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Phylogenetic relationships of Pelobatoidea re-examined using mtDNA

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Abstract

Pelobatoidea is a clade of ancient anurans with obscure relationships to the remaining clades of frogs. We used partial sequences of two mitochondrial genes (cytochrome *b* and 16S RNA) from all Pelobatoidea subclades, including all species of Pelobatidae and Pelodytidae and four outgroup taxa (*Xenopus*, *Ascaphus*, *Discoglossus*, and *Rana*), to propose a phylogenetic hypothesis for relationships within Pelobatoidea. Maximum likelihood and Bayesian analyses support the monophyly of Pelobatoidea, but our hypothesis of internal relationships differs substantially from all previous hypotheses. Megophryidae is sister to *Pelobates*, and this clade is sister to *Pelodytes*. The most basal clade within Pelobatoidea is formed by *Scaphiopus* and *Spea*. The family Pelobatidae, as previously defined is not monophyletic, and it is split into Eurasian spadefoot toads *Pelobates* which retain the name Pelobatidae and North American spadefoot toads *Scaphiopus* and *Spea* which comprise the revived taxon Scaphiopodidae. Our analysis uncovers the existence of morphologically cryptic taxa within previously recognized species of the genus *Spea* and reveals marked genetic differentiation within Iberian *Pelodytes*. We discuss biogeographic implications and the evolution of fossoriality in the light of the new phylogenetic hypothesis.

Keywords: Amphibia; Anura; Pelobatoidea; Phylogeny; Evolution; Biogeography; Mitochondrial DNA

1. Introduction

Pelobatoidea is a morphologically conservative group of ancient primitive frogs that have obscure relationships to the remaining clades of Anura (Brattstrom, 1957; Ford and Cannatella, 1993; Hay et al., 1995; Lynch, 1973; Noble, 1924). Elucidating the phylogenetic relationships of basal anurans has proved difficult using either morphological or molecular data sets (Ford and Cannatella, 1993; Hay et al., 1995). The most recent morphological (Ford and Cannatella, 1993; Gao and Wang, 2001) and molecular (Hay et al., 1995; Ruvinsky and Maxon, 1996) hypotheses of relationships for the Anura deeply disagree in the position of Pelobatidae. According to the morphological hypothesis of Ford and Cannatella (1993), Pipoidea is sister to Pelobatoidea forming Mesobatrachia, and together with Neobatrachia form the Pipanura. Sequentially basal to this clade are Discoglossidae, Bombinatoridae, and Ascaphidae. The morphological hypothesis of Gao and Wang (2001) also suggests a basal position for *Ascaphus* and *Leiopelma* but considers Pelobatoidea sister to Discoglossidae (including Bombinatoridae). Pipoidea is sister to the Pelobatoidea and Discoglossoidea clade, but Neobatrachia is not represented. According to the molecular hypotheses, the Pelobatoidea are sister to the clade formed by Ascaphidae, Discoglossoidea, and Pipoidea rendering a monophyletic Archaeobatrachia (Hay et al., 1995). Archaeobatrachia is in turn sister to Neobatrachia.

The recent Pelobatoidea comprise three groups usually treated at the family level, Pelobatidae, Pelodytidae, and Megophryidae (Frost, 1985). The family Pelobatidae has two main groups, Old World spadefoot toads (*Pelobates*) from Europe, Morocco, and western Asia,

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and New World spadefoot toads (*Scaphiopus* and *Spea*) from North America. *Pelobates* includes four species: *Pelobates cultripes*, *Pb. fuscus*, *Pb. syriacus*, and *Pb. varaldii* (Barbadillo et al., 1997; Gislen, 1936; Roček, 1980); *Scaphiopus* is represented by three species: *Scaphiopus couchii*, *Sc. holbrookii, Sc. hurterii*; and *Spea* by four species: *Spea bombifrons*, *Sp. hammondii*, *Sp. intermontana*, *Sp. multiplicata* (Conant and Collins, 1991; Duellman, 1955; Frost, 1985; Tanner, 1989). Pelodytidae (parsley frogs), represented by the genus *Pelodytes*, is found in Europe and western Asia and includes three species: *Pd. caucasicus*, *Pd. ibericus*, and *Pd. punctatus* (Golubev, 1980; Kuzmin, 1997; Mazin et al., 1980; Sánchez-Herráiz et al., 2000). Megophryidae, the most diversified group within Pelobatoidea (about eight genera and 80 species), lives in tropical montane southeast Asia (Duellman and Trueb, 1994; Lathrop, 1997).

Pelobatoidea has not been consistently recognized as a natural group (Lynch, 1973; Roček, 1980), and no fewer than 12 hypotheses of evolutionary relationships have been proposed for subsets of Pelobatoidea (Barbadillo et al., 1997; Cannatella, 1985; Estes, 1970; Ford and Cannatella, 1993; Gao and Wang, 2001; Henrici, 1994; Kluge, 1966; Roček, 1980; Sage et al., 1982; Wiens and Titus, 1991; Lathrop, 1997; Maglia, 1998). Within Pelobatoidea, the monophyly of each of *Pelobates*, *Scaphiopus*, *Spea*, *Pelodytes*, and Megophryidae has not been questioned (Ford and Cannatella, 1993). The relationships within and among these groups remain controversial and biogeographic hypotheses are inconclusive despite the existence of a well known and extensive fossil record (Roček and Rage, 2000; Sanchiz, 1998a).

The present study is the first to examine molecular evidence to elucidate the phylogenetic relationships among the Pelobatoidea. This work is the most inclusive study of the pelobatoids, using all recognized species of Pelobatidae and Pelodytidae. Nearly 1000 base pairs of 16S rRNA (16S) and cytochrome *b* (cyt *b*) sequence data from mitochondrial DNA were analyzed. We focused attention on the relationships within and among the four genera of Pelobatidae and Pelodytidae. Our hypotheses are used to discuss biogeography and evolution of fossoriality in Pelobatoidea.

