Contributions to Zoology, 75 (3/4) 109-120 (2006)

Historical biogeography of Western Palaearctic pelobatid and pelodytid frogs: a molecular phylogenetic perspective

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Key words: Phylogeny, paleobiogeography, speciation, regression-based molecular clock, Bayesian molecular dating, Messinian salinity crisis, 16S, 12S.

Abstract

Spadefoot toads (*Pelobates*) and Parsley frogs (*Pelodytes*) are an enigmatic group of Western Palaearctic anurans. In the genus *Pelobates*, a fossorial lifestyle has enforced a conserved bauplan that masks their intraspecific evolutionary history. We used partial sequences of the mitochondrial 16S and 12S rRNA genes to infer a paleobiogeographic scenario of speciation events in these two anuran genera. Based on two alternative, mutually exclusive calibrations of the Iberian-African split within *Pelobates* (*Pb. cultripes* and *Pb. varaldii*), the disjunction of the Betic Cordillera ca. 14-16 million years ago (mya), and the end of the Messinian Salinity crisis 5.33 mya, we inferred alternative scenarios for species evolution within both genera applying regression-based dating and Bayesian molecular dating. *Pelobates* and *Pelodytes* are both monophyletic genera. Interspecific relationships among spadefoot toads are poorly resolved, and only an Iberian-African *Pb. cultripes/Pb. varaldii* clade consistently emerges from our analyses. An evolutionary scenario based on the Messinian divergence of African and Iberian *Pelobates* lineages becomes plausible in the light of geological and paleontological data. Consequently, *Pelobates* species are likely to have originated from the Miocene. Speciation around the Oligocene/Miocene boundary is inferred for the Iberian-Caucasian *Pelodytes*, and a Messinian divergence has to be invoked to explain intraspecific diversification of Iberian parsley frogs. There is indication that the different *Pb. syriacus* lineages may not form a monophylum.

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Introduction

Frog diversity is very unevenly distributed among major lineages. According to current species counts (www.amphibiaweb.org as of November 2005), the monophyletic frog crown group (Neobatrachia) contains more than 5000 species, whereas the basal lineages, subsumed in the Archaeobatrachia, include only 200 species. Whether archaeobatrachians are paraphyletic (e.g., Ford and Cannatella 1993) or monophyletic (e.g., Feller and Hedges 1998) has long been disputed, but recent studies using large datasets of nuclear DNA sequences point to a paraphyly of archaeobatrachians (Hoegg *et al.* 2004; San Mauro *et al.* 2005; Roelants and Bossuyt 2005). Biogeographically, several families of the Archaeobatrachia show an exclusively Holarctic distribution (such as Pelobatidae, Ascaphidae), with some being restricted to the Palearctic realm (Discoglossidae, Bombinatoridae, Pelodytidae, Megophryidae). Three of these

families, the Megophryidae, Pelobatidae and Pelodytidae were formerly subsumed in a single family named Pelobatidae (e.g., Gilsen 1936). However, molecular data showed non-monophyly of the former Pelobatinae, resulting in the assignment of familial rank to each of these groups (e.g. Ford and Cannatella 1993; Hoegg *et al.* 2004), and to the Scaphiopodidae, containing the Nearctic spadefoot toads, *Scaphiopus* and *Spea* (e.g., Roelants and Bossuyt 2005). Hence, the Pelobatidae consist of a single genus, the Western Palearctic spadefoot toads (*Pelobates* Wagler), similar to the Pelodytidae which contain a single genus, the Western Palaearctic parsley frogs (*Pelodytes*).

Within *Pelobates*, four species are currently recognised: *Pb. fuscus* (Laurenti, 1768) (with two subspecies, the widespread *Pb. f. fuscus* and the northern Italian endemic *Pb. f. insubricus* Cornalia, 1873), *Pb. varaldii* Pasteur and Bons 1959 (Morocco, Africa), *Pb. cultripes* Cuvier 1929 (mainly the Iberian Peninsula) and *Pb. syriacus* Boettger, 1889 (with four recognised subspecies, *Pb. s. syriacus*, *Pb. s. boettgeri* Mertens 1923, *Pb. s. balcanicus* Karaman 1928 and *Pb. s. transcaucasicus* Delwig 1928). Spadefoot toads are the most fossorially adapted European amphibians. They prefer sandy areas where they burrow themselves during the daytime, using their large and sharp metatarsal tubercles.

