

Phylogeny and Comparative Substitution Rates of Frogs Inferred from Sequences of Three Nuclear Genes

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Phylogenetic relationships among major clades of anuran amphibians were studied using partial sequences of three nuclear protein coding genes, Rag-1, Rag-2, and rhodopsin in 26 frog species from 18 families. The concatenated nuclear data set comprised 2,616 nucleotides and was complemented by sequences of the mitochondrial 12S and 16S rRNA genes for analyses of evolutionary rates. Separate and combined analyses of the nuclear markers supported the monophyly of modern frogs (Neobatrachia), whereas they did not provide support for the monophyly of archaic frog lineages (Archaeobatrachia), contrary to previous studies based on mitochondrial data. The Neobatrachia contain two well supported clades that correspond to the subfamilies Ranoidea (Hyperoliidae, Mantellidae, Microhylidae, Ranidae, and Rhacophoridae) and Hyloidea (Bufonidae, Hylidae, Leptodactylidae, and Pseudidae). Two other families (Heleophrynidae and Sooglossidae) occupied basal positions and probably represent ancient relicts within the Neobatrachia, which had been less clearly indicated by previous mitochondrial analyses. Branch lengths of archaeobatrachians were consistently shorter in all separate analyses, and nonparametric rate smoothing indicated accelerated substitution rates in neobatrachians. However, relative rate tests confirmed this tendency only for mitochondrial genes. In contrast, nuclear gene sequences from our study and from an additional GenBank survey showed no clear phylogenetic trends in terms of differences in rates of molecular evolution. Maximum likelihood trees based on Rag-1 and using only one neobatrachian and one archaeobatrachian sequence, respectively, even had longer archaeobatrachian branches averaged over all pairwise comparisons. More data are necessary to understand the significance of a possibly general assignment of short branches to basal and species-poor taxa by tree-reconstruction algorithms.

Introduction

Anurans (frogs and toads) form by far the largest order of the living amphibians (Lissamphibia) with 4,899 species (Amphibiaweb, July 2003). Although the monophyly of each of the three lissamphibian orders (anurans, salamanders, and caecilians) is widely accepted (Duellman and Trueb 1986; Hedges and Maxson 1993; Hay et al. 1995; Zardoya and Meyer 2001, Meyer and Zardoya 2003), the relationships within these groups are still debated. Morphological studies of anurans did not provide a clear answer on the phylogenetic relationships, especially among the basal groups (Griffiths 1963; Laurent 1979; Duellman and Trueb 1986; Ford and Cannatella 1993). The derived anuran *bauplan* makes the recognition of frogs unambiguous, but it also led to a limited morphological evolutionary plasticity (Wallace, Maxson, and Wilson 1971; Emerson 1988). A high degree of homoplasy is found among clades that radiated into similar adaptive zones (e.g., Maxson and Wilson 1974; Bossuyt and Milinkovitch 2000; Vences et al. 2000a).

Early workers (e.g., Duellman 1975) subdivided the anurans into two suborders: Archaeobatrachia (containing the families Leiopelmatidae, Discoglossidae, Pipidae, Rhinophrynidae, Pelobatidae, and Pelodytidae) and Neobatrachia (superfamilies Hyloidea, Microhyloidea, and

Ranoidea) (table 1). The Archaeobatrachia were generally found to be paraphyletic in phylogenetic reconstructions (e.g., Duellman and Trueb 1986). Later studies proposed a third suborder Mesobatrachia consisting of Pipoidea and Pelobatoidea (table 1). Molecular studies based on mitochondrial data sets (Hedges and Maxson 1993; Hay et al. 1995; Feller and Hedges 1998) indicated monophyly of a suborder Archaeobatrachia *sensu* Duellman (1975), and this classification (Archaeobatrachia including Mesobatrachia) is followed herein.

Although the monophyly of archaeobatrachians has been disputed, there is general consensus that the Neobatrachia are a monophyletic group. Neobatrachian clades are characterized by several derived character states (e.g., Duellman and Trueb 1986; Ford and Cannatella 1993; Haas 2003) and are much more species-rich than archaeobatrachians (4,693 vs. 206 species; Amphibiaweb, July 2003). In the fossil record of extant anuran families, archaeobatrachians are known from the Middle Jurassic (Discoglossidae), whereas the oldest neobatrachian fossils are leptodactylids from the Late Cretaceous (Sanchiz 1998; Báez 2000; Roček 2000; Roček and Rage 2000). According to mitochondrial data (Hay et al. 1995; Ruvinsky and Maxson 1996; Feller and Hedges 1998), neobatrachians mainly consist of two large monophyletic clades, Hyloidea (previously named Bufonidea) and Ranoidea, with current centers of diversity in the Neotropics and in the Old World, respectively (Feller and Hedges 1998). In contrast, morphological phylogenies (Duellman and Trueb 1986; Ford and Cannatella 1993; Haas 2003) typically found the Hyloidea to be paraphyletic. Few families, such as the Sooglossidae and the Microhylidae, were not consistently classified as members of one of the major clades.

Although all frogs are characterized by a somewhat limited morphological variability, indications exist that molecular evolution is faster in neobatrachian frogs. The

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Key words: Amphibia, Anura, Archaeobatrachia, Neobatrachia, Ranoidea, Hyloidea, Rag-1, Rag-2, rhodopsin, substitution rates.

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Mol. Biol. Evol. 21(7):1188–1200. 2004

doi:10.1093/molbev/msh081

Advance Access publication February 12, 2004

Table 1
Classification of Anurans Used in Recent Morphological and Molecular Studies

Duellman (1975)	Laurent (1979)	Hay et al. (1995)
<u>Archaeobatrachia</u>	<u>Archaeobatrachia</u>	<u>Archaeobatrachia</u>
Discoglossoidae	Discoglossoidae	Leiopelmatidae
Leiopelmatidae	Leiopelmatidae	Discoglossidae
Discoglossidae	Discoglossidae	Pipoidea
Pipoidea	<u>Mesobatrachia</u>	Rhinophrynidae
Pipidae	Pipoidea	Pipidae
Rhinophrynidae	Pipidae	Pelobatoidea
Pelobatoidea	Rhinophrynidae	Pelobatidae
Pelobatidae	Pelobatoidea	Pelodytidae
Pelodytidae	Pelobatidae	<u>Neobatrachia</u>
<u>Neobatrachia</u>	Pelodytidae	Ranoidea
Bufonoidea	<u>Neobatrachia</u>	Ranidae
Myobatrachidae	Bufonoidea	Mantellidae
Leptodactylidae	Rheobatrachidae	Microhylidae
Bufoidea	Myobatrachidae	Hyperoliidae
Brachycephalidae	Sooglossidae	Sooglossidae
Rhinodermatidae	Leptodactylidae	Bufonoidea
Dendrobatidae	Phyllobatidae	Myobatrachidae
Pseudidae	Bufoidea	Heleophrynidae
Hylidae	Brachycephalidae	Leptodactylidae
Centrolenidae	Rhinodermatidae	Pseudidae
Microhyloidea	Pseudidae	Dendrobatidae
Microhyloidae	Hylidae	Rhinodermatidae
Ranoidea	Centrolenidae	Bufoidea
Sooglossidae	Pelodytidae	Hylidae
Ranidae	Microhyloidea	Centrolenidae
Hyperoliidae	Microhylidae	
Rhacophoridae	Ranidae	
	Hyperoliidae	
	Ranidae	
	Hemisidae	

published molecular phylogenetic trees of frogs, based on mitochondrial and nuclear rRNA genes, usually had distinctly longer branch lengths in neobatrachians than in basal frogs (Hedges, Moberg, and Maxson 1990; Hay et al. 1995; Feller and Hedges 1998). The neobatrachian mitochondrial genome is characterized by unique genomic rearrangements (Macey et al. 1997; Sumida et al. 2001), a feature that can be correlated with higher substitution rates at the nucleotide level (Shao et al., 2003). This possible tendency of accelerated rates in neobatrachians merits further exploration, because tree-reconstruction artifacts such as long-branch attraction (Felsenstein 1978) or erosion (Fuellen, Wägele, and Giegerich 2001) are widespread phenomena when some taxa have particularly fast or slow substitution rates (Philippe and Laurent 1998; Philippe, Germot, and Moreira 2000).

