. For most of the 53 ingroup branches, the average *RRP* (estimated as the average posterior probability of rooting the ingroup along a particular branch) is proportional to their length, but in several cases, a high variance is observed, with the incidence of excessively high posterior probabilities for some of the artificial outgroups (e.g., the maximum rooting probability using an artificial outgroup is 0.216 for the terminal branch leading to *Silurana tropicalis*). In addition, external branches tend to display a somewhat higher *RRP* than internal branches of similar length. However, the ingroup branch leading to Amphicoela shows an average *RRP* of 0.0098, and the highest observed rooting probability using an artifical outgroup is 0.0224. This is still > 44 times smaller than the *PP* = 1.0 inferred with the empirical outgroup taxa, implying a high ratio of *PP* over *RRP*. The four alternative root positions indicated in Fig. 2 have similarly low average *RRP*s (0.0188, 0.0066, 0.0052, and 0.0226, respectively), but even their minimum observed rooting probabilities using an artificial outgroup are still larger than their zero *PP*s, using the empirical out group. These ratios suggest that the rooting probability distribution across the ingroup topology obtained using salamanders and caecilians seems hardly influenced by random rooting artifacts caused by the large evolutionary distance between ingroup and outgroup taxa.

Evaluation of Alternative Phylogenetic Hypotheses

We estimated constrained ML trees for 21 alternative hypotheses (Fig. 5), which altogether represent a combination of seven alternative rooting scenarios (Figs. 2 and 5a to g), the nonmonophyly of the five well-supported anuran clades (Fig. 5g to k), and five previously published phylogenies (Duellman and Trueb, 1986; Ford and Cannatella, 1993; Hay et al., 1995; Haas, 2003; Pugener et al., 2003; Figs. 2 and 5a, e, l to n). Two of these disagree with our ML topology in more than one aspect. Ford and Cannatella's tree (1993) differs from our ML tree (compare Fig. 5l with Fig. 3) in corroborating (1) a paraphyletic Amphicoela, with *Ascaphus* diverging first; (2) a paraphyletic Discoglossoidea, with Bombinatoridae diverging first; and (3) the sister group relationship of Pelobatoidea and Pipoidea (= Mesobatrachia). Likewise, the cladograms proposed by Haas (2003) differ from our ML tree (compare Fig. 5m with Fig. 3) by supporting (1) a reversed branching order for the origins of Discoglossoidea and Pipoidea; (2) a paraphyletic arrangement of pelobatoid lineages; and (3) an (*Alytes* + (*Bombina* + *Discoglossus*)) clade, instead of (*Bombina* + (*Alytes* + *Discoglossus*)). The rejection of a topology containing a combination of such conflicting relationships does not necessarily justify the rejection of each of these relationships individually. As a more conservative approach, we constructed and evaluated null topologies for these six alternative relationships separately as well (Fig. 5g, o to s). Finally,