Systematic relationships among *Pelobates* species have recently been studied by García-Paris *et al.* (2003). They showed that a *Pb. cultripes*-*Pb. varaldii* clade is sister to a *Pb. fuscus* - *Pb. syriacus* clade. However, their sampling did not allow for detailed assessment of intraspecific phylogeny, and their approach did not include a molecular dating of speciation events in *Pelobates* or *Pelodytes*.

The intrageneric taxonomy of *Pelobates* has changed several times, mainly with regard to the eastern *Pb. syriacus* group. The validity of the *Pb. syriacus* subspecies has been repeatedly questioned (e.g. Eiselt and Schmidtler 1973, Terentiev and Chernov 1965, Kuzmin 1999), and Eiselt (1988) even considered *Pb. syriacus* a monotypic species. Morphological evolutionary stasis, probably linked to their fossorial lifestyle, may account for the lack of diagnostic morphological characters among *Pelobates* taxa (e.g. between *Pb. varaldii* and *Pb. cultripes*; Busack *et al.* 1985). However, based on a morphological analysis, Ugurtas *et al.* (2002) recently emphasised that Anatolian and European *Pb. syriacus* comprise at least three taxa: *Pb. s. syriacus*, *Pb. s. balcanicus* and a third lineage that they collected in Serbia. In addition, Borkin *et al.* (2001, 2003) discovered two well-differentiated *Pb. f. fuscus* lineages with non-overlapping genome sizes (measured by the total amount of nuclear DNA per cell). This finding was supported by allozyme data, so they regarded them as differentiated at the species level (provisionally named the "western" and the "eastern" form of *P. f. fuscus*). Again, they attributed this cryptic species diversity to the morphological stasis of spadefoot toads.

Parsley frogs live in all kinds of stagnant and slow-moving waters of the Iberian Peninsula and the Caucasus mountains (Gasc *et al.* 1997; in contrast to the Iberian species the Caucasian parsley frog prefers habitats with semi-current water). Their distribution is disjunct. While *Pd. caucasicus* (Boulenger, 1896) is restricted to the Caucasus, two species, *Pd. ibericus* Sánchez-Herráiz, Barbadillo, Machordom and Sanchiz, 2000 and *Pd. punctatus* (Daudin, 1803) are originally Iberian endemics, with the latter having dispersed postglacially into large parts of France (Gasc *et al.* 1997). This Caucasus-Iberian disjunction is mirrored by the almost identical distribution of *Mertensiella caucasica* and *Chioglossa lusitanica*, two sister taxa within the Salamandridae (Titus and Larson 1995, Veith *et al.* 1998).

In the present paper we use molecular sequence data of mitochondrial genes to analyse the intrageneric phylogenetic relationships of *Pelobates* and *Pelodytes*. Based on two different calibrations of a molecular clock, we infer paleogeographic scenarios of their evolution in the circum-Mediterranean region.

Materials and methods

We sequenced 34 specimens from eight taxa of *Pelobates* and *Pelodytes* (see appendix). For hierarchical outgroup rooting we included *Scaphiopus* (from GeneBank), several archaeobatrachian representatives (Pipidae, Megophryidae, Discoglossidae, Leiopelmatidae and Ascaphidae; partially from GenBank), two Neobatrachian species of the Ranidae (GenBank) and Bufonidae (GenBank), and the newt *Triturus vulgaris* (GenBank).

DNA extraction, sequencing and sequence alignment

DNA was extracted from ethanol-preserved tissue samples using the QiAmp tissue extraction kit (Qiagen). We amplified via polymerase chain reaction (PCR) fragments of two mitochondrial genes coding for fractions of the 16S rRNA and 12S rRNA (Tab. 1).

16S: 16SA (light chain; 5' - CGC CTG TTT ATC AAA AAC AT - 3') and 16SB (heavy chain; 5' - CCC GTC TGA ACT CAG ATC ACG T - 3') of Palumbi *et al.* (1991) amplified a ca. 580 bp section of the mitochondrial 16S rRNA gene homologous to positions 3976-4554 of the *Xenopus laevis* mitochondrial genome (Roe *et al.* 1985).