Recent work has shown that nuclear protein-coding single-copy genes outperform mitochondrial sequences in reconstructing ancient relationships (e.g., Springer et al. 2001). This is probably also true for amphibians, in which mitochondrial genes have been found to have a limited resolution in some cases (Graybeal 1993, 1994). In this study we obtained sequences of three markers (Rag-1, Rag-2, and rhodopsin) from 26 species of anurans that represent most major frog lineages. We submitted these sequences to separate and combined analyses and investigated possible artifacts in tree reconstruction as well as variations in substitution rate estimation.

Materials and Methods

Taxon Sampling and DNA Extraction

Taxa were selected to cover all major groups within the archaeobatrachians, mesobatrachians, and neobatrachians (table 2). Coelacanth, human, chicken, and salamander sequences were used as outgroup. GenBank accession numbers are given in table 2.

DNA was extracted from muscle tissue stored at -80°C or fixed in 70% ethanol using the ATL extraction buffer (Qiagen, Germany) and additional Proteinase K (final concentration 1 mg/ml). After homogenization, DNA was purified by a standard phenol/chloroform procedure followed by ethanol precipitation (Sambrook, Fritsch, and Maniatis 1989).

DNA Amplification and Sequencing

Fragments of nuclear DNA were amplified by applying conditions of a long range PCR (Cheng et al. 1994). For Rag-1, a combination of degenerated primers designed for sharks (Martin 1999) and amphibians (R. Zardoya, personal communication) were used to obtain overlapping fragments for a total length of 1,482 bp. Rag-2 fragments were amplified with primers that range over a 829-bp fragment of the 5' end of the coding region (Rag2A.F35 TGG CCI AAA MGI TCY TGY CCM ACW GG, Rag2.Lung.35F GGC CAA AGA GRT CYT GTC CIA CTG G, Rag2.Lung.320R AYC ACC CAT ATY RCT ACC AAA CC). Rhodopsin exon 1 sequences were obtained using a forward primer Rhod.ma (AAC GGA ACA GAA GGY CC) and a reverse primer Rhod.md (GTA GCG AAG AAR CCT TC).

PCR was performed in 25 μl reactions containing 0.5–1.0 units of REDTaq DNA Polymerase (Sigma, Taufkirchen, Germany), 0.01 units of *Pwo* DNA polymerase (Roche, Mannheim, Germany), 50 ng genomic DNA, 10 pmol of each primer, 15 nmol of each dNTP, 50 nmol additional MgCl_2 , and the REDTaq PCR reaction buffer (onefold concentrated: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.1 mM MgCl_2 , and 0.01% gelatine). Cycle conditions were adapted from a long range PCR protocol (Barnes 1994) with an initial denaturation step at 94°C for 5 min, followed by ten cycles with 94°C for 30 s, annealing temperatures increasing by 0.5°C per cycle from 50°C to 55°C but decreasing annealing time by 5 s per cycle from 90 to 40 s and extending for 4 min at 68°C . An additional 20 cycles were performed at 94°C for 10 s, 55°C for 40 s, and 68°C for 4 min. The final extension was done at 68°C for 5 min.

PCR products were purified either directly via spin columns (Qiagen) or over gel (1% agarose) using the gel purification kit (Qiagen). Sequencing was performed either directly using the corresponding PCR primers or after cloning into the pCR2.1/TOPO vector (Invitrogen, Karlsruhe, Germany) using the M13 primers (forward and reverse).

DNA sequences of both strands were obtained using the BigDye Terminator cycle-sequencing ready reaction kit (Applied Biosystems, Inc., Foster City, CA) on an ABI 3100 capillary sequencer following the manufacturer's instructions.

Table 2
Systematic Overview of Species Sampling and Genbank Accession Numbers

	Rag-1	Rag-2	rhodopsin	12S rDNA	16S rDNA
Coelacanthiformes					
<i>Latimeria chalumnae</i>	—	—	AH007712	U82228	U82228
<i>Latimeria menadoensis</i>	AY323779	AF369087	—	—	—
Amniota					
<i>Homo sapiens</i>	NM_000448	BC022397	NM_000539	NC_001807	NC_001807
<i>Gallus gallus</i>	AF143730	M58531	D00702	AY235571	AY235571
Amphibia					
Urodela					
Salamandra					
<i>Ambystoma mexicanum</i>	AY323752	AY323782	—	X86223	X86257
<i>Ambystoma tigrinum</i>	—	—	U36574	—	—
<i>Pleurodeles waltl</i>	AJ010258	AY323800	—	—	—
<i>Cynops pyrrhogaster</i>	—	—	AB043890	—	—
<i>Mertensiella luschani</i>	AY323753	AY323797	—	AF154053	AF154053
Anura (Salientia)					
Ascaphidae					
<i>Ascaphus truei</i>	AY323754	—	AY323730	X86225	X86257
Bombinatoridae					
<i>Bombina orientalis</i>	AY323756	AY323783	AY323732	AY333658	AY333696
Discoglossidae					
<i>Alytes muletensis</i>	AY323755	AY323781	AY323731	AY333671	AF224729
<i>Discoglossus sardus</i>	AY323757	AY323785	AY323733	AY333674	AY333712
Pelobatidae					
<i>Pelobates cultripes</i>	AY323758	—	AY323736	AY494053	AY494052
Scaphiopodidae					
<i>Scaphiopus couchii</i>	AY323759	AY323804	AY323738	AY330910	AY330897
Megophryidae					
<i>Megophrys</i> sp.	AY323760	AY323796	AY323737	AY330907	AY330895
Pipidae					
<i>Xenopus laevis</i>	L19324	L19325	S62229	M10217	AY341727
<i>Pipa parva</i>	AY323761	AY323799	AY323734	AY333652	AY333690
<i>Hymenochirus boettgeri</i>	—	—	AY323735	AY341634	AY341726
Heleophrynidae					
<i>Heleophryne regis</i>	AY323764	AY323786	AY323739	—	AF215501
<i>Heleophryne natalensis</i>	—	—	—	X86237	—
Sooglossidae					
<i>Nesomantis thomasseti</i>	AY323778	AY323798	AY323744	AY330908	AY330889
Microhylidae					
<i>Kaloula pulchra</i>	AY323772	AY323790	—	AY330902	AY330893
<i>Kaloula taprobanica</i>	—	—	AF249100	—	—
Hyperoliidae					
<i>Hyperolius viridiflavus</i>	AY323769	AY323789	AY323740	AF215440	AF215223
<i>Heterixalus tricolor</i>	AY323768	AY323787	AY323741	AF215434	AF215220
Ranidae					
<i>Rana temporaria</i>	AY323776	AY323803	AF249119	AF124135	AF124103
Rhacophoridae					
<i>Polypedates maculatus</i>	AY323777	AY323802	—	AF215358	AF215184
<i>Polypedates cruciger</i>	—	—	AF249124	—	—
Mantellidae					
<i>Mantidactylus</i> sp.	AY323775	AY323794	AY323742	AY330906	AY330888
<i>Mantidactylus wittei</i>	AY323774	AY323795	AY323743	AY330904	AF317691
Leptodactylidae					
<i>Leptodactylus fuscus</i>	AY323770	AY323791	AY323746	AY263215	AY263226
<i>Leptodactylus mystacinus</i>	AY323771	AY323792	AY323747	AY330905	AF124129
Pseudidae					
<i>Pseudis paradoxa</i>	AY323773	AY323801	AY323748	AY330909	AY330896
Hylidae					
Hylinae					
<i>Hyla cinerea</i>	AY323766	AY323788	AY323749	X86238	AY330892
Pelodryadinae					
<i>Litoria caerulea</i>	AY323767	AY323793	AY323751	AY330903	AY330894
<i>Agalychnis callidryas</i>	AY323765	AY323780	AY323750	AY330898	AY330890
Bufo					
<i>Bufo bufo</i>	AY323762	—	U59921	—	—
<i>Bufo regularis</i>	AY323763	AY323784	AY323745	AY330899	AY330891