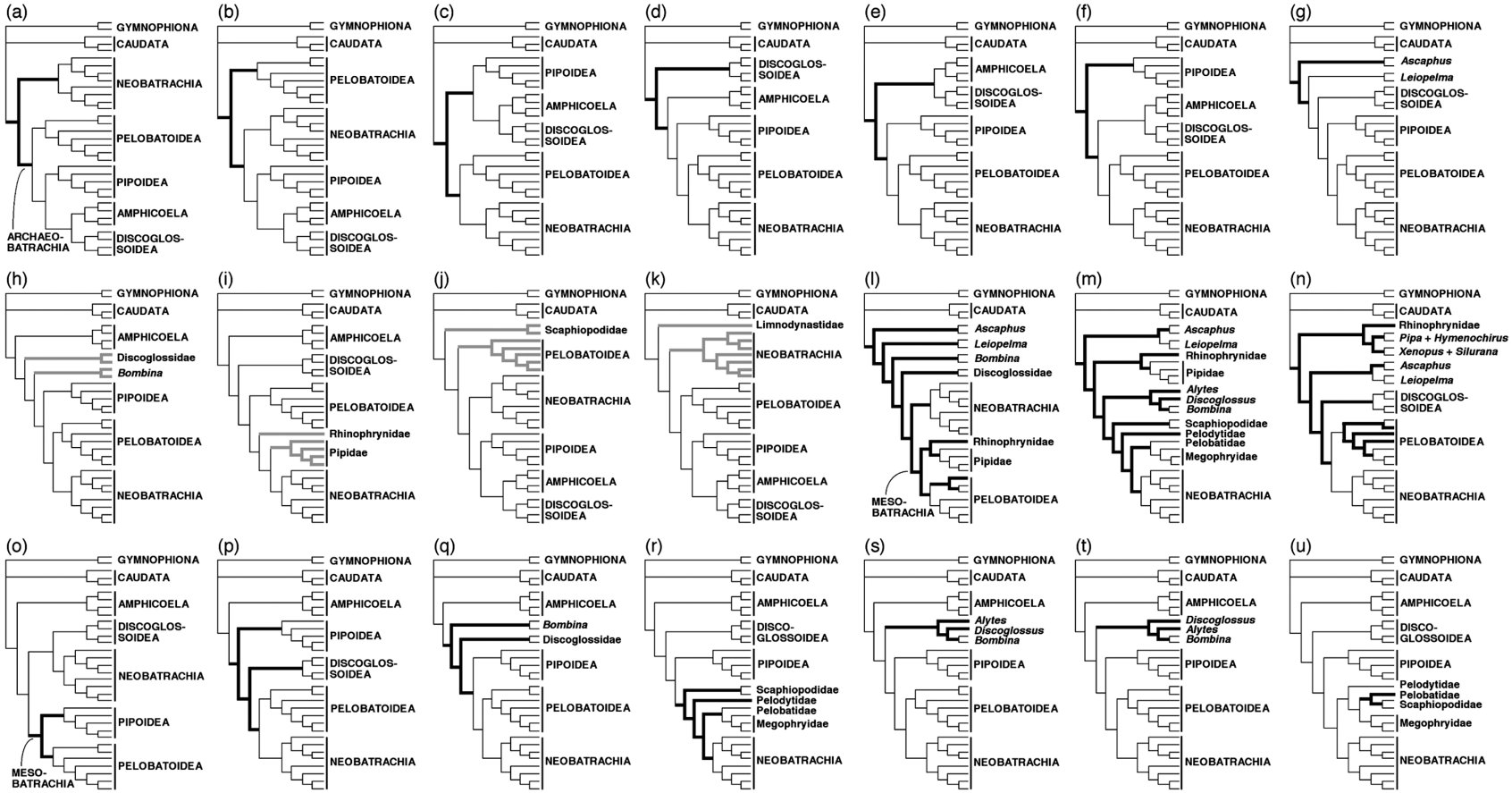

FIGURE 5. Topologies evaluated by the AU test and parametric bootstrapping. Branches in bold indicate the constraints used for ML optimization of the null hypothesis. (a) Ingroup root specifying Archaeobatrachia and Neobatrachia as sister clades (Hedges and Maxson, 1993; Hay et al., 1995; Dutta et al., 2004; Hertwig et al., 2004); (b) ingroup root on branch between Pelobatoidea and all other frogs; (c) ingroup root on branch between (Pelobatoidea + Neobatrachia) and all other frogs; (d) ingroup root on branch between Discoglossoidea and all other frogs; (e) ingroup root on branch between (Amphicoela + Discoglossoidea) and all other frogs (Laurent, 1979; Duellman and Trueb, 1986); (f) ingroup root on branch between Pipoidea and all other frogs (Maglia et al., 2001; Pugener et al., 2003); (g) ingroup root on branch between *Ascaphus* and all other frogs (= idem ML tree for nonmonophyly of Amphicoela; Ford and Cannatella, 1993); (h) nonmonophyly of Discoglossoidea; (i) nonmonophyly of Pipoidea; (j) nonmonophyly of Pelobatoidea; (k) nonmonophyly of Neobatrachia; (l) phylogeny proposed by Ford and Cannatella (1993); (m) phylogeny proposed by Haas (2003); (n) phylogeny proposed by Pugener et al. (2003); (o) sister group relationship of Pipoidea and Pelobatoidea (= Mesobatrachia; Laurent, 1979; Ford and Cannatella, 1993); (p) origin of Pipoidea prior to origin of Discoglossoidea (Haas, 2003); (q) origin of Bombinatoridae prior to origin of Discoglossidae (Ford and Cannatella, 1993); (r) paraphyletic arrangement of pelobatoid families according to Haas (2003); (s) monophyly of (*Bombina* + *Discoglossus*) with the exclusion of *Alytes* (Haas, 2003); (t) monophyly of (*Alytes* + *Bombina*) with the exclusion of *Discoglossus* (Hertwig et al., 2004); (u) monophyly of American and Eurasian spadefoot toads (*Pelobates* + (*Spea* + *Scaphiopus*)) (e.g., Ford and Cannatella, 1993).

TABLE 4. Alternative rooting and branching hypotheses with corresponding log-likelihoods, test statistics, and *P*-values inferred from the approximately unbiased (AU) and parametric bootstrap (PB) tests.