12S: 12SA-L (light chain: 5' - AAA CTG GGA TTA GAT ACC CCA CTA T - 3') and 12SB-H (heavy chain: 5' - GAG GGT GAC GGG CGG TGT GT - 3') of Goebel *et al.* (1999) amplified a ca. 490 bp section of the mitochondrial 12S rRNA gene homologous to positions 2510-2997 of the *Xenopus laevis* mitochondrial genome (Roe *et al.* 1985).

Polymerase chain reaction cycling procedures were as follows: 16S rRNA gene: denaturation for 45 s at 94°C, primer annealing for 45 s at 55°C, extension for 60 s at 72°C; after 35 cycles final step at 72°C for 10 min.; 12S rRNA gene: denaturation for 45 s at 92°C (initial denaturation for 120 s at 94°C), primer annealing for 60 s at 50°C, extension for 90 s at 72°C; after 35 cycles final step at 72°C for 10 min.

PCR products were purified using the "High Pure PCR Product Purification Kit" (Roche diagnostics). We sequenced single-stranded fragments using the "Big dye terminator cycle sequencing kit" of Applied Biosystems Inc. on an ABI 377 automatic sequencer using standard protocols.

Sequences were aligned automatically using the Clustal X software for Windows, version 1.81 (Thompson *et al.*, 1997). Alignment penalties were arbitrarily set to 12 for gap opening and to 5 for gap extension. Redundant haplotypes were excluded from analyses.

Phylogenetic analyses

We tested for partition (=gene) combinability (Huelsenbeck *et al.*, 1996) using the maximum parsimony procedure of Farris *et al.* (1994; incongruence of length differential test, ILD) as implemented in Paup* (Swofford, 2001; 100 replicates, heuristic search using the tree bisection-reconnection (TBR) branch-swapping algorithm). Recently, the ILD test has been criticised (e.g., Yoder *et al.*, 2001; Barker and Lutzoni, 2002). We therefore also analysed each gene separately and compared the resulting topologies with the combined analysis.

To avoid topology formation by variation in base composition among taxa (Steel *et al.*, 1993) we applied a χ^2-test as implemented in PAUP*. Unequal base frequencies among taxa would necessitate a LogDet transformation of sequence differences that allows consistent recovery of the correct tree when sequences evolve under simple asymmetric models that can vary between lineages (Lockhart *et al.*, 1994). Equal base distribution among taxa would allow application of a specific substitution model, as was recently recommended by Sullivan and Swofford (2001).

Additionally, we applied a hierarchical likelihood ratio test for the goodness-of-fit of nested substitution models (ingroup taxa only; for details see Huelsenbeck and Crandall, 1997) using the program Modeltest version 3.02 of Posada and Crandall (1998). To test for the possibility that some types of nucleotide substitutions have become saturated, we plotted uncorrected p distances versus molecular distances derived from the specific substitution model (transitions and transversions, separately for each gene).

We defined the newt *Triturus vulgaris* as the outgroup and subjected our alignment to four different methods of phylogenetic reconstruction: (i) neighbor joining; (NJ; Saitou and Nei, 1987) using the specific substitution model; (ii) maximum parsimony with gaps treated as fifth character state (within ingroups, gaps occurred only in the 16S rDNA and mostly consisted of single indels); 10 random additions of haplotypes; transitions and transversions were given equal weight; heuristic search with the TBR branch swapping algorithm; (iii) maximum likelihood (ML; Felsenstein, 1981) based on the specific substitution model, (iv) Bayesian inference (Rannala and Yang, 1996, Huelsenbeck *et al.*, 2001), which is based upon the notion of posterior probabilities of a phylogenetic tree. With the exception of the Bayesian approach (MrBayes; Huelsenbeck and Ronquist, 2001), all analyses were done with Paup* (Swofford, 2001).