2. Materials and methods

2.1. Sampling design

We obtained sequences of 16S (520 bp) and cyt *b* (385 bp) for 1–3 specimens of all species of Pelobatidae and Pelodytidae (except *Scaphiopus holbrookii* for which only 16S data were gathered). We also obtained molecular data for 1–2 specimens of *Leptolalax pelodytoides*, *Brachytarsophrys feae*, and *Megophrys lateralis* (Table 1).

Because the phylogenetic position of Pelobatoidea within Anura is controversial, we selected outgroups representing all major clades of frogs: Pipoidea (represented by the already published sequence of *Xenopus laevis* GenBank NC001573, Roe et al., 1985), Neobatrachia (represented by *Rana iberica*), Discoglossoidea (represented by *Discoglossus galganoi*), and Ascaphidae (represented by *Ascaphus montanus* and *A. truei*). All trees were rooted with the two species of *Ascaphus* (Ritland et al., 2000) because morphological evidence suggest that Ascaphidae is basal to all anurans (Ford and Cannatella, 1993). Alternatively we also use *Rana*, because previous molecular evidence suggest that Neobatrachia are the most distantly related taxa included in this study (Hay et al., 1995) (see Section 4). Both rooting strategies allowed the positions of the other outgroup species, particularly *Xenopus*, to remain free with respect to the ingroup, since the interrelationships among Pelobatoidea and Pipoidea are subject of debate (Ford and Cannatella, 1993; Gao and Wang, 2001; Hay et al., 1995; Roček, 1980).

2.2. Amplification and sequencing

Tissues for this study were obtained from various sources, including recent field collections and donations of several researchers and institutions (see Acknowledgments). A large proportion of the samples were obtained from the frozen tissue collection of the Museum of Vertebrate Zoology, University of California, Berkeley.

Whole genomic DNA was extracted from small amounts of frozen or ethanol-preserved tissues using NaCl following a protocol modified from Miller et al. (1988). We sequenced 580 base pairs of the large 16S subunit ribosomal mtDNA gene corresponding roughly to positions 2510–3059 in the human mitochondrial genome (Anderson et al., 1981); and 353–385 base pairs of the cytochrome *b* gene, starting from codon 7 of the *Xenopus* cyt *b* gene (Roe et al., 1985) for Pelobatidae and Pelodytidae. These genes were selected in order to recover maximum phylogenetic information for the terminal nodes and the base of the tree. Amplification was done via the polymerase chain reaction (PCR) (Saiki et al., 1988), using the primers "MVZ15" (Moritz et al., 1992) and "cyt *b*2" (Kocher et al., 1989) for cyt *b*, and the primers "16Sar" and "16Sbr" (Palumbi et al., 1991) for 16S. PCRs consisted of 38 cycles with a denaturing temperature of 92 °C (1 min), annealing at 48–50 °C (1 min), and extension at 72 °C (1 min) in a Techne PHC-1 thermocycler. PCRs were run in a total volume of 25 μl, using 0.5 pmol of each primer.

Double strand templates were cleaned using QIAquick PCR purification kit (QIAgen). We used 1.0–5.5 μl of PCR product for cycle sequencing in 10 μl

Table 1
Samples used in this study, and GenBank accession numbers

Sample	Species name	Locality	Voucher	GenBank Accession Nos.	
				16S	Cyt *b*
1	*Leptolalax pelodytoides*	VIET-NAM: Vinh Phu Prov.: Tam Dao	MVZ 223641	AY236797	AY236764
2	*Leptolalax pelodytoides*	VIET-NAM: Vinh Phu Prov.: Tam Dao	MVZ 223642	AY236798	AY236765
3	*Brachytarsophrys feae*	VIET-NAM: Vinh Phu Prov.: Tam Dao	MVZ 223683	AY236799	—
4	*Megophrys lateralis*	VIET-NAM: Vinh Phu Prov.: Tam Dao	MVZ 223691	AY236800	AY236766
5	*Pelobates cultripes*	SPAIN: Avila: Fresnedilla	MGP photo voucher	AY236801	AY236767
6	*Pelobates cultripes*	SPAIN: Cádiz: Tarifa	(No voucher)	AY236802	AY236768
7	*Pelobates cultripes*	SPAIN: Huelva: La Matilla	(No voucher)	AY236803	AY236769
8	*Pelobates cultripes*	SPAIN: Badajoz: Garbayuela	MGP photo voucher	AY236804	AY236770
9	*Pelobates fuscus*	CZECH REPUBLIC: Southern Moravia: Znojmo	MVZ 233602	AY236805	AY236771
10	*Pelobates fuscus*	CZECH REPUBLIC: Southern Moravia: Znojmo	MVZ 233601	AY236806	AY236772
11	*Pelobates syriacus*	TURKEY: Bursa: Osman Gazi.	MVZ 234658	AY236807	AY236773
12	*Pelobates varaldii*	MOROCCO: Alcazarquivir	MNCN (uncatalogued)	AY236808	AY236774
13	*Pelobates varaldii*	MOROCCO: Alcazarquivir	MNCN (uncatalogued)	AY236809	AY236775
14	*Pelobates varaldii*	MOROCCO: Rabat Prov.: 10.5 km E of Rabat	MVZ 175957	AY236810	AY236776
15	*Pelodytes caucasicus*	GEORGIA: Borzhomi	MVZ 218724	AY236811	AY236777
16	*Pelodytes ibericus*	SPAIN: Huelva: 10 km N Niebla	MGP photo voucher	AY236812	AY236778
17	*Pelodytes ibericus*	SPAIN: Badajoz: Fuentes de León	MGP photo voucher	AY236813	AY236779
18	*Pelodytes punctatus*	SPAIN:Barcelona: El Garraf	MNCN 20176	AY236814	AY236780
19	*Pelodytes punctatus*	SPAIN: Burgos: Masa	(No voucher)	AY236815	AY236781
20	*Pelodytes punctatus*	SPAIN: Teruel: Corbalán	MGP photo voucher	AY236816	AY236782
21	*Pelodytes punctatus*	SPAIN: Toledo: Navalrincón	MGP photo voucher	AY236817	AY236783
22	*Spea bombifrons*	USA: Arizona: Cochise Co.: US Hwy 80 near NM	MVZ 138976	AY236818	AY236784
23	*Spea intermontana*	USA: California: Inyo Co.: Deep Springs College	MVZ 234190	AY236819	AY236785
24	*Spea hammondii*	USA: California: San Diego Co.: Hwy 76 near Pala Jcn.	MVZ 145193	AY236820	AY236786
25	*Spea hammondii*	USA: California: San Diego Co.: Hwy 76 near Pala Jcn.	MVZ 145197	AY236821	AY236787
26	*Spea hammondii*	USA: California: Alameda Co.: Corral Hollow Rd.	MVZ 149995	AY236822	AY236788
27	*Spea multiplicata*	USA: Arizona: Cochise Co.: Portal Rd.	MVZ 150038	AY236823	AY236789
28	*Spea multiplicata*	MEXICO: Michoacán: near Uruapan	MVZ 164769	AY236824	AY236790
29	*Scaphiopus couchii*	MEXICO: Baja California Sur: San Bartolo	MVZ 161886	AY236825	AY236791
30	*Scaphiopus couchii*	USA: Arizona: Cochise Co.: near Rodeo	MVZ 145179	AY236826	AY236792
31	*Scaphiopus holbrookii*	USA: Florida: Hillsborough Co.: Tampa	MVZ 16193	AY236827	—
32	*Scaphiopus hurterii*	USA: Oklahoma:Payne Co.: nr jcn of Hwy 1 and 18	MVZ 145203	AY236828	AY236793
33	*Ascaphus truei*	USA: Oregon: Benton Co.: near Philomath	MVZ 187732	AY236829	AY236794
34	*Ascaphus montanus*	USA: Idaho: Valley Co.: 1 3.5 km N of Knox	MVZ 187733	AY236830	AY236795
35	*Discoglossus galganoi*	SPAIN: Zamora: Aliste	MGP photo voucher	AY236831	AF128897
36	*Rana iberica*	SPAIN: La Coruña: Caaveiro	MGP photo voucher	AY236832	AY236796