ML tree[a]	−ln L	δ ln *L*	*P*-value AU test	*P*-value PB test	Rejection[b]
Unconstrained (Fig. 3)	52,100.00	0.00	0.794	–	– –
Rooted on branch between Neobatrachia and Archaeobatrachia (a) (= reciprocal monophyly of Archaeobatrachia and Neobatrachia)	52,111.47	11.47	0.300	<0.002	– +
Rooted on branch between Pelobatoidea and all other frogs (b)	52,114.77	14.77	0.080	<0.002	– +
Rooted on branch between (Pelobatoidea + Neobatrachia) and all other frogs (c)	52,109.44	9.44	0.379	<0.002	– +
Rooted on branch between Discoglossoidea and all other frogs (d)	52,115.89	15.89	0.043	<0.002	+ +
Rooted on branch between (Amphicoela + Discoglossoidea) and all other frogs (e)	52,114.99	14.99	0.072	<0.002	– +
Rooted on branch between Pipoidea and all other frogs (f)	52,114.31	14.31	0.101	<0.002	– +
Rooted on branch between *Ascaphus* and all other frogs (g) (= idem nonmonophyly of Amphicoela)	52,115.64	15.64	0.054	<0.002	– +
Nonmonophyly of Discoglossoidea (h)	52,122.38	22.38	0.038	<0.002	+ +
Nonmonophyly of Pipoidea (i)	52,127.38	27.38	0.040	<0.002	+ +
Nonmonophyly of Pelobatoidea (j)	52,171.89	71.89	<0.001	<0.002	+ +
Nonmonophyly of Neobatrachia (k)	52,218.10	118.10	<0.001	<0.002	+ +
Phylogeny proposed by Ford and Cannatella (1993) (l)	52,197.97	97.97	<0.001	<0.002	+ +
Phylogeny proposed by Haas (2003) (m)	52,243.54	143.54	<0.001	<0.002	+ +
Phylogeny proposed by Pugener et al. (2003) (n)	52,127.84	27.84	0.019	<0.002	+ +
Sister-clade relationship of Pipoidea and Pelobatoidea (= Mesobatrachia) (o)	52,111.41	11.41	0.193	< 0.002	– +
Origin of Pipoidea prior to origin of Discoglossoidea (p)	52,101.12	1.12	0.718	0.092	– –
Origin of Bombinatoridae prior to origin of Discoglossidae (q)	52,123.31	23.31	0.017	<0.002	+ +
Paraphyletic arrangement of pelobatoid families according to Haas (2003) (r)	52,198.35	98.35	<0.001	<0.002	+ +
(*Alytes* + (*Bombina* + *Discoglossus*)) (s)	52,143.41	43.41	<0.001	<0.002	+ +
(*Discoglossus* + (*Alytes* + *Bombina*)) (t)	52,142.82	42.82	<0.001	<0.002	+ +
(*Pelobates* + (*Scaphiopus* + *Spea*)) (u)	52,156.73	56.73	<0.001	<0.002	+ +

[a] Letters in parentheses refer to the topologies depicted in Figure 5.

[b] A '+ +' score indicates rejection by the AU test at $\alpha = 0.05$ and by the PB test at a Bonferroni-corrected $\alpha = 0.00238$; '– +' indicates rejection by the PB test alone; '– –' indicates failure of rejection by either test.

we evaluated two topologies corroborating a (*Discoglossus* + (*Alytes* + *Bombina*)) arrangement (Hertwig et al., 2004), and the traditionally postulated (*Pelobates* + (*Spea* + *Scaphiopus*)) arrangement, respectively (Fig. 5t, u).

Results of the AU and the PB tests are summarized in Table 4. Thirteen alternative hypotheses are rejected under the assumptions of both tests, at $\alpha = 0.05$ and at a Bonferroni-corrected $\alpha = 0.00238$, respectively. These include the root position along the branch separating Discoglossoidea from all other frogs (Fig. 5d); the nonmonophyly of Discoglossoidea, Pipoidea, Pelobatoidea, and Neobatrachia (Fig. 5h to k); phylogenies proposed by Ford and Cannatella (1993), Haas (2003), and Pugener et al. (2003) (Fig. 5l to n); and alternative phylogenetic arrangements of discoglossoid and pelobatoid taxa (Fig. 5q to u). The six remaining root positions (Fig. 5a to c, e to g); as well as the Mesobatrachia hypothesis (Fig. 5o) could not be rejected based on the AU test, but receive *P*-values <0.002 from the PB tests. A single tree, postulating the reversed branching order of Discoglossoidea and Pipoidea (Fig. 5p), cannot be rejected significantly under the assumptions of either test. The latter outcome is consistent with the low bootstrap value and posterior support for the branch separating Amphicoela and Discoglossoidea from all other anurans (Figs. 2 and 3).