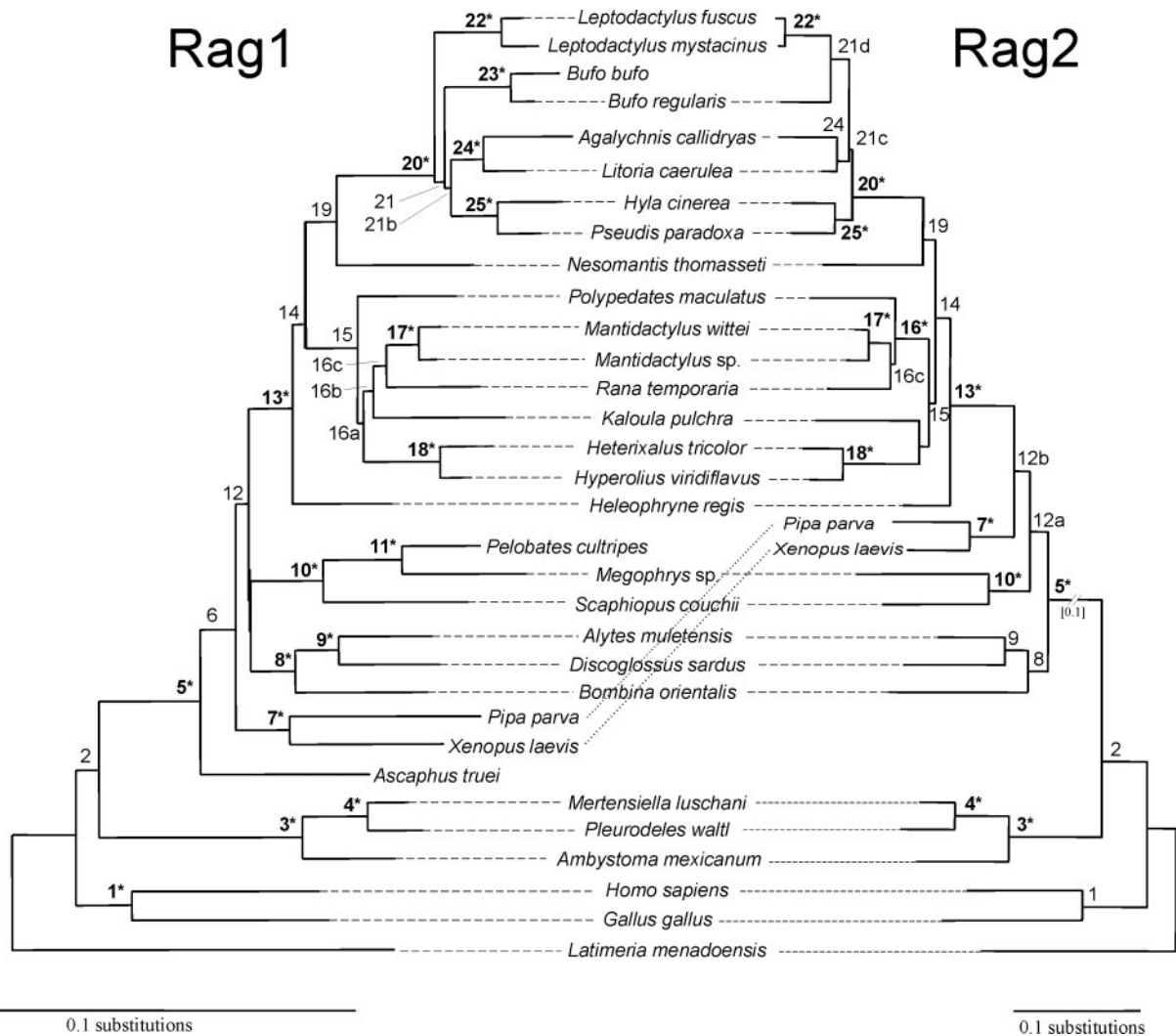


FIG. 1.—Phylogenetic trees as obtained by ML analyses. Nodes are numbered; the corresponding bootstrap proportions can be found in table 3 (Rag-1) and table 4 (Rag-2). Asterisks and boldface indicate bootstrap support values over 90%. Note that the branch leading to node 5* in the Rag-2 tree was shortened for optical reasons by 0.1 substitutions (= 1 bar length).

To compare our data with previously published hypotheses (Hay et al. 1995), we also amplified and sequenced fragments of the mitochondrial 12S and 16S rRNA genes, using primers and PCR protocols as specified in Vences et al. (2000b).

Phylogenetic Analysis

For all three nuclear genes, we plotted the transition-transversion ratio as determined by MEGA 2.1 (Kumar et al. 2001) against the sequence divergence (Kimura-2-parameter model) to test our data sets for saturation. Based on these saturation plots (data not shown), we excluded third codon positions from the Rag-1 and Rag-2 data sets; the rhodopsin sequences showed no saturation effects, and third positions of this gene were therefore not excluded from the phylogenetic analyses. For analyses of the 12S and 16S rDNA data sets, we excluded all regions that could not be aligned reliably as well as all gapped positions (alignment available from the authors upon request).

We calculated phylogenetic trees using each marker separately (fig. 1) and using the three nuclear genes in a combined approach (fig. 2). Homogeneity of the data sets was tested with the incongruence-length difference test (Farris et al. 1995) as implemented in PAUP*. Neighbor-Joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) analyses were performed using PAUP* (Swofford 1998). The best fitting models of sequence evolution for ML analyses were obtained by Modeltest 3.06 (Posada and Crandall 1998). Heuristic searches were performed using 10 replicates of a stepwise addition of taxa.