Robustness of NJ and MP tree topologies was tested by bootstrap analyses (Felsenstein, 1985), with 2000 replicates each (Hedges, 1992). Due to computational constraints, we used Quartet Puzzling (Strimmer and von Haeseler, 1996) with 100,000 permutations to infer reliability values for ML tree topologies. To gain confidence in Puzzle support values we compared them to ML bootstrap values derived from 100 replicates. We applied the Bayesian method using the general time reversible model of nucleotide substitution (GTR; Rodríguez *et al.*, 1990) with the proportion of invariable sites estimated from the data. We ran four simultaneous Metropolis-coupled Monte Carlo Markov chains for 500,000 generations. We repeated Bayesian analysis four times and plotted likelihood values against generation number to ensure that the likelihood converged at the same value (Leaché and Reeder, 2002) and to determine the necessary burn-in. All runs with the Bayesian approach converged at a likelihood of ca. -lnL =4660, indicating that this marks a global optimum. We sampled a tree every 100 generations and calculated Bayesian posterior probabilities for 4000 trees by omitting the first 1000 trees (burn-in).

For simplicity, we use the term "support values" when referring to bootstrap values for NJ and MP, puzzle support values for ML and Bayesian posterior probabilities. We regard bootstrap proportions and Puzzle support values of ≥70% as indicators for sufficiently resolved topologies since they usually correspond to a probability of ≥95% that the corresponding clade is valid (Hillis and Bull 1993, but see constraints of this interpretation discussed therein). We accept Bayesian posterior probabilities ≥95% as indicators of sufficient node support, since they correspond to a ≤5% probability of committing a type I error (i.e. of drawing an erroneous conclusion).

Molecular clock calibration

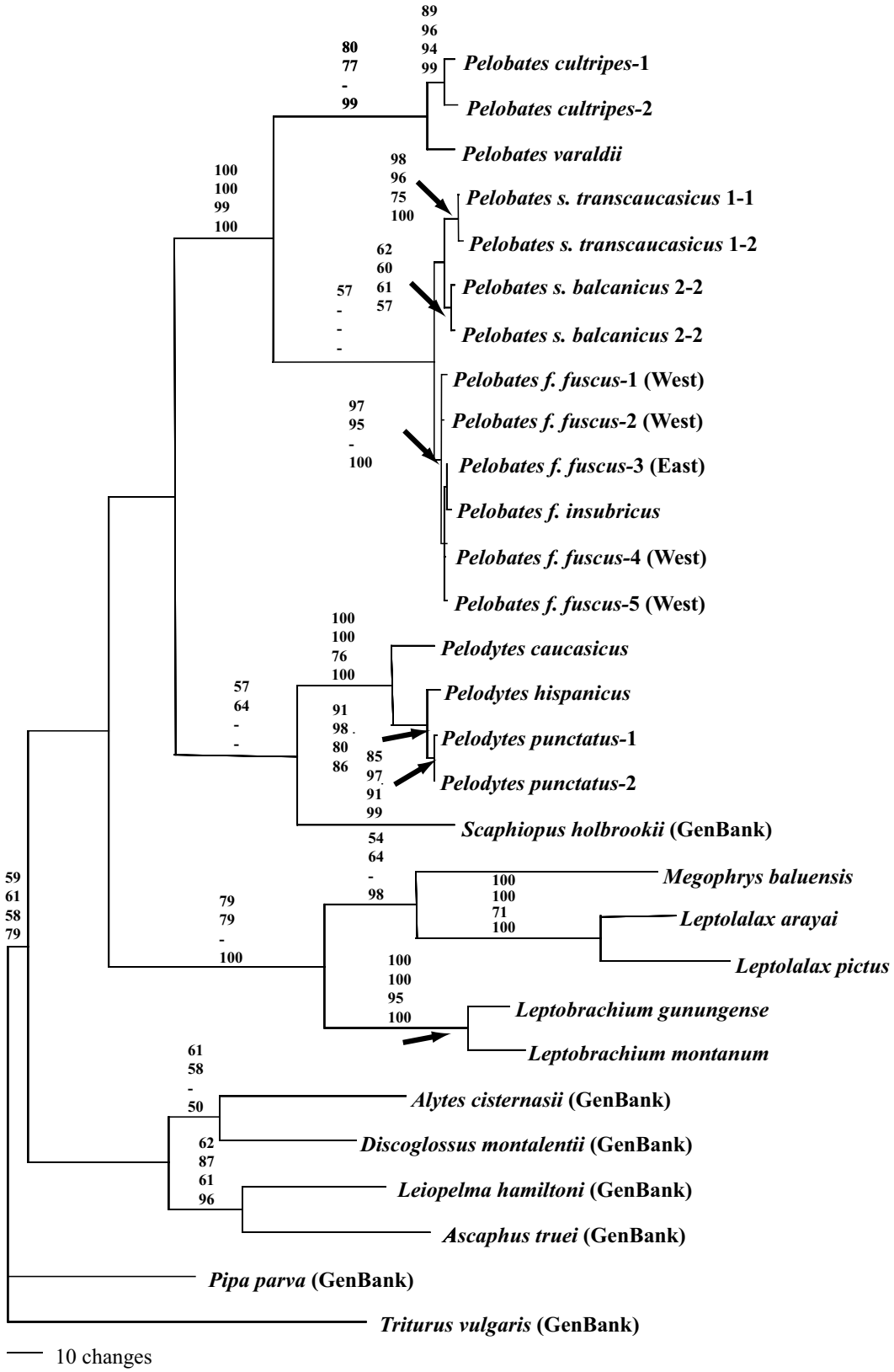
We tested for rate constancy between *Pelobates* and *Pelodytes* using a likelihood ratio test (Tree Puzzle; Schmidt *et al.*, 2002) and defining *Discoglossus montalentii* as the single outgroup (TrN substitution model with eight gamma rate categories). This test compares the log-likelihood of the most likely tree with and without a molecular clock enforced. In addition we applied the Tajima χ^2-test (Tajima, 1993) for substitution rate heterogeneity among all possible 153 pairs of ingroup haplotypes (including *Scaphiopus*), again using *D. montalentii* as the outgroup.