Estimation of Divergence Times

Based on fossil and tectonic evidence, we imposed the following time constraints on seven internal nodes:

1. The split between birds and mammals (diapsid versus synapsid reptiles) was set at 310 ± 10 Mya (Benton, 1997).
2. A minimum age of 338 Mya for the divergence between Lissamphibia and Amniota based on the aïstopod fossil †*Lethiscus stocki*, of Viséan age (Ruta et al., 2003).
3. A minimum age of 161 Mya for the origin of the cryptobranchoid salamanders (represented here by *Hynobius*), based on the fossil †*Chunerpeton tianyiensis*, of Bathonian age (Gao and Shubin, 2003).
4. A minimum age of 164 Mya for the origin of Discoglossoidea, based on the fossil †*Eodiscoglossus oxoniensis*, of Bathonian age (Evans et al., 1990; Yuan et al., 2000; Rocek, 2000).
5. A minimum age of 151 Mya for the origin of Pipoidea, based on the fossil †*Rhadinosteus parvus*, of Kimmeridgian age (Henrici, 1998).
6. A minimum age of 100 Mya for the divergence of the South American genus *Pipa* from African Pipidae, corresponding to the final separation of the respective continents (Smith et al., 1994; Scotese, 2001).
7. A minimum age of 82 Mya for the split between North America's *Ascaphus*, and New Zealand's *Leiopelma*, corresponding to the detachment of New Zealand from Antarctica and the loss of any terrestrial passage to the former (Lawver et al., 1991, Cooper et al., 2001).

Bayesian relaxed-clock analyses on the ML tree, using either all seven, or only the first two (nonamphibian)

TABLE 5. Comparison of divergence age estimates (Mya) and 95% credibility intervals (between brackets) for the major crown-group frog lineages.

Node	ML tree, 7 calibration points	ML tree, 2 calibration points	Pipoidea prior to Discoglossoidea, 7 calibration points
Root of anuran crown, origin of Amphicoela	225.1 (197.9–256.7)	224.4 (189.2–259.6)	226.8 (194.5–261.7)
Origin of Discoglossoidea	211.0 (183.6–243.5)	209.9 (173.4–245.9)	207.1 (173.6–243.2)
Origin of Pipoidea	204.3 (177.4–237.3)	203.1 (166.4–240.1)	211.0 (177.6–246.9)
Origin of Ascaphidae and Leiopelmatidae	182.8 (153.7–214.7)	183.6 (148.4–219.9)	180.1 (147.8–215.6)
Origin of Rhinophrynidae and Pipidae	176.3 (153.2–207.5)	175.0 (141.4–209.5)	179.3 (148.5–213.5)
Origin of Pelobatoidea and Neobatrachia	185.2 (158.5–217.4)	184.1 (149.2–219.6)	186.4 (154.0–221.2)

calibration points (see Materials and Methods), yielded nearly identical nodal time estimates (differing by less than 3 million years). Likewise, very similar age estimates (differing by less than 4 million years) were extracted from the ML-optimized topology corroborating the origin of Pipoidea prior to the origin of Discoglossoidea (Fig. 5p). The resulting divergence age estimates and 95% credibility intervals for the major anuran branching events (Table 5) situate the origin of the anuran crown-group at approximately 225 Mya, in the Upper Triassic. The subsequent origins of Discoglossoidea and Pipoidea approach the Triassic–Jurassic transition (200 to 210 Mya), suggesting that the diversification of extant frogs was well in progress by the early Jurassic.

DISCUSSION

Our likelihood-based analyses recover an evolutionary scenario that corroborates the paraphyly of archaeobatrachian frogs and the nested position of Neobatrachia. The credibility of the inferred root, specifying Amphicoela as the sister group of all other living anurans, is strengthened by additional examination of three potential sources of phylogenetic bias. The ambiguous outcome of the nonparametric and parametric tree selection tests precludes more conclusiveness on the anuran root, but both tests unanimously favor the rejection of several controversial branching hypotheses. The observed discrepancy between the AU test and the PB tests is most likely a result of the different forms of null hypothesis applied by both tests (Aris-Brosou, 2003). This complicates straightforward comparison of their respective *P*-values (Buckley, 2002), which may imply either a high type-1 error rate in the PB tests (making them appear too liberal), a high type-2 error rate in the AU test (making it appear too conservative), or a combination of both. Although PB tests have been described as potentially more powerful than nonparametric tests (Goldman et al., 2000), recent explorations of their performances record an increased risk of type-1 errors, related to the use of oversimplified models (Buckley, 2002; Aris-Brosou, 2003). Besides using the relatively parameter-rich GTR+Γ+I model for our tests, we have attempted to reduce the risk of model misspecification by evaluating the effects of latent SSRV. The AU test is designed to combine accurate control of type-1 errors with correction for the overconservative tree selection by the SH test (Shimodaira, 2002). However, given the relative novelty of the AU test, information on its robustness to deviations from its basic assumptions (such as the asymptotic theory (Shimodaira, 2002) or to model misspecification is still limited (Aris-Brosou, 2003). This complicates evaluation of its appropriateness in specific empirical cases. In summary, the outcome of both tests should be interpreted with caution, as potential rejection biases cannot be ruled out in either case.