MGP, M. García-París photo voucher collection; MVZ, Museum of Vertebrate Zoology, Berkeley, California; MNCN, Museo Nacional de Ciencias Naturales, CSIC, Madrid, Spain.

reaction volumes using the Perkin–Elmer Ready Reaction Kit to incorporate dye-labeled dideoxy terminators. Thermal cycling was performed using standard conditions. Cycle sequencing products were ethanol precipitated and separated on a 6% polyacrylamide gel using an ABI 377 DNA sequencer (Applied Biosystems).

2.3. Sequence alignment and analyses

All sequences were compiled using Sequence Navigator version 1.0.1 (Applied Biosystems). 16S sequences were aligned using Clustal X (Aladdin Systems, Heidelberg, Germany) with default gap costs and then refined manually by comparing them to published

secondary structure models for 16S (Ortí and Meyer, 1997).

Observed proportional sequence divergence (*p*-distance) and corrected sequence divergence (Kimura 2-parameter; Kimura, 1980) in pairwise comparisons and the number of transitions and transversions were obtained using the computer program PAUP*4.0b10 (Swofford, 2002). We plotted *p*-distance (y) versus corrected (K2p) estimates of proportional sequence divergence (x) for first, second, and third codon positions, and for transitions and transversions separately, to test for the possibility that some types of nucleotide substitutions have become saturated.

2.4. Phylogenetic analysis

The analyses were performed using the combined data set, which included 17 species (32 samples) of Pelobatoidea and five outgroups for two genes: 16S and cyt *b* (Table 1). Additionally, 16S sequences of *Scaphiopus holbrookii* and *Brachytarsophrys feae* were also included in the combined analysis following recommendations by Wiens and Reeder (1995). A set of 33 contiguous bases of the 16S with difficult alignment across taxa was excluded. Gaps were treated as missing data. Additional analyses on the 16S and cyt *b* data sets were performed independently.

We used Model Test 3.06 (Posada and Crandall, 1998) to find the best model of evolution that fit the data for subsequent Maximum Likelihood analyses (ML: Felsenstein, 1981, 1993). The GTR model of evolution with gamma parameter and proportion of invariable positions was used for ML analyses (Gu et al., 1995; Swofford et al., 1996; Yang, 1994). ML analyses with empirical base frequencies were performed using PAUP*. We used nonparametric bootstrapping (100 pseudoreplicates) (bs) to assess the stability of internal branches (Felsenstein, 1985; Felsenstein and Kishino, 1993) (Table 2). Shimodaira–Hasegawa parametric tests (Shimodaira and Hasegawa, 1999) using bootstrap with full optimization (1000 bs replicates), were used to test for the monophyly of selected taxa (Leaché and Reeder, 2002) as implemented in PAUP*.