Robustness of NJ and MP tree topologies were tested by bootstrap analyses (Felsenstein 1985) with 2,000 replicates each (Hedges 1992); only 100 ML bootstrap replicates were calculated, because of computational constraints. Bayesian inference was conducted with MrBayes 2.0 (Huelsenbeck and Ronquist 2001) using the GTR model with 200,000 generations, sampling trees every 10th generation, and calculating a consensus tree

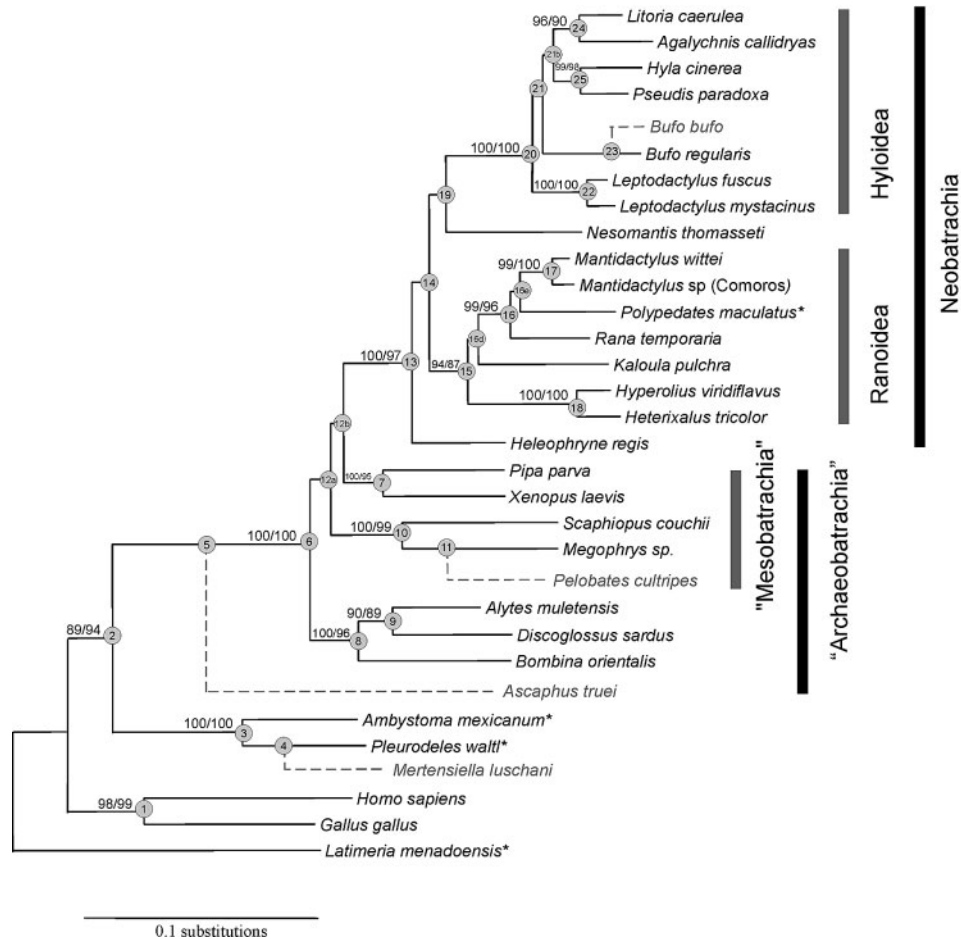


FIG. 2.—Phylogenetic tree obtained from ML analyses of combined data sets (Rag-1, Rag-2, and rhodopsin). Nodes are numbered; values shown are the ML and MP bootstrap values from the nucleotide data set. Further bootstrap proportions are listed in table 5. Asterisks indicate hybrid sequences, composed of sequences from closely related species (Table 2). Grey dashed lines indicate taxa that were added to the tree based on the analysis of only a subset of these sequences (fig. 1).

after omitting the first 5,000 trees (“burn-in” set at 50,000 based on empirical evidence).

Phylogenetic analyses of the corresponding data set of combined amino acids were performed with MEGA 2.1 (Kumar et al. 2001; Neighbor-Joining and maximum parsimony), Tree-Puzzle 5.0 (Schmidt et al. 2002; maximum likelihood) and MrBayes 2.0 (Huelsenbeck and Ronquist 2001; Bayesian inference). Tree-Puzzle and MrBayes analyses were performed under the JTT model (Jones, Taylor, and Thornton 1992).

Comparisons of Branch Lengths

Branch lengths from the ML tree of the Rag-1 nucleotide data set were calculated down to the *Ascaphus* split, by adding up single internal and terminal branches. Pairwise comparisons were done with ML settings of the complete Rag-1 data set, creating a subset of sequences consisting of the outgroup (*Latimeria menadoensis*, *Homo sapiens*, *Gallus gallus*, *Mertensiella luschani*, *Pleurodeles waltl*, *Ambystoma mexicanum*) and one neobatrachian and one archaeobatrachian sequence.

Comparisons of Substitution Rates

To estimate the substitution rates of the different genes in the resulting phylogeny, we defined the preferred ML topology (fig. 2) as a constraint and constructed ML phylograms for each of the four data partitions in PAUP*, using specific substitution models suggested by Modeltest. The phylograms were subjected to nonparametric rate-smoothing (NPRS) using the program r8s (Sanderson 1997). The obtained substitution rates for each node were used as independent characters in correlation analyses and analyses of variance (ANOVA). Relative rate tests (Takezaki, Rzhetsky, and Nei 1995) were performed using Phyltest (Kumar 1996) under the Kimura-2-parameter substitution model. Two analyses of long-branch attraction indicators were carried out following Stiller and Hall (1999). First, a chi-square test for deviant nucleotide was performed using Tree-Puzzle. Second, we tabulated the number of unique substitutions, i.e., instances in which a nucleotide at a given position in one sequence was different and invariable in all other sequences, including the outgroups (autapomorphies).

The GenBank/EMBL database was screened for genes of which at least one neobatrachian and one

Table 3
Support Values for Rag-1 Nucleotide Topology

Node	Bayesian	ML	NJ	MP	Clade Name
1	98	73	83	74	Amniotes
2	—	—	53	43	Amphibia
3	100	100	100	100	Caudata
4	100	95	99	98	Salamandridae
5	100	95	99	96	Anura
6	100	72	55	59	
7	98	71	91	83	Pipidae
8	100	96	83	82	
9	100	87	94	95	Discoglossidae
10	100	93	94	89	Pelobatoidea
11	100	99	97	98	
12	50	41	—	—	
13	100	92	91	72	Neobatrachia
14	71	43	—	—	
15	100	68	76	73	Ranoidea
16a	41	—	—	—	
16b	76	48	—	—	
16c	86	63	73	45	Ranidae
17	100	89	99	99	Mantellidae
18	100	100	100	99	Hyperoliinae
19	65	47	36	60	
20	100	100	100	100	Hyloidea
21a	54	—	50	40	
21b	63	—	—	36	
22	100	99	100	100	Leptodactylidae
23	100	100	100	100	Bufoidea
24	100	86	75	73	
25	100	92	91	95	

NOTE.—Numbers refer to labeled nodes in figure 1. Bayesian: Posterior probabilities as obtained from MrBayes analyses; ML: Maximum likelihood bootstrap proportions; NJ: Neighbor-Joining bootstrap proportions; MP: Maximum parsimony bootstrap proportions.

archaeobatrachian sequence was available. Sequences were downloaded and Blast searches carried out to find homologous sequences of outgroup species. Sequences that could not be reliably aligned or for which gene duplication events were observed in preliminary phylogenetic reconstructions were excluded. Alignments were done by ClustalX (Thompson et al. 1997) and afterwards refined manually. Gaps and ambiguous sites were excluded from all alignments. Statistical analyses (Spearman rank correlation, ANOVA) were carried out using the program SPSS for Windows, version 10.