Anuran Phylogenetic Relationships

The phylogeny proposed here is fairly compatible with morphological evidence. For example, the branching sequence of the major extant frog lineages displayed in Figure 3 shows large congruence with phylogenies proposed by Lynch (1973) and Haas (1997). Nevertheless, our phylogeny differs in several important aspects from recent hypotheses derived from both adult and larval morphology.

Amphicoela.—Although disputed by recent morphological analyses of mainly larval traits (Maglia et al., 2001; Pugener et al., 2003), the basal divergence of *Ascaphus* (tailed frogs) and *Leiopelma* (New Zealand frogs) receives support from several morphological character states that are unique in frogs, but bear relative similarity to those in stem fossils and salamanders. These include a metamorphic reorientation of the palatoquadrate that seems intermediate to those in salamanders and other frogs (Bell and Wasserzug, 2003) and a bilaterally paired sphenetmoid similar to that of stem frogs, which is single in other crown-group archaeobatrachians (Gao and Wang, 2001). In addition, the presence of nine presacral vertebrae represents a transitional stage between the 9 to 14 vertebrae observed in stem fossils and the 5 to 8 vertebrae of the remaining crown-group frogs. This is consistent with a stable trend of vertebral reduction throughout frog evolution, which has provided the necessary axial rigidity for efficient jumping (Shubin and Jenkins, 1995). The apparent lack of unambiguous synapomorphies has led to the postulation of paraphyly of Amphicoela, with *Ascaphus* as the sister group of all other living frogs (*Ascaphus* vs. Leiopelmatanura; Ford and Cannatella, 1993; Green and Cannatella, 1993). Our phylogenetic analyses and PB tests question this hypothesis, suggesting that shared characters of Leiopelmatanura (e.g., elongated arms on the sternum and an anteriorly ossifying sphenetmoid) are convergences, or that the lack of these structures in *Ascaphus* represents secondary losses. The reidentification of the Jurassic anurans †*Notobatrachus degiustoi* and

†*Vieraella herbstii* as stem anurans (Báez and Basso, 1996; Yuan et al., 2000; Gao and Wang, 2001) has excluded Amphicoela from the Mesozoic fossil record. Nevertheless, the numerous similarities between these fossils and modern Amphicoela may testify for an exceptional case of bradytely. Intriguingly, this suggests that the last common ancestor of living frogs may have had an appearance that was very similar to those of present-day *Ascaphus* and *Leiopelma*.

Discoglossoidea.—Ford and Cannatella (1993) postulated a paraphyletic arrangement of Bombinatoridae and Discoglossidae, with the sharing of a bicondylar sacrococcygeal articulation and the presence of an episternum as arguments for the grouping of Discoglossidae with a (Mesobatrachia + Neobatrachia) clade. Other phylogenetic reconstructions have corroborated the monophyly of the Discoglossoidea but diversely supported a split between *Alytes* and (*Bombina, Discoglossus*) (Haas, 2003), between *Discoglossus* and (*Alytes* + *Bombina*) (Clarke, 1988; Hertwig et al., 2004), or between *Bombina* and (*Alytes* + *Discoglossus*) (Yuan et al., 2000; Gao and Wang, 2001; Pugener et al., 2003; Hoegg et al., 2004). Both our nonparametric and parametric tests favor the rejection of discoglossoid paraphyly, and of the (*Alytes* + *Bombina*) and (*Bombina* + *Discoglossus*) clades. A morphological synapomorphy supporting the (*Alytes* + *Discoglossus*) grouping is a rodlike epipubis, which is either absent or more platelike in all other frogs (Pugener et al., 2003). Conversely, our results suggest that inspiratory sound production, postulated as a unique apomorphy of a (*Bombina* + *Discoglossus*) clade (Haas, 2003), either evolved twice independently in Discoglossoidea, or reversed in *Alytes*.