Bayesian phylogenetic analyses were conducted with MrBayes 2.0 (Huelsenbeck and Ronquist, 2001). The GTR model of evolution with gamma parameter and

Table 2
Support values for Bayesian and MP nodes shared by the combined data ML phylogeny

Node	Bayesian	ML	MP	MP (no 3rd)	Decay	Clade name
1	100	100	100	100	50	
2	—	—	—	59	—	
3	**100**	**99**	**87**	**78**	**12**	Mesobatrachia
4	**99**	**85**	—	**53**	**2**	Pelobatoidea
5	**100**	**94**	**88**	**79**	**7**	Scaphiopodidae
6	**100**	**98**	**96**	**77**	**10**	*Scaphiopus*
7	100	100	100	98	9	
8	100	97	100	99	16	
9	**100**	**100**	**100**	**100**	**10**	*Spea*
10	78	80	79	—	3	
11	100	97	98	77	11	
12	100	93	100	84	11	
13	100	99	100	97	6	
14	77	52	—	—	2	
15	**100**	**100**	**100**	**100**	**32**	Pelodytidae
16	100	99	100	100	27	
17	97	85	97	50	6	
18	100	100	99	68	5	
19	100	96	95	—	3	
20	73	77	93	83	2	
21	90	70	—	—	4	
22	**100**	**100**	**100**	**100**	**20**	Pelobatidae
23	100	98	91	56	7	
24	100	100	100	100	25	
25	100	99	100	91	18	
26	100	99	100	99	15	
27	98	65	61	65	1	
28	100	100	100	68	12	
29	71	70	70	—	1	
30	**100**	**96**	**78**	**95**	**7**	Megophryidae
31	100	100	99	99	12	
32	100	100	100	100	56	

Node numbers correspond to those in Fig. 1. Dashes represent nodes with non-parametric bootstrap support lower than 50%. Nodes corresponding to relevant taxonomic groups are indicated in bold.

proportion of invariable positions was used also for this analysis. Analyses were initiated with random starting trees and run for 1,000,000 generations. The Markov chains were sampled each 100 generations. Of the resulting 10,000 trees, 2500 were discarded as "burn-in." Support values are presented in Table 2.

Maximum parsimony (MP: Swofford, 1998) phylogenies were estimated using the heuristic search algorithm for each tree-building methodology. We used 20 repeated randomized input order of taxa for all MP analyses to minimize the effect of entry sequence on the topology of the resulting cladograms. MP analyses were conducted without the steepest descent option, and with accelerated character transformation (ACCTRAN) optimization, tree bisection-reconnection (TBR) branch swapping, and zero-length branches collapsed to yield polytomies. We used nonparametric bootstrapping (1000 pseudoreplicates) and decay indices (*d*) (Table 2) to assess the stability of internal branches in the resulting topologies (Bremer, 1994; Felsenstein, 1985; Felsenstein and Kishino, 1993). Nonparametric bootstrap values and decay indices generally are a conservative measure of the probability that a recovered group represents a true clade (Hillis and Bull, 1993; Li, 1997; Zharkikh and Li, 1992). For the cyt *b* data, we used two different schemes of analyses, equal weighting for all codon positions and exclusion of third positions, in order to eliminate the misleading phylogenetic effect of third position saturation (Moritz et al., 1992). Each base position was treated as an unordered character with four alternative states.

3. Results

3.1. Characteristics of individual genes

Cyt b. Thirty four sequences of 307–385 bp (all but five had 385 bp) of the cyt *b* gene were obtained, 208 characters were variable, and 190 of these characters were phylogenetically informative. Sequence divergence (*p*) within the ingroup was as high as 30.8% (*Leptolalax pelodytoides* compared to *Pelodytes punctatus*). Substantial divergence was found even within certain taxa currently recognized as single species (e.g., *S. couchii*, as high as 6.2%, and *Spea hammondii* 9.9%). The smallest divergence between two species was from *S. bombifrons* to *S. intermontana* (2.3%). Base composition was slightly A + T biased (58%). There was an excess of thymine for first and second codon positions. For third codon position cytosine was present in high amounts (38.5%), adenine and thymine were present in similar proportions (28.1 and 28.5%), and guanine was rare (4.7%). Transitions account for 60% of all substitutions and TC transitions outnumber AG transitions by about 3:1. The empirical ratio of transitions to transversions was 2.19. The saturation plots of uncorrected sequence divergence against corrected sequence divergence divided by codon position indicated saturation at third position transitions (data available from authors). The $g1$ statistic indicated that significant phylogenetic signal was present: $g1 = -0.60$; $P < 0.01$; mean ± SD tree length = 1676.89 ± 62.79.

Analysis of molecular evolution of the cyt *b* shows that the sequences for this analysis have a typical "mitochondrial" behavior (Zhang and Hewitt, 1996). Most variable sites are in the third codon position as is typical for protein coding regions and the reading frame is conserved. The number of amino acid changes across sequences is very limited suggesting that random base changes, as would be expected for non-functional nuclear copies, are not occurring.

16S. Thirty six sequences of 580 bp of the 16S gene were analyzed, 260 characters were variable, and 193 of these characters were phylogenetically informative. Sequence divergence within the ingroup was as high as 18.5% (between *Leptolalax pelodytoides* and *Megophrys lateralis*). The highest divergence within species was 2.2% between the two populations of *Spea hammondii*. The smallest divergence between two species was from *Sp. bombifrons* to *Sp. hammondii* (0.7%). Base composition was also A + T biased (55.3%). Transitions account for 55% of all substitutions and TC transitions outnumber AG transitions by about 2:1. The mean ratio of transitions to transversions for all pairwise species comparisons was 1.6%. Scatterplots of uncorrected versus corrected sequence divergence suggest that transitions and transversions are not saturated (data available from authors). The alignment of the ingroup required accommodation of 5–8 gaps per sequence. Most indels were 1 bp in length and maximum indel length was 12 bp. The $g1$ statistic indicated that significant phylogenetic signal was present: 16S: $g1 = -0.53$; $P < 0.01$; mean ± SD tree length = 1505.28 ± 48.29.