We used the specific substitution model to calculate pairwise corrected molecular distances among haplotypes. We calibrated two molecular clocks. Calibration I is based on the hypothesis that the population of the ancestor of Ibero-African *Pelobates* was separated by the formation of the Strait of Gibraltar (Busack, 1986; García-Paris *et al.*, 2003) into the Iberian *Pelobates cultripes* and the African *P. varaldii*. This event was precisely dated to 5.33 mya, when the reflooding of the Mediterranean basin marked the end of the Messinian Salinity Crisis (Krijgsman *et al.*, 1999). Calibration II is based on the Betic Crisis. This vicariance event has often been invoked to explain divergent evolution of African and Iberian lineages (García-París and Jockusch 1999, Fromhage *et al.* 2004, Veith *et al.* 2004). During the Betic Crisis 16-14 mya, marine transgressions through the Betic Strait (corresponding to today's Guadalquivir basin in southern Iberia) separated the Betic-Rif mountain belt, which developed during, and partly in response to, Late Mesozoic and Tertiary convergence between Africa and Iberia (Lonergan and White, 1997), from the Iberian mainland. Promoted by the further orogenic uplift of the Alboran basin between Iberia and Africa, the southernmost part of the insular Betic region connected to the African continent and formed today's Rif Mountains (De Jong, 1998).

We applied the bootstrap method to compute variances for molecular distances between two sequences. This requires no assumption about the underlying distribution of evolutionary distances except that each site evolves independently, an assumption that is usually met when the number of sites is >100 (Nei and Kumar, 2000). We used the software MEGA, version 2.1 (Kumar *et al.*, 2001), to estimate times of divergence of splits. 95% confidence limits of estimates of divergence time were calculated *via* 1000 bootstrap replicates.

Since estimation of rate constancy using the likelihood approach as implemented in Tree Puzzle may

Fig. 1. Maximum parsimony tree of Western Palearctic Pelobatidae and Pelodytidae based on 809 bp of the 16S and 12S rRNA genes (TrN substitution model); support values >50% are given for MP (upper), NJ (upper half), ML using quartet puzzling (lower half) and Bayesian inference (lower). ▶