Pipoidea.—The numerous unique synapomorphies recorded in pipoid frogs (Maglia et al., 2001; Yeh, 2002; Pugener et al., 2003) are indicative for a dramatic rate acceleration in the morphological evolution of this lineage. Because the fossorial Rhinophrynidae and the strictly aquatic Pipidae represent invasions of very different adaptive zones, these synapomorphies seem to reflect an earlier, major shift to a new ontogenetic niche. Most changes are related to profound cranial remodeling and a derived type of filter-feeding tadpole with barbels and without keratinized mouthparts (Yeh, 2002; Pugener et al., 2003). This modified anatomy is likely to complicate homology assessment in morphological traits among frogs (Barry Clarke, personal communication), which may explain why the phylogenetic position of Pipoidea is a point of ongoing controversy. Indeed, morphology-based phylogenies using larval and/or adult characters have variously supported a sister group relationship with (1) Pelobatoidea (= the Mesobatrachia hypothesis, Ford and Cannatella, 1993); (2) a (Pelobatoidea + Neobatrachia) clade (Lynch, 1973; Duellman and Trueb, 1986; Haas, 1997); (3) a (Discoglossoidea + (Pelobatoidea + Neobatrachia)) clade (Haas, 2003); or (4) all other extant frogs (Maglia et al., 2001; Pugener et al., 2003). Molecular studies as well failed to reach a consensus on the phylogenetic position of pipoids and corroborated either the Mesobatrachia hypothesis (Hillis et al., 1993; García-París et al., 2003), a sister group relationship with the Neobatrachia (Kjer, 1995; Hoegg et al., 2004), or a trichotomy with Amphicoela and Discoglossoidea (Hay et al., 1995). The complexity of this issue is manifested in our topology tests by the inability to discriminate between alternate branching orders for Discoglossoidea and Pipoidea and failure of our AU test to reject the Mesobatrachia hypothesis or a basal divergence of Pipoidea. According to our ML tree, the absence of keratinous mouthparts in pipoid larvae (also lacking in salamanders; Pugener et al., 2003) may represent a reversed condition, and apparent synapomorphies of the Mesobatrachia (i.e., closure of the frontoparietal fontanelle and the loss of taeniae tecti; Ford and Cannatella, 1993) may have evolved twice independently, or reversed along the stem lineage of Neobatrachia.

A striking morphological trend in the evolution of frogs is the accelerated degeneration of free ribs during ontogenesis. Our results indicate that either ribs in adults are lost independently in Rhinophrynidae and in the ancestor of Pelobatoidea and Neobatrachia, or that a preceding degeneration is spectacularly reversed in Pipidae, although in a modified form (i.e., in adults, large ribs are fused to the transverse processes of the vertebrae). Evidence for the presence of free ribs in several primitive pipoid fossils (Henrici, 1998; Rocek, 2000) seems to favor the former scenario. The reduction of ribs in amphibian groups has been associated with a shift from coelom-driven to buccal-pump respiration (Duellman and Trueb, 1986), but the preservation of ribs may eventually have adaptive benefits for aquatic specialists. Ribs provide important adhesion points for respiratory musculature and therefore facilitate pulmonary ventilation in an environment that prevents efficient cutaneous or buccal respiration. Interestingly, other pipid features, such as large lungs with cartilagenous enforcements and an enlarged sternum, are consistent with this hypothesis.

Pelobatoidea and Neobatrachia.—Although not recovered by any earlier molecular study, the identification of pelobatoid frogs as the closest living relatives of the neobatrachian radiation is consistent with several morphological studies. One of the most prominent synapomorphies supporting their grouping is a distinct type of tadpole with single rows of keratodonts on the oral labia and a single sinistrally positioned spiracle (Orton, 1957; Maglia et al., 2001; Pugener et al., 2003; Haas, 2003). Remarkably, Pelobatoidea and Neobatrachia also possess palatine bones (Duellman and Trueb, 1986), also occurring in salamanders and basal amphibians. According to our ML tree, the absence of these structures in Amphicoela, Discoglossoidea, and Pipoidea suggests a single regain in an ancestor of Pelobatoidea and Neobatrachia, or at least three recurrent losses during anuran evolution. Within Pelobatoidea, our analyses and tests corroborate recent molecular (García-París et al., 2003, Hoegg et al., 2004; Hertwig et al., 2004) and morphological (Pugener er al., 2003; Haas, 2003) evidence against the traditional grouping of the spadefoot toad genera

Scaphiopus, *Spea* (North America), and *Pelobates* (Europe and adjacent Asia).

Pangaean Diversification of Crown-Group Frogs

The divergence time estimates presented here have several important implications for our perception of early anuran evolution. First, the estimated Upper-Triassic age for the deepest crown-group split documents the rise of this group approximately 55 million years earlier than its first appearance in the fossil record (Evans et al., 1990; Rocek, 2000; Fig. 6). Interestingly, this estimate predates several Jurassic stem anurans as well (e.g., †*Prosalirus* and †*Vieraella*), thus setting a lower limit to the origin of these extinct lineages. As a consequence, frogs may have obtained their specialized anatomy much earlier than currently evidenced by the fossil record. The inferred date estimates also corroborate an overlap of the early differentiation of crown-group anurans with mass extinction events at the end of the Triassic, which generated major shifts in the composition of