3.2. Phylogenetic relationships

The maximum likelihood analysis of the combined data set when rooted with *Ascaphus* (see Section 4) resulted in a tree ($\ln L = -7443.491$) where all samples of Pelobatoidea form a monophyletic group (Fig. 1) and all genera included in the analysis are monophyletic. Within *Spea*, *Sp. multiplicata* is basal to an assemblage formed by *Sp. hammondii*, *Sp. intermontana*, and *Sp. bombifrons*. *Spea hammondii* is not monophyletic, the population of San Diego County, California (samples 24 and 25), is sister to *Sp. bombifrons* while the population of Alameda, California (sample 26), is sister to a clade formed by *Sp. intermontana*, *Sp. bombifrons*, and *Sp. hammondii* from San Diego County. *Scaphiopus*, represented in our analysis by three species, is monophyletic. *Pelodytes* is monophyletic, with *Pd. caucasicus* sister to

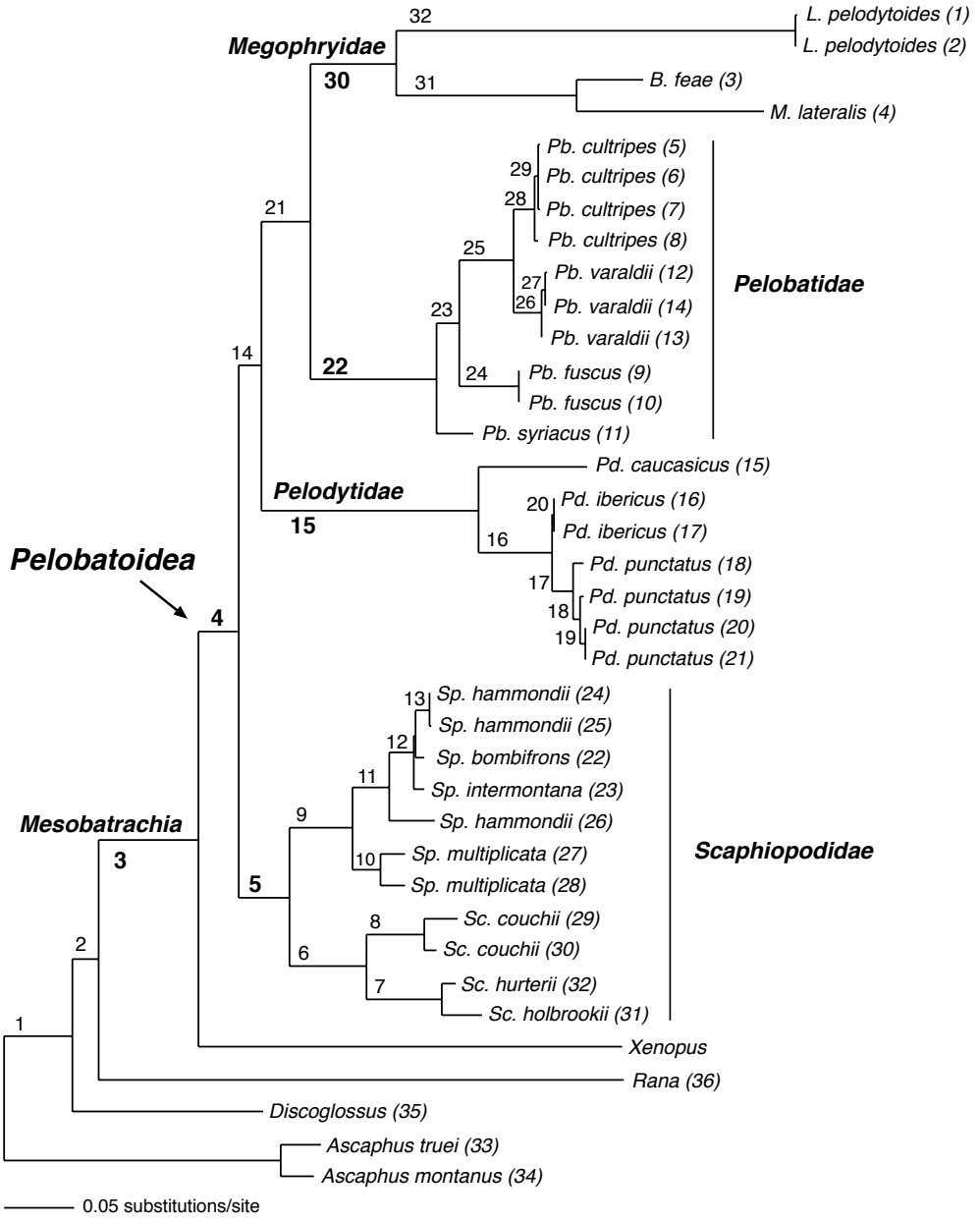

Fig. 1. Tree based on maximum likelihood analysis of the combined data set (ln $L = -7443.491$) (see text for comparisons to other analyses). Support values (MP, ML, Bayesian porterior clade probabilities, and decay indices) for the numbered nodes are provided in Table 2. Nodes representing relevant taxonomic groups are indicated in bold.

an Iberian clade formed by *Pd. ibericus* and *Pd. punctatus*. *Pelobates* is also monophyletic with the southern European *Pb. cultripes* and the north African *Pb. varaldii* as sister taxa. Support values for individual nodes are shown in Table 2.

The relationships among genera of Pelobatoidea differ from currently considered hypotheses. Pelodytidae and Megophryidae are monophyletic taxa, but the monophyly of Pelobatidae, as previously considered, is broken with the genus *Pelobates* sister to Megophryidae, which in turn is sister to Pelodytidae with the *Scaphiopus Spea* clade basal to them (Fig. 1). When we forced all the samples of Pelobatidae (*Pelobates*, *Scaphiopus*, and *Spea*) to form a monophyletic group, the tree obtained (ln $L = -7756.848$) differs significantly ($P = 0.046$) from the tree shown in Figure 1, based on Shimodaira–Hasegawa parametric test (Shimodaira and Hasegawa, 1999), so monophyly of Pelobatidae can be conclusively rejected.