Pelobates cultripes-1
Pelobates cultripes-2
Pelobates varaldii
Pelobates s. transcaucasicus 1-1
Pelobates s. transcaucasicus 1-2
Pelobates s. balcanicus 2-2
Pelobates s. balcanicus 2-2
Pelobates f. fuscus-1 (West)
Pelobates f. fuscus-2 (West)
Pelobates f. fuscus-3 (East)
Pelobates f. insubricus
Pelobates f. fuscus-4 (West)
Pelobates f. fuscus-5 (West)
Pelodytes caucasicus
Pelodytes hispanicus
Pelodytes punctatus-1
Pelodytes punctatus-2
Scaphiopus holbrookii (GenBank)
Megophrys baluensis
Leptolalax arayai
Leptolalax pictus
Leptobrachium gunungense
Leptobrachium montanum
Alytes cisternasii (GenBank)
Discoglossus montalentii (GenBank)
Leiopelma hamiltoni (GenBank)
Ascaphus truei (GenBank)
Pipa parva (GenBank)
Triturus vulgaris (GenBank)
10 changes

strongly depend on the choice of outgroup (in our case some possible outgroups resulted in a rejection of the assumption of rate constancy) we also applied Bayesian molecular dating (Thorne *et al.*, 1998) using the multidivtime package of Thorne and Kishino (2002). This approach accounts for rate heterogeneity among lineages. We consistently used default settings of multidivtime as recommended by Rutschmann (2004). Again we defined two calibrations: the vicariance of Europe from Africa following the end of the Messinian salinity crisis 5.33±0.02 million years ago (Krijgsman *et al.* 1999), and the Betic cordillera disjunction between 16 and 14 mya (Lonnergan & White, 1997). To avoid biased estimates of divergence times due to overrepresentation of some lineages (Yoder & Yang 2004) we only included one sequence per species, except for *P. fuscus*, where we included a representative of each genome size form.

Results

Alignment statistics and model selection

According to the ILD test, data partitions contained no incongruent phylogenetic signals, so we combined 12S and 16S fragments into a single alignment. It consistently contained 809 bp for all specimens, with 333 variable and 245 parsimony informative sites (143 and 98, respectively, when regarding ingroup taxa only). Empirical base frequencies were $\pi_A = 0.356$, $\pi_C = 0.233$, $\pi_T = 0.249$ and $\pi_G = 0.162$. The strong anti-G bias indicates that no nuclear pseudocopies of the genes have been analysed (Zhang and Hewitt, 1996). Bases were homogeneously distributed among ingroup haplotypes (χ^2-test: $p = 1.00$). The substitution model that fitted our alignment best was the Tamura-Nei model (Tamura and Nei, 1983) with a proportion of invariable sites of I = 0.3674, a gamma distribution shape parameter $\alpha = 0.6172$ and substitution $rate_{[A-G]} = 3.0767$ and $rate_{[C-T]} = 6.1846$ ($-\ln L_{TrN+I+G} = 4690.2271$).

Clade formation

Only a few topologies were sufficiently supported by all tree-building approaches (Fig. 1): (i) monophyly of each of *Pelobates*, *Pelodytes*, *Leptobrachium* and *Leptolalax*; (ii) monophyly of *Pb. varaldii* and *Pb. cultripes*; (iii) monophyly of *Pd. ibericus* and *Pd. punctatus*.

Basal split among families are poorly resolved. *Scaphiopus* stands together with *Pelodytes*, although with low support values. Within *Pelobates*, two well-differentiated lineages of *Pb. syriacus* (ssp. *transcaucasicus* and *balcanicus*) emerged, with no support for monophyly of the species.

Molecular clock calibration and application

Plots for uncorrected distances against corrected TrN+I+G distances (Fig. 2) showed signs of saturation for neither transitions (ti's) nor transversions (tv's). The log-likelihood ratio test with *D. montalentii* as outgroup indicated rate constancy among all taxa of *Pelobates* and *Pelodytes*. The log-likelihood of the clocklike tree (-logL=2332.95) was not significantly smaller than the log-likelihood of the tree with no clock enforced (-logL=2342.67; the simpler (clocklike) tree is therefore not rejected on a significance level of 5%). In addition, no pair of ingroup haplotypes showed a significant deviation from substitution rate constancy when applying the Tajima test.