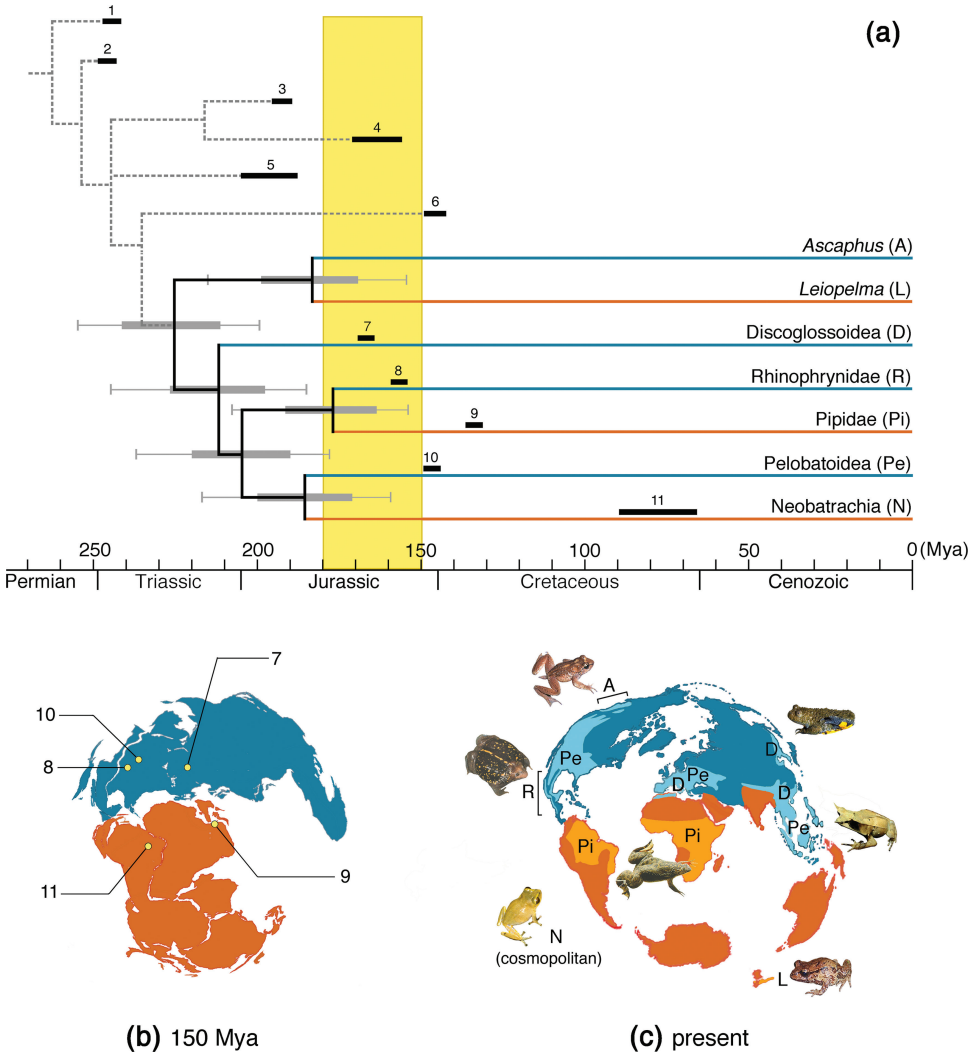

FIGURE 6. (a) The deep-time evolutionary history of frogs. Solid branches specify lineages dated using a relaxed molecular clock. The horizontal bars and thin horizontal lines at internal nodes indicate standard deviations and 95% credibility intervals, respectively. Dashed branches represent stem lineages (Ford and Cannatella, 1993; Báez and Basso, 1996; Yuan et al., 2000; Gao and Wang, 2001) with unknown divergence ages. Numbered bars represent the oldest known fossils for accompanying lineages. Nonanuran Salientia: 1, †*Triadobatrachus massinoti*; 2, †*Czatkobatrachus polonicus*; Stem Anura: 3, †*Prosalirus bitis*; 4, †*Notobatrachus degiustoi*; 5, †*Vieraella herbstii*; 6, †*Mesophryne beipiaoensis*; Crown Anura: 7, †*Eodiscoglossus oxoniensis* (Discoglossoidea); 8, †*Rhadinosteus parvus* (?Rhinophrynidae); 9, †*Shomronella jordanica*, †*Cordicephalus gracilis*, and †*Thoraciliacus rostriceps* (Pipidae); 10, *incertae sedis* within Pelobatidae (Evans and Milner, 1993); 11, †*Baurubatrachus pricei* (Leptodactylidae; Báez and Perí, 1989). Three independent splits between a Laurasia- (blue) and Gondwana- (red) associated branch coincide with the onset of Pangaean breakup (yellow window) (Gurnis, 1988; Smith et al., 1994; Scotese, 2001). (b) Plate-tectonic reconstruction of continents at full completion of this geological episode (Smith et al., 1994), and spatial distribution of early crown-group fossils. Numbers refer to fossil taxa in (a). The localities are fully consistent with the formation of distinct anuran communities on the northern and southern hemisphere. (c) Present-day distribution ranges of the major frog lineages still largely reflect the north–south rifting of Laurasia and Gondwana. Letter codes refer to lineages in (a). Although Neobatrachia have attained a cosmopolitan distribution, their early evolution is unambiguously associated with Gondwana.