When rooting with *Ascaphus*, Pelobatoidea is sister to *Xenopus* (Pipoidea) rendering a monophyletic Mesobatrachia (Table 2), and this clade is in turn sister to *Rana* (Neobatrachia), and *Discoglossus* is basal to all of them (Fig. 1). The alternative use of *Rana* as rooting (Hay et al., 1995) yielded identical relationships for Mesobatrachia, but *Ascaphus* and *Discoglossus* form a clade

basal to Mesobatrachia. All further results will be presented using *Ascaphus* for rooting.

Bayesian analysis resulted in a consensus tree (50% majority rule) with identical topology to the ML tree for the ingroup (Fig. 1). Nodes corresponding to Mesobatrachia, Pelobatoidea, Scaphiopodidae, Pelodytidae, Pelobatidae, and Megophryidae are highly supported (Bayesian support 99–100) (Table 2). The position of the outgroups differ from ML in that *Discoglossus* is sister to Mesobatrachia (Pipoidea plus Pelobatoidea), while *Rana* is basal to *Discoglossus* plus Mesobatrachia (Bayesian support 54) (not shown).

Maximum parsimony analysis using equal weighting and all positions included, yielded a single tree ($L =$ 1545 steps; 383 characters were parsimony informative; $CI = 0.476$, $RI = 0.749$) (not shown). The tree differs from the ML tree in the relative position of *Sp. hammondii* from San Diego Co. which is sister to a clade formed by *Sp. bombifrons* plus *Sp. intermontana* rather than to *Sp. bombifrons* alone as in the ML tree. The topology of the outgroups is identical to the Bayesian tree, where *Discoglossus* is sister to Mesobatrachia (Pipoidea plus Pelobatoidea) and *Rana* is basal to *Discoglossus* plus Mesobatrachia. Support values for individual nodes based on 1000 nonparametric bootstrap pseudoreplicates are shown in Table 2. Analyses performed excluding cyt *b* third positions produced 24 equally parsimonious trees ($L = 864$ steps; 258 characters were parsimony informative; $CI = 0.571$, $RI = 0.749$). The strict consensus tree (not shown) is mostly unresolved at the base of Pelobatoidea, where a sister taxa relationship between Megophryidae and Pelobatidae is present, although bootstrap support was lower than 50% for this grouping. The outgroup arrangement in this analysis is like that shown in Fig. 1. Nonparametric bootstrap support values for nodes shared with ML analyses are shown in Table 2.

We performed additional analyses of the 16S rDNA data set comprising a total of 36 sequences. The topology obtained in the ML analysis (not shown), only differs from the combined ML analysis (Fig. 1) in the structure of the *Pd. punctatus* and *Sp. multiplicata* clades whose respective monophyly is broken. Analyses based on the cyt *b* data set, consistently included the outgroup taxa *Rana* and *Discoglossus* within the ingroup (ML, MP equally weighted and MP with 3rd positions excluded), although there is no boostrap support for any major grouping except for genera.

4. Discussion

4.1. Phylogenetic relationships of Pelobatoidea

Pelobatoidea has not been consistently recognized as a natural group (Lynch, 1973; Roček, 1980) and although our analyses support its monophyly, the bootstrap support for the clade is low based on MP analyses. Relationships of Pelobatoidea to the other major clades of frogs are under discussion, and the most recent morphological (Ford and Cannatella, 1993; Gao and Wang, 2001) and molecular (Hay et al., 1995; Ruvinsky and Maxon, 1996) hypotheses of relationships for the Anura disagree. The limited sampling found in most of the analyses could pose problems involving long branch effects. Also, the lack of testing for monophyly of most primitive groups, which generally include taxa that diverged very early in anuran history, increases the possibility of missing important branches. Rooting is also a major problem for anuran phylogenies based on molecular data, since both of its living relatives, Caudata and Gymnophiona, are so distantly related that the effect of using either one for rooting is similar to the result of a mid-point rooting. The morphological study by Ford and Cannatella (1993) considered Ascaphidae as the basal-most taxon within Anura, rendering a paraphyletic Archaeobatrachia, with Neobatrachia as the sister taxon to a Pelobatoidea-Pipoidea clade. Alternatively, molecular analyses by Hay et al. (1995) placed Neobatrachia as the sister taxon of a monophyletic Archaeobatrachia. Although our sampling is appropriate for the Pelobatoidea it is not adequate nor intended to sort out the relationships among the major clades of frogs. The use of Ascaphidae or Neobatrachia as outgroups implies a subjective decision that affects all further analyses. In our study the relationships within Pelobatoidea are not affected by the use of either taxon as rooting, but relationships among outgroups are. Using *Ascaphus* as rooting, *Xenopus* (Pipoidea) is sister to Pelobatoidea, in agreement with the morphological hypothesis of Ford and Cannatella (1993). Rooting with *Rana* (Neobatrachia), Pelobatoidea are sister to an archaeobatrachian clade congruent with the molecular hypothesis of Hay et al. (1995).

Our hypothesis recognizes the monophyly of Pelodytidae and Megophryidae but not Pelobatidae (*Pelobates*, *Scaphiopus*, and *Spea*). These results are in partial agreement with Roček's (1980) hypothesis, who rejected the monophyly of Pelobatidae and erected the family Scaphiopodidae for the North American *Spea* and *Scaphiopus*, retaining Pelobatidae for the Eurasian *Pelobates*. In our analyses, the North American spadefoot toads (*Spea* and *Scaphiopus*) appear as the most basal clade of Pelobatoidea, although the support for this placement is relatively low. The Eurasian *Pelobates* is sister to Megophryidae (Fig. 1), also with little bootstrap support in the MP analyses. A Shimodaira–Hasegawa parametric test (Shimodaira and Hasegawa, 1999) comparing the ML topology shown (Fig. 1) to the ML topology obtained by constraining all the samples of Pelobatidae (*Pelobates*, *Scaphiopus*, and *Spea*) to form a monophyletic group, indicates that