Results

Rag-1 and Rag-2 Analyses

The Rag-1 data set consisted of 1,482 nucleotide positions and 32 species (26 ingroup and 6 outgroup taxa) with 28 sequences obtained in this study. Of 988 included positions (3rd positions excluded), 206 were parsimony informative. For Rag-2, we obtained sequences from 23 species and the final data set contained 29 sequences (23 ingroup and 6 outgroup taxa) with a total length of 829 bp. Of 546 included positions (3rd positions excluded), 236 were parsimony informative. For both data sets, saturation plots warranted an exclusion of third codon positions for subsequent phylogenetic analyses. The Rag-1 data set contains sequences from *Pelobates cultripes* and *Ascapheus truei*, for which no Rag-2 sequences were available. Figure 1 shows the ML trees of Rag-1 and Rag-2, tables 3 and 4 give

Table 4
Support Values for Rag-2 Nucleotide Topology

Node	Bayesian	ML	NJ	MP	Clade Name
1	99	65	90	84	Amniotes
2	98	79	77	69	Amphibia
3	100	100	100	100	Caudata
4	100	98	100	100	Salamandridae
5	100	100	100	100	Anura
7	100	99	100	97	Pipidae
8	76	63	83	48	Discoglossidae
9	87	51	57	40	
10	100	97	91	89	Pelobatoidea
12a	56	—	—	—	
12b	74	43	66	42	
13	100	99	100	98	Neobatrachia
14	54	43	—	48	
15	95	56	—	—	Ranoidea
16	100	93	80	82	
16c	47	48	90	—	
16d	58	50	60	—	
17	100	98	99	95	Mantellidae
18	100	100	100	100	Hyperoliinae
19	84	57	—	—	
20	100	100	100	100	Hyloidea
21c	61	—	—	—	
21d	97	58	75	50	
22	100	100	100	100	Leptodactylidae
24	98	71	75	63	
25	100	96	95	94	

NOTE.—Numbers refer to labeled nodes in figure 1. Bayesian: Posterior probabilities as obtained from MrBayes analyses; ML: Maximum likelihood bootstrap proportions; NJ: Neighbor-Joining bootstrap proportions; MP: Maximum parsimony bootstrap proportions.

the corresponding bootstrap proportions (ML, NJ, MP) and posterior probabilities (Bayesian inference) for each node.

We find that several of the main nodes in analyses of both data sets are well supported: the monophyly of Caudata, Salamandridae, Anura, and Neobatrachia. Within the paraphyletic archaeobatrachians, the nodes for the Pipidae, Pelobatoidea, and Discoglossidae (only for Rag-1) received strong support. The Bombinatoridae are grouped with the Discoglossidae. Within the Pelobatoidea, *Megophrys* forms a monophyletic group with *Pelobates* (fig. 1, node 11). The basal position of *Ascapheus truei* within the Anura is mainly supported by ML based methods (fig. 1, node 6). The relationships between Pipidae, Pelobatoidea, and Discoglossidae remain unresolved, and, importantly, no indications for archaeobatrachian monophyly are found. Our data neither supported nor rejected a basal position of pipids as found in a recent morphological study (Pugener, Maglia, and Trueb 2003).

Within the neobatrachians there is clear support for two major clades. One of these corresponds to the Ranoidea (fig. 1, node 15), but relationships within this group are not well resolved. The second clade contains all hyloid taxa except for *Nesomantis* and *Heleophryne*; these taxa form a unanimously supported monophyletic group (fig. 1, node 20), but relationships between the single families are not resolved (fig. 1, nodes 21a–d). A clear monophyletic group is formed by *Hyla cinerea* and *Pseudis paradoxa* (fig. 1, node 25), which suggests paraphyly of the Hylidae with respect to *Agalychnis callidryas* and *Litoria caerulea* if Pseudidae is to be recognized as a distinct family. Only weak support is

Table 5
Support Values for Combined Data Set Based on Nucleotide Data (nt) and Amino Acid Data (aa)

Node	Bayesian (nt)	ML (nt)	NJ (nt)	MP (nt)	Bayesian (aa)	ML (aa)	NJ (aa)	MP (aa)	Clade Name
1	100	98	99	99	100	97	96	99	Amniota
2	100	89	74	94	100	86	59	62	Amphibia
3	100	100	100	100	100	95	100	100	Caudata
6	100	100	100	100	100	82	100	100	Anura
7	100	100	100	95	100	97	99	90	Pipidae
8	100	100	100	96	100	87	99	92	
9	100	90	96	89	100	95	91	74	
10	100	100	100	99	100	88	92	62	Pelobatoidea
12a	86	61	—	—	72	—	—	—	
12b	74	47	—	—	—	—	—	—	
13	100	100	100	97	100	98	100	99	Neobatrachia
14	88	60	—	—	51	—	—	55	
15	100	94	96	87	100	40	81	76	Ranoidea
16	100	99	100	96	100	43	93	74	
16d	61	55	—	42	—	—	—	—	
16e	52	—	—	44	—	—	—	—	
17	100	99	100	100	100	72	99	99	Mantelliidae
18	100	100	100	100	100	78	100	100	Hyperoliidae
19	84	48	—	61	77	61	56	59	
20	100	100	100	100	100	61	99	100	Hyoidea
21	80	48	—	—	68	—	—	—	
21b	96	56	49	50	100	46	58	81	
22	100	100	100	100	100	78	100	100	Leptodactylidae
24	100	96	98	90	100	77	94	85	
25	100	99	100	98	100	90	93	97	

NOTE.—Numbers refer to labels nodes on figure 2. Bayesian: Posterior probabilities as obtained from MrBayes analysis; ML: Maximum likelihood bootstrap proportions, NJ: Neighbor-Joining bootstrap proportions; MP: Maximum parsimony bootstrap proportions. (nt) are values obtained from analysis of nucleotide data set; (aa) are values obtained from analysis of amino acid data set.

found for a basal position of *Heleophryne* and *Nesomantis* as a part of the Hyloidea, but these taxa show strong divergences from other hyloids.

Additional Data Sets

For rhodopsin, the data set comprised 32 taxa with a length of 315 nucleotides, all of which were retained for the analyses. Due to the limited number of positions, the analysis recovers most of the major clades (e.g., Neobatrachia), but in general is poorly resolved (data not shown). The combined 12S/16S data set, after exclusion of all hypervariable regions and gapped positions contained 458 nucleotides, of which 173 were parsimony-informative. Among the clades supported by high bootstrap values (>70%) are the Neobatrachia and the Hyloidea (excluding *Nesomantis* and *Heleophryne*). None of the analyses (ML, NJ, MP) resulted in a monophyletic grouping of the Archaeobatrachia.

Phylogenetic analyses of corresponding amino acid data sets of Rag-1, Rag-2, and rhodopsin yielded similar results as the nucleotide data sets (data not shown).

Combined Nuclear Data

For increased resolution with more positions (Leconte et al. 1994), we combined the three nuclear markers (Rag-1, Rag-2, and rhodopsin) and obtained a final data set containing 28 species and 2,616 nucleotide positions. Because our aim was to understand frog phylogeny based on nuclear genes, we did not further combine these data with the mitochondrial sequences. After exclusion of the

third positions of Rag-1 and Rag-2, the data set consisted of 1,849 nucleotides, of which 569 were parsimony-informative. The incongruence-length difference test (Farris et al. 1995) did not reject data combinability, although incongruence was close to significance ($P = 0.07$). Modeltest suggested a GTR+I+G substitution model as best fit for the combined data.