Calibrating a molecular clock based on molecular distances and using the end of the Messinian Salinity Crisis (calibration I) for the vicariance of *Pb. cultripes* and *Pb. varaldii* results in mean separation times among basal *Pelobates* lineages of 12.12 my (Tab. 1). Separation of the Eastern and Western genome size lineages of *Pb. f. fuscus* are estimated to the Pleistocene ca. 1.69 mya. However, contemporary separation is within their lower 95% CI's. The split between Iberian *Pelodytes* is dated exactly to the end of the Messinian Salinity Crisis 5.33 mya, while separation of Iberian and Caucasian *Pelodytes* lineages is much older (22.3 mya). Divergence time estimates based on Bayesian methods (Tab. 2) are not in line with those based on molecular distances (Tab. 1). The split between the eastern and western genome size lineages of *Pb. fuscus* is estimated to 2.4 mya, while the basal split among *Pelobates* is dated to 6.8 mya. Estimates among *Pelodytes* lineages are much younger under a Bayesian framework, with the intra-Iberian split estimated to only 1.15 mya and the Iberian-Caucasian separation being estimated to the late Pliocene 2.87 mya.

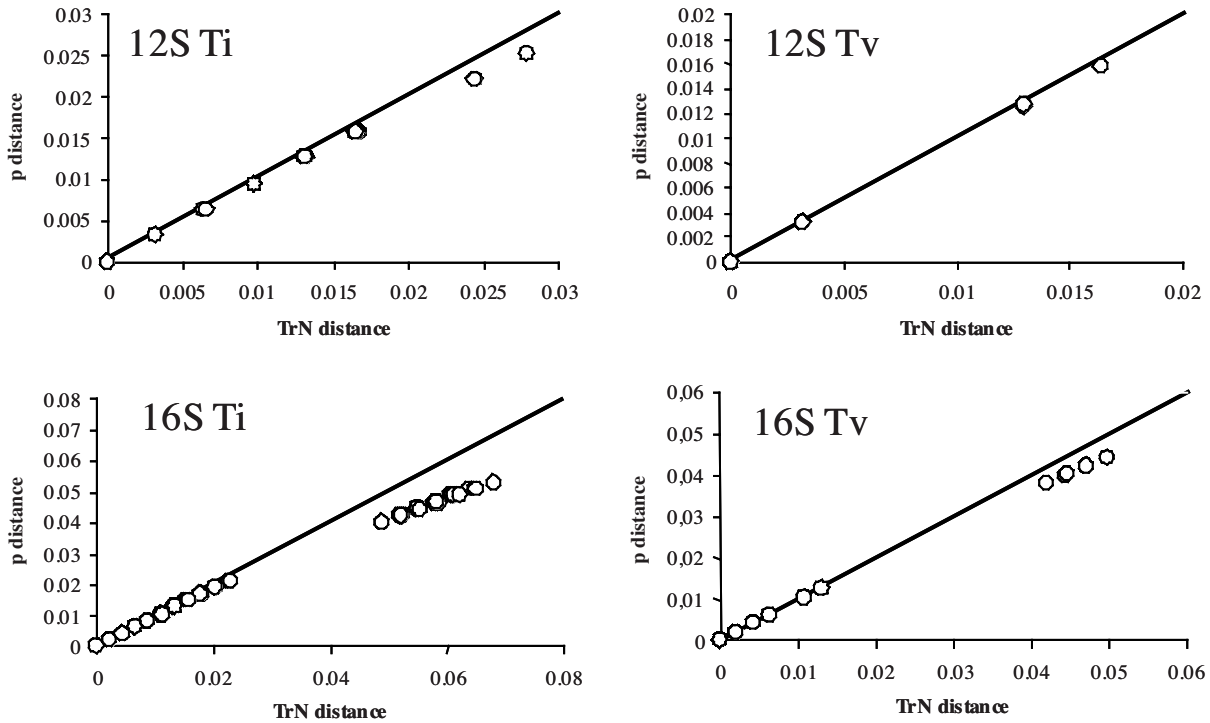


Fig. 2. Saturation plots of uncorrected distances against TrN+I+G molecular distances for different genes and portions of genes; deviation from the bisector of an angle indicates the degree of saturation.

Discussion

Phylogeny of Pelobatidae and Pelodytidae

The phylogenetic hypotheses (Fig. 1) do not provide an adequate resolution of the basal splits among archaeobatrachian representatives. In contrast to García-Paris *et al.* (2003) but in agreement with the nuclear DNA sequence analysis of Hoegg *et al.* (2004) and Roelants and Bossuyt (2005), our data do not support a Mesobatrachia clade that includes the Pipidae, since our representative, *Pipa parva