the topologies are significantly different. Therefore, the monophyly of Pelobatidae is statistically rejected based on our sampling. The support for most of the basal nodes within Pelobatoidea are not high and relationships among the different clades are likely subjected to change by using different molecular data sets, however in no case we have found a topology in which the Eurasian and the North American spadefoot toads form a monophyletic group. Theses results are in agreement with Roček's (1980) proposal of family recognition for the North American taxon, Scaphiopodidae. We believe that given the antiquity and the long history of independence of the North American and Eurasian pelobatoid lineages, about 110 Ma according to immunological estimates (Sage et al., 1982), neither the current molecular data set (too few characters supporting the old basal splitting pattern) nor previous morphological studies (Cannatella, 1985; Lathrop, 1997; Maglia, 1998; Maglia et al., 2001) are sufficient to demonstrate a sister taxon relationship between the North American and the Eurasian pelobatids. Therefore our preferred taxonomic treatment for these groups is at the family level avoiding the possible conflicts and the misleading evolutionary implications resulting from retaining a non-monophyletic family Pelobatidae. An alternative to the four family taxonomic scheme is to unify Scaphiopodidae, Pelobatidae, Megophryidae and Pelodytidae into a single taxon, named Pelobatidae, but no improvement is achieved in morphological or ecological predictability.

Our phylogenetic hypothesis supports the monophyly of all genera currently recognized within Pelobatoidea. The recent discovery and study of a new species of *Pelodytes* in the Iberian Peninsula (Salvador and García-París, 2001; Sánchez-Herráiz et al., 2000) suggested the existence of previously hidden genetic diversity within this seemingly conservative genus. No hypothesis of relationships has been proposed for the species of Pelodytidae, but given the relatively recent genetic differentiation found between *Pd. ibericus* and *Pd. punctatus*, along the Pliocene-Pleistocene boundary ($D_{\mathrm{Nei}} = 0.15$ to 0.19) a sister relationship among them was expected (Sánchez-Herráiz et al., 2000). Our study supports such a sister relationship (decay 9–23, bs 99–100%) with *Pd. caucasicus* basal to them. Our results also indicate the existence of local differentiation among Iberian populations of *Pd. punctatus*, with the Catalonian population divergent from all others. These results are in close agreement with previous protein data (Sánchez-Herráiz et al., 2000) suggesting that *Pd. punctatus* is in need of detailed phylogeographic study.

The family Pelobatidae, in its current new sense, includes four living species in the genus *Pelobates*. Relationships among species of *Pelobates* have been extensively debated (Barbadillo et al., 1997; Busack et al., 1985; Cannatella, 1985; Estes, 1970; Gislen, 1936; Lathrop, 1997). None of these hypotheses was fully resolved, except that of Barbadillo et al. (1997), which used osteological characters and genetic data. *Pelobates varaldii* and *Pb. cultripes* were sister taxa, as previously suggested by Busack et al. (1985), and the clade formed by *Pb. cultripes* and *Pb. varaldii* was sister to *Pb. syriacus*, with *Pb. fuscus* basal to the entire clade. Our hypothesis based on mtDNA supports the clade *Pb. varaldii—Pb. cultripes*, but places *Pb. syriacus* basal to the entire *Pelobates* clade. The systematics of *Pb. fuscus* are in need of revision and the taxon might be represented by more than one species (Borkin et al., 2001).

The high genetic divergence (this study), larval period differences (Buchholz and Hayes, 2000, 2002) and morphological differences (Cannatella, 1985; Maglia, 1998) between *Scaphiopus* and *Spea*, indicate that these genera names are biologically useful at this taxonomic level (Dubois, 1987). Previous studies of relationships within *Scaphiopus* (Cannatella, 1985; Lathrop, 1997) found that *Sc. couchii* is sister to a clade formed by *Sc. holbrookii* and *Sc. hurterii*. Our 16S data support this conclusion, providing further support for the recognition of *Sc. hurterii* as an independent taxon. Six previous studies hypothesized relationships within *Spea*. Four studies, using allozymes or morphology, are consistent with our hypothesis in which *Sp. multiplicata* is basal, and *Sp. hammondii* is sister to a clade formed by *Sp. bombifrons* and *Sp. intermontana* (Kluge, 1966; Sattler, 1980; Tanner, 1939; Wiens and Titus, 1991). Hypotheses not congruent with our data are those of Northen (1970) who suggested that *Sp. intermontana* is basal to the *Spea* clade based on the morphology of frontoparietals and of Brown (1966) who suggested that the clade of *Sp. multiplicata* and *Sp. hammondii* is sister to a clade of *Sp. bombifrons* and *Sp. intermontana* based on advertisement call characteristics. Discerning the relationships among *Spea* requires further sampling due to the existence of unrecognized taxa within *Sp. hammondii* which are not sister to each other (Fig. 1), and within *Sp. intermontana* (Wiens and Titus, 1991).

Sequence divergence among genera of Megophryidae is very high, and although our limited sampling suggest the group is monophyletic, further extensive analyses including many more taxa are needed.

5. Biogeography

A comparison of mtDNA divergences within and between Scaphiopodidae, Pelodytidae, Megophryidae, and Pelobatidae, suggests that the ancestral pelobatoid lineage split into four main clades in a relatively short period of time, not far from their split from the lineage leading to Pipoidea. Fossil remains generally

accepted as pelobatoids (but see Roček, 2000) are knowm in North America as early as the Upper Jurassic of North America (Evans and Milner, 1993; Sanchiz, 1998a). This record points to a very ancient origin for pelobatoid differentiation clearly older than 155 Ma.