The topology obtained from the combined data set is in global agreement with the trees from the analyses of single genes (fig. 2; table 5). The archaeobatrachians are again resolved as paraphyletic and so are the mesobatrachians, but with weak support. The monophyly of the neobatrachians is well supported with a potential basal position of *Heleophryne regis*. *Nesomantis thomasseti* is grouped with the Hyloidea, but this node is still lacking support. *Pseudis paradoxa* and *Hyla cinerea* are clustering as a clear monophyletic group, as are *Litoria* and *Agalychnis*.

Branch Lengths, Substitution Rates, and Long-Branch Attraction

All sequences passed the 5% chi-square tests comparing the nucleotide composition of each sequence to the frequency distribution assumed in the maximum likelihood model, except for two outgroup sequences (*Homo sapiens* and *Gallus gallus*) in the rhodopsin data set. Archaeobatrachians had relatively higher numbers of unique substitutions (fig. 3).

Branches were distinctly longer in neobatrachians as compared to archaeobatrachians in the combined ML tree (fig. 2) as well as in separate Rag-2 (fig. 1), rhodopsin, and

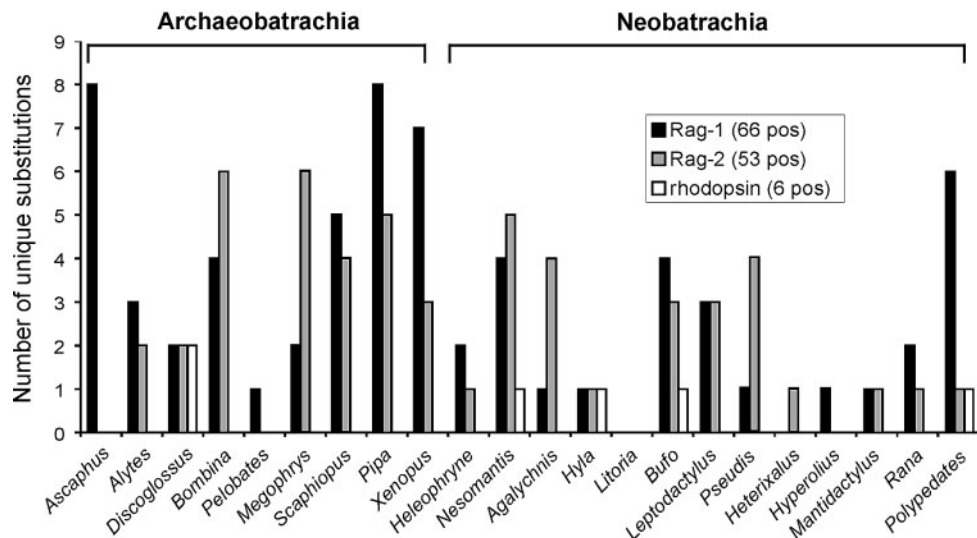


FIG. 3.—Distribution of unique substitutions (instances in which a nucleotide was different and invariable in all other sequences, including the outgroups) among archaeobatrachian and neobatrachian samples.

rDNA trees (not shown). This effect was less obvious in the separate Rag-1 tree (fig. 1). However, averaging branch lengths (to the basal node of the Anura) for archaeobatrachians and neobatrachians resulted in the latter having distinctly longer branches (fig. 4). This result was reversed, however, when the taxon sampling was reduced to only one archaeobatrachian and one neobatrachian (mean of all possible pairwise combinations; fig. 4). The branches of the outgroup species were also conspicuously shorter, especially for the combined data set (fig. 2).

A similar effect was observed when comparing the substitution rates estimated using nonparametric rate-smoothing, a method that strongly considers branch lengths. Substitution rates estimated for the nodes of the ML phylogenetic tree were highly correlated among genes. Spearman rank coefficients were positive for all five pairwise correlations, and these were significant ($P < 0.05$, after Bonferroni correction) for four of them (not significant for the correlation between Rag-1 and rDNA). Mean substitution rates of archaeobatrachians were lower than those of neobatrachians in all four gene partitions, and this difference was significant ($P < 0.005$; t -tests with Bonferroni correction) for Rag-2, rhodopsin, and rDNA. A multifactorial ANOVA with data partition (genes) and major location of nodes in phylogeny (Neobatrachia vs. Archaeobatrachia) provided highly significant evidence ($P < 0.001$) for an influence of the latter category on substitution rates.

However, the results from relative rate tests were not congruent with these phylogeny-based estimates (fig. 5). Significantly higher rates ($P < 0.05$) of neobatrachians were found for Rag-2 and 12S/16S rDNA. In contrast, in Rag-1 and rhodopsin the mean branch lengths, equivalent to the number of substitutions from the common ancestor, were longer in archaeobatrachians, although the differences were statistically not significant.

Relative rate tests of other genes for which at least one archaeobatrachian and one neobatrachian were available from GenBank (see Appendix) also failed to yield any

distinct trend. Comparison of the complete mitochondrial sequences of *Xenopus laevis* (archaeobatrachian) and *Rana nigromaculata* (neobatrachian) using *Mertensiella luschani* (salamander; Zardoya et al. 2003) as outgroup revealed significantly faster neobatrachian substitution rates in coding sequences, and rRNAs as well as tRNAs. Among the nuclear genes, faster neobatrachian rates were found in c-mos, CNBP, rhodopsin (complete cds), trans-thyretin, and calreticulin. Faster archaeobatrachian rates were found in 18S rDNA, arginase, CFTR, glucagen receptor, POMC, and ADP-ATP-translocator. Relative rate tests indicated that the rate differences were significant in the calreticulin and CFTR genes.

Discussion

Major Clades of Frogs

The present study used, for the first time, a large data set of single-copy protein coding nuclear genes to assess the phylogeny among major frog lineages. Our taxon sampling included most relevant clades that had been identified by previous studies (Hay et al. 1995; Feller and Hedges 1998), except for the basal Leiopelmatidae. Additional archaeobatrachian families to be included in future nuclear phylogenies are the Pelodytidae, which probably belong into the Pelobatoidea, and the Rhinophrynidae, which are probably related to the Pipidae. Among neobatrachians, we missed the Australian Myobatrachidae, which sometimes are thought to be related to heleophrynids (Duellman and Trueb 1986). The remaining neobatrachian families almost certainly belong to either the Hyloidea or Ranoidea clades (Duellman and Trueb 1986; Dubois 1992; Ford and Cannatella 1993; Hay et al. 1995; Vences et al. 2000b).

We could confirm neither the assumed monophyly of Archaeobatrachia (Hay et al. 1995) nor the existence of the clade Mesobatrachia (Laurent 1979; Ford and Cannatella 1993). These unsolved basal relationships may indicate that archaeobatrachians are remnants of an ancient fast radiation

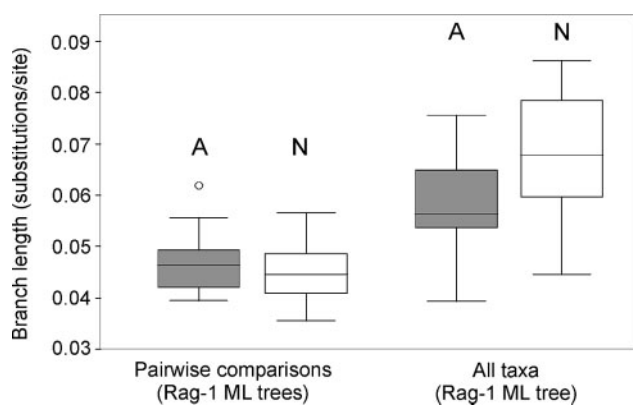


FIG. 4.—Comparison of branch lengths of archaeobatrachians (A) vs. neobatrachians (N) from the basal node of the Anura in ML trees based on Rag-1 sequences and containing different sets of taxa. The left boxplots show the branch lengths from trees that contained all outgroup specimens, one archaeobatrachian and one neobatrachian (means of all possible pairwise comparisons). The right plots show the branch lengths from the ML tree containing all taxa and shown in figure 1. The dotted circle represents an outlier value as automatically identified by the boxplot calculation with the SPSS program.

(Duellman and Trueb 1986) rather than a clade induced by the breakup of Pangaea (Feller and Hedges 1998).

The placement of *Ascaphus truei* as the most basal lineage of all anurans, as found in this study, had previously been proposed based on morphological data (Ford and Cannatella 1993), in contrast to assignment to the superfamily Discoglossoidae (*Ascaphus* and Discoglossidae) (Duellman 1975; Laurent 1979). So far, molecular phylogenies have not found this position for *Ascaphus* but propose a monophyletic archaeobatrachian clade also including this species. The Pipoidea (Pipidae and Rhacophoridae) as a sistergroup to all other anurans as proposed by larval morphological characteristics (Pugener, Maglia, and Trueb 2003), is not confirmed or rejected by our data. The strongly supported placement of *Bombina* with the Discoglossidae in our trees rejects the existence of the Bombinatoridae and Discoglossanura as sistergroups, as proposed by a study based on morphological characters (Ford and Cannatella 1993). We could confirm the relationships within the Pelobatoidea, as recently suggested in a study based on mitochondrial DNA (Megophryidae clustering with Pelobatidae rather than with Scaphiopodiidae; García-París, Buchholz, and Parra-Olea 2003).

In contrast to the archaeobatrachians, the Neobatrachia clearly form a monophyletic group that is highly supported by all methods applied. Within this clade, the basal position of the South African family Heleophryinidae as a sistergroup of all other neobatrachians is not highly supported by bootstrap analyses, but repeatedly found in different analyses. Previous studies either could not resolve the position of this family (Ford and Cannatella 1993) or grouped the Heleophryinidae within the Hyloidea (Hay et al. 1995; Ruvinsky and Maxson 1996). However, a placement of *Heleophryne* among archaeobatrachians as suggested by larval characters (Haas 2003) is highly unlikely. A further neobatrachian with an isolated position is *Nesomantis thomasseti*. This species is a representative of the Sooglossidae, a family only found on the Seychelles

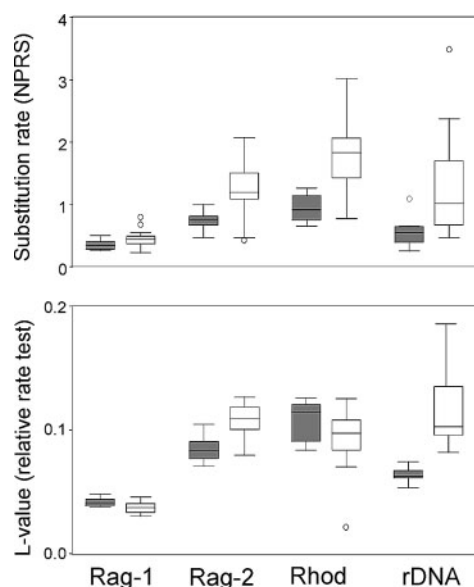


FIG. 5.—Boxplots of substitution rates $\times 10^{-3}$ (upper graph) of terminal taxa as calculated by nonparametric rate-smoothing (Sanderson 1997) of the corresponding ML phylogram, and branch lengths (L-values; lower graph) of taxa averaged over all possible pairwise comparisons between archaeobatrachians and neobatrachians in relative rate tests using Phyltest (Kumar 1996). Values for archaeobatrachians ($N=7$) are marked by grey boxes, and values for neobatrachians ($N=15$) are marked by white boxes. NPRS indicated consistently faster rates in the crown-group (Neobatrachia), whereas in the pairwise comparisons this was apparent only for Rag-2 and the mitochondrial 12S and 16S rDNA sequences. Dotted circles represent outlier values as automatically identified by the boxplot calculation with the SPSS program.

archipelago. Its basal position within the Hyloidea as indicated by our trees' position was previously proposed by some morphological studies (Laurent 1979; Ruvinsky and Maxson 1996), whereas other authors grouped it with the Ranoidea (Duellman and Trueb 1986). There is little doubt that heleophryinids and sooglossids represent independent ancient lineages within the Neobatrachia; the unresolved placement might be a result of a fast radiation event at the basis that also involved the recently discovered Nasikabatrachidae (Biju and Bossuyt 2003). All remaining neobatrachian representatives studied here were unambiguously placed in either one of the two major superfamilies (Hyloidea and Ranoidea). The close relationships of hyloids are especially surprising because they share no unequivocally identified morphological synapomorphies (Duellman and Trueb 1986; Ford and Cannatella 1993).

Interestingly, within the hyloids, all our molecular markers grouped *Hyla cinerea* (Hylidae) together with *Pseudis paradoxa* (Pseudidae). This grouping agrees with the phylogeny proposed by Duellman (2001), in which Hylinae and Pseudidae were the sistergroup of a clade containing Phyllomedusinae and Hemiphractinae. However, in our analysis, *Agalychnis* (Phyllomedusinae, South America) and *Litoria* (Pelodyadinae, Australia) formed a separate, highly supported clade, whereas Duellman (2001) placed pelodyadids as the most basal hyloid subfamily. Our results support that the intercontinental divergence (Australia-South America) is not only deeply

nested within the superfamily Hyloidea (Ruvinsky and Maxson 1996) but also within the family Hylidae, and the results thus provide evidence for yet another instance of convergent evolution of the typical treefrog morphology (Bossuyt and Milinkovitch 2000). Other relationships among hyloid families remain unsolved, and the same is true for most relationships within the second large neobatrachian clade, the Ranoidea. Some ranoid relationships supported by our trees agree with those suggested by mitochondrial data, such as the monophyly of hyperoliines (Richards and Moore 1996; Emerson et al. 2000; Vences et al. 2003). The placement of mantellids and rhacophorids as sister clades (Bossuyt and Milinkovitch 2000; Emerson et al. 2000; Richards et al. 2000) is found in our combined tree (fig. 2) but only with very weak support.

So far, our data set of nuclear genes seems to be too small to resolve short internal branches with strong support. As proposed by studies on mammals, a higher number of genes can give further insights (Murphy et al. 2001). Additional studies might need to increase the amount of sequence data to enhance the level of resolution and the confidence in particular nodes.

Acceleration of Evolutionary Rates in the Neobatrachia?

It is well known that tree-reconstruction algorithms are affected by unequal substitution rates of sequences. Long-branch attraction (Felsenstein 1978) leads to a false robust grouping of the longest branches, irrespective of the underlying phylogeny. Because the outgroup is a long branch per se, this can lead to attraction towards the outgroup and basal placement of any ingroup species with fast rates (Philippe and Laurent 1998; Brinkmann and Philippe 1999; Philippe and Germot 2000). On the other hand, Fuellen, Wägele, and Giegerich (2001) used the term “erosion” to refer to the fact that short branches may also attract each other because of the “leftover” similarity in shared old character states that “eroded” away in fast evolving lineages.

However, we are convinced that these phenomena played only a minor role in our data set. Of the so-called long-branch attraction indicators (Stiller and Hall 1999), the chi-square test did not detect a bias in the nucleotide composition in any of the amphibian sequences studied. Unique substitutions were more frequent in the basal (archaeobatrachian) branches. But the disparity of unique substitutions per taxon was much lower than in other data sets in which long-branch attraction has been demonstrated to play a major role (Stiller and Hall 1999), and the observed difference can easily be explained by phylogenetic structure and by the more ancient archaeobatrachian splits as confirmed by the fossil record (Sanchiz 1998). Furthermore the generally good agreement of analyses based on different genes, and their concordance with morphological phylogenies (Duellman and Trueb 1986; Ford and Cannatella 1993; Haas 2003) and the fossil record (Sanchiz 1998) makes it unlikely that the topology is strongly affected by long-branch attraction. The weakly supported basal position of *Heleophryne* within the Neobatrachia as found in our study could be reinforced by their short branches that indicate slower substitution

rates, more similar to those of archaeobatrachians (figs. 1 and 2).

Neobatrachians have much higher species diversity (and thus probably a faster recent speciation rate including more bottleneck events) than extant archaeobatrachians. Their highest diversity is found in the tropics, whereas archaeobatrachians mainly populate temperate areas (Feller and Hedges 1998). Because amphibians are poikilothermic, this unequal geographic distribution might have led to shorter generation times and higher metabolic rates in the evolution of neobatrachians. These biological patterns might have played a role in accelerating the mitochondrial substitution rates of neobatrachians (Li, Tanimura, and Sharp 1987; Martin and Palumbi 1993), which are significantly faster than those of archaeobatrachians, according to our results. Any such reasoning, however, needs to explain why no consistent trend is found in nuclear genes; according to the data analyzed herein, neobatrachians do not have consistently faster substitution rates in nuclear genes, despite their longer branches in the corresponding trees. As a possible explanation, rate accelerations and decelerations over limited time intervals may remain undetectable in nuclear DNA, but they have an important impact on mitochondrial DNA that evolves much faster (Brown, George, and Wilson 1979; Vawter and Brown 1986).

Based on branch lengths of phylograms, our four data sets were unambiguous in indicating an acceleration of substitution rates in neobatrachians. Although relative rate tests strongly confirmed this tendency for mitochondrial genes, no clear picture was apparent from the three nuclear genes studied in detail. Also, a multi-gene comparison using sequences available from GenBank did not yield any consistent trend. Because we excluded third codon positions of Rag-1 and Rag-2 and hypervariable regions of rDNA, and because trees based on amino acid sequences showed the same branch length differences (data not shown), we can exclude saturation as a factor to explain these observations. For our nuclear encoded data set, the disparity of branch lengths between Archaeobatrachia and Neobatrachia does not seem to reflect significant differences in evolutionary rates. We suspect that tree reconstruction algorithms tend to regularly assign shorter branches to basal taxa when these are placed paraphyletically towards a taxon-rich and well-supported crown group. This is also especially evident from the very short branches of the outgroups in our trees, even though these partly represent very species-rich, homoiothermous groups (mammals and birds) that should be expected to have fast substitution rates (Martin and Palumbi 1993). The potential impact of this phenomenon on tree-based molecular clock methods appears to be an interesting problem to be addressed in future studies.

Appendix: Accession Numbers of Sequences Used for Relative Rate Tests

Complete mitochondrial sequences: Outgroup: *Mertensiella luschani*, AF154053; Archaeobatrachia: *Xenopus laevis*, M10217; Neobatrachia: *Rana nigromaculata*, AB043889.

18S rDNA: Outgroup: *Ambystoma macrodactylum*, AF212178; Archaeobatrachia: *Xenopus borealis*, X59733, *X. laevis*, X59734; Neobatrachia: *Rana nigromaculata*, AB099628.

POMC: Outgroup: *Homo sapiens*, NM000939; *Mus musculus* NM008805; Archaeobatrachia: *Xenopus laevis* X59369, X59370, *Spea multiplicata* AF115251, Neobatrachia: *Rana catesbeiana*, X15510, *R. ridibunda*, M62770, *Bufo marinus*, AF194966

Arginase: Outgroup: *Oncorhynchus mykiss*, AY056477, *Homo sapiens*, BC020753, *Mus musculus*, NM 007482, *Rattus norvegicus*, NM 017134; Archaeobatrachia: *Xenopus laevis*, BC043635; Neobatrachia: *Rana catesbeiana*, U26351

Glucagon receptor: Outgroup: *Homo sapiens*, NM 000160, *Mus musculus*, BC031885, *Rattus norvegicus*, NM 172092, Archaeobatrachia: *Xenopus laevis* AF318178, Neobatrachia: *Rana pipiens*, AF318179, *R. tigrina rugulosa*, AF179589

Calreticulin: Outgroup: *Danio rerio*, NM 131047, *Homo sapiens*, NM 004343, *Mus musculus*, NM 007591, *Rattus norvegicus*, NM 022399, *Oryctolagus cuniculus*, J05138, Archaeobatrachia: *Xenopus laevis*, BC044068, Neobatrachia: *Rana rugosa*, D78589

ADP-ATP translocator: Outgroup: *Danio rerio*, AF506216, *Mus musculus*, BC004570, *Rattus norvegicus*, D12771, Archaeobatrachia: *Xenopus laevis*, BC043821, AF231347, Neobatrachia: *Rana rugulosa*, AB093536–9, AB008456–63

Transthyretin: Outgroup: *Homo sapiens*, BC005310, *Rattus norvegicus*, NM 012681, Archaeobatrachia: *Xenopus laevis*, AB026996, Neobatrachia: *Rana catesbeiana*, AB006134

Rhodopsin: Outgroup: *Latimeria chalumnae*, AH007712, *Gallus gallus*, D00702, *Homo sapiens*, NM 000539, *Ambystoma tigrinum*, U36574, Archaeobatrachia: *Xenopus laevis*, S62229, Neobatrachia: *Rana temporaria*, U59920, *R. catesbeiana*, S79840, *R. pipiens*, S49004

C-mos: Outgroup: *Danio rerio*, AB032727, *Gallus gallus*, M19412, *Rattus norvegicus*, X52952, Archaeobatrachia: *Xenopus laevis*, X13311, Neobatrachia: *Rana japonica*, AB026181

CFTR: Outgroup: *Fundulus heteroclitus*, AF000271, *Homo sapiens*, NM 000492, *Mus musculus*, NM 021050, Archaeobatrachia: *Xenopus laevis*, U60209, X65256, Neobatrachia: *Bufo bufo*, AY026761

CNBP: Outgroup: *Danio rerio*, AY228240, *Homo sapiens*, BC014911, *Rattus norvegicus*, NM 022598, *Mus musculus*, AK075760, *Gallus gallus*, AF035676, Archaeobatrachia: *Xenopus laevis*, Y07751, Neobatrachia: *Bufo arenarum*, AF144698

Acknowledgments

We are indebted to R. Zardoya and Diego San Mauro for the Rag-1 and rhodopsin sequences of *Ascaphus truei* and *Pelobates cultripes* and to Marius Burger, Lutz Fromhage, Frank Glaw, Joachim Kosuch, Gaetano Odierna, Annemarie Ohler, Michael Veith, and Thomas

Ziegler, who supplied crucial samples. Arie van der Meijden helped with statistical analysis and Ilse Eistetter provided laboratory assistance. This research was supported by grants from the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie, and the University of Konstanz to A.M.

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Manolo Gouy, Associate Editor

Accepted December 18, 2003