vertebrate diversity (Benton, 1997). This evokes the possibility that extant frogs started diversifying as an opportunistic radiation, launched in the aftermath of this bottleneck episode by sudden niche availability. Such scenario would be analogous to contemporaneous patterns postulated for the ascent of dinosaurs (Benton, 1997; Sereno, 1999) and lizards (Evans, 2003).

Second, an entirely post-Pangaean diversification of living Anura seems improbable in the light of our data, as the 95% credibility intervals for the origins of four extant frog lineages fall prior to the initial north–south breakup of Pangaea (Fig. 6). This suggests that cladogenesis of extant frogs was well underway on this continent when it still formed a single landmass. The fossil record is currently too incomplete to substantiate this scenario, but a potential occurrence of basal crown-group frogs on Pangaea had been perceived previously (Savage, 1973; Duellman and Trueb, 1986; Shubin and Jenkins, 1995), based on the multicontinental recovery of stem fossils and the disjunct distribution of modern Amphicoela. An arid climate belt that covered large parts of the equatorial region of inland Pangaea (Scotese, 2001) most likely created an unfavorable environment for drought-intolerant amphibians. However, frogs may have diversified and reached a widespread distribution along the peripheral zones, where humid tropical and temperate climate conditions prevailed.

Finally, our phylogeny and divergence age estimates reveal a biogeographical pattern in crown-group frogs that strongly reflects the initial fragmention of Pangaea. The previously proposed hypothesis of a single vicariant event that isolated Archaeobatrachia on Laurasia and Neobatrachia on Gondwana (Feller and Hedges, 1998) is incompatible with the paraphyletic arrangement of the former. Moreover, a Laurasian origin of Archaeobatrachia would require two additional long-range dispersals into Gondwana after its breakup from Laurasia, in order to explain the present-day restriction of *Leiopelma* to New Zealand and occurrence of Pipidae in Africa and South America. Instead, our ML tree, in combination with the geographic distribution of fossil and/or present-day representatives, corroborates three major splitting events between a Laurasia- and a Gondwana-associated lineage, represented by *Ascaphus* and *Leiopelma*, Rhinophrynidae and Pipidae, and Pelobatoidea and Neobatrachia, respectively (Fig. 6). Curiously, our dating estimates situate each of these splits very close to the onset of Pangaean breakup, approximately 180 Mya (Gurnis, 1988; Smith et al., 1994; Scotese, 2001). This multiple contemporariness is suggestive for the formation of distinct anuran assemblages in both hemispheres, as a result of either synchronized continent-scale vicariance or Pangaean provincialism that originated shortly before the breakup.

Evolutionary branching patterns reflecting tectonic movements are ubiquitous in vertebrates (e.g., Savage, 1973; Krause et al., 1997; Feller and Hedges, 1998; Macey et al., 2000; Cooper et al., 2001; Gao and Shubin, 2003; García-París et al., 2003; Biju and Bossuyt, 2003), but nearly all documented cases are related to the progressive fragmentation of Gondwanan or Laurasian landmasses in the Cretaceous and Tertiary. In contrast, evidence for lineage diversification mediated by the initial Laurasia-Gondwana rifting is remarkably scarce. For modern vertebrates, the possibility of vicariance related to this event has been postulated for the divergence of scleroglossan and iguanian lizards (Estes, 1983, but see Evans, 2003) and the contentious split between caecilians and salamanders (Feller and Hedges, 1998). The observation of three nearly simultaneous splits in extant frogs alone outlines the possibility that this geological event has played a key role in the allopatric diversification of many terrestrial faunal assemblages. The initial north–south breakup of Pangaea should therefore be considered a potentially significant factor in the biogeographical interpretation of fossil data and the reconstruction of mesozoic biodiversity patterns.

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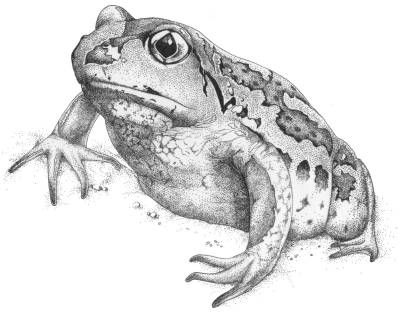

European spadefoot toad, *Pelobates fuscus*. Like its three congeners, this burrowing archaeobatrachian spreads a distinct garlic smell when handled. Drawing by Kim Roelants.