The split separating Scaphiopodidae from the morphologically diverse assemblage of Megophryidae, Pelodytidae, and Pelobatidae occurred between mid-Cretaceous, based on immunological estimates (Sage et al., 1982), and the early Eocene, when Scaphiopodidae was already differentiated (Henrici, 2000). The putative vicariant event separating Scaphiopodidae is the break up of Laurasia by the formation of the Atlantic Ocean during the mid-Cretaceous. The fossil record of Scaphiopodidae extends from the early Eocene to the Holocene of North America (Henrici, 2000; Sanchiz, 1998a), and the oldest records correspond to *Scaphiopus guthriei*, a species previously included within the North American "*Eopelobates*" (with quotes, *sensu* Sanchiz, 1998a) and recently transferred to *Scaphiopus* (Henrici, 2000; Roček, 1980). The taxonomic position of the North American "*Eopelobates*" *grandis* has also been questioned by Roček (1980) who argued that it corresponds to Scaphiopodidae. The North American *Scaphiopus* and *Spea* separated over 20–29 Ma between the Oligocene and the Miocene based on fossil data (Kluge, 1966), although the recent attribution of "*Eopelobates*" *guthriei* to *Scaphiopus* changes this view (Henrici, 2000). Immunological estimates suggest that extant taxa within *Scaphiopus* diverged 21 million years ago and species within *Spea* diverged in the last six million years (Sage et al., 1982). The relatively recent diversification of *Spea* compared to that of *Scaphiopus* may explain the stronger morphological differentiation reached within *Scaphiopus*.

Fossil Pelodytidae are found in North America (*Tephrodytes* and *Miopelodytes*) from the Oligocene to Miocene and in Europe (*Pelodytes*) from the Eocene to Holocene (Henrici, 1994; Roček and Rage, 2000; Sanchiz, 1998a). Within *Pelodytes*, the Caucasian–Iberian split was likely a slow process resulting from the old extinction (pre-Miocene) of a series of geographically intermediate linking populations (Sanchiz, 1998b). The Iberian Peninsula has been a center of speciation for Pelodytidae since the Miocene (Sanchiz, 1978), allowing for the recolonization of central Europe by *Pd. punctatus* during the Pleistocene, likely from the Iberian source. If the Laurasian break-up divided Scaphiopodidae from other pelobatoids, a trans-Atlantic colonization of America by Pelodytidae is necessary, and it must have occurred before the Middle Eocene, at which point the European genus *Pelodytes* was already well differentiated from the American pelodytid taxa (Roček and Rage, 2000; Sanchiz, 1978; Sanchiz, 1998a).

Pelobatidae, including *Eopelobates* (without quotes, *sensu* Sanchiz, 1998a), *Macropelobates* and *Pelobates*, is exclusively Eurasian. *Eopelobates* is known from the European Eocene to the Pliocene, *Macropelobates* is known from central Asian Oligocene and perhaps Miocene, and *Pelobates* is represented from the Oligocene-Miocene boundary to the Holocene (Sanchiz, 1988) of Europe and Anatolia. Roček and Rage (2000), departing from Sanchiz's (1988) opinion, attribute *Macropelobates* to Scaphiopodidae. The pelobatid *Liaobatrachus*, known from the Mesozoic of China (Shu'an and Qiang, 1998), provides further support for rapid, ancient divergence of the Pelobatoidea. Fossil remains of *Pb. fuscus*, disregarding earlier questionable reports, are known at least since the early Pliocene (Sanchiz, 1998a). A sister taxon relationship between *Pb. cultripes* and *Pb. varaldii* is well supported, and their split may correspond to the late Miocene, shortly before the formation of the Strait of Gibraltar, during the Miocene–Pliocene boundary (5.5 Ma), as previously suggested by Busack et al. (1985).

The Turgai strait, separating Europe and Asia from the Jurassic to the late Eocene, may represent an ancient vicariant event which isolated ancestors of the Megophryidae, which are now found in temperate-tropical regions of southeastern Asia, from Pelobatidae. However, Pelobatidae of uncertain adscription (*Macropelobates, Uldzinia*) are known from the Lower Oligocene of Mongolia (Gubin, 1996; Sanchiz, 1998a), and other recently discovered Pelobatidae, including *Liaobatrachus*, are known from the Mesozoic of China (Shu'an and Qiang, 1998), making this hypothesis questionable.

6. Evolution of fossoriality

Pelobates, *Scaphiopus*, and *Spea* all have similar fossorial habits and a similar general morphology, combining the presence of well-developed metatarsal spades with co-ossification of the head skin with the skull. Previous phylogenetic hypotheses and discussions of fossoriality suggested digging is plesiomorphic to all spadefoot toads (Ford and Cannatella, 1993; Noble, 1924). According to our phylogenetic hypothesis, the basal position of Scaphiopodidae within Pelobatoidea might support fossoriality as the primitive condition for the entire clade (Ford and Cannatella, 1993), a condition that subsequently was lost in Pelodytidae and Megophryidae and retained in Pelobatidae. However, Gislen (1936) and Bragg (1961) argued for independent origins of fossoriality in spadefoot toads. Fossoriality has evolved many times independently in numerous anuran families, and in no case has a reversal from fossoriality been identified as a precedent for a hypothetical reversal in Pelodytidae and Megophryidae.

Also, fossoriality is thought to originate in desert or semiarid climatic conditions (Bragg, 1961; Gislen, 1936; Noble, 1924). Pelobatoid remains from the Jurassic and Cretaceous and the ancient divergence suggested by our mtDNA data reveal that it is an old lineage which diverged before the aridification of North America or Europe in the mid-Cenozoic. Thus, the existence of a common ancestor with a digging morphology in such humid conditions would be unlikely (but see Zweifel, 1956, for an alternative focused in the relationships within *Scaphiopus*). Supporting this claim is morphological evidence from the oldest fossils of Scaphiopodidae (previously included in "*Eopelobates*"), which do not show fossorial modifications characteristic of either Scaphiopodidae or *Pelobates* (Henrici, 2000). For these reasons, regardless of the question of spadefoot monophyly, we suggest fossoriality evolved independently in Scaphiopodidae and Pelobatidae. A striking precedent of parallel evolution of the digging phenotype is found in the paraphyletic *Tomopterna* (Bossuyt and Millinkovitch, 2000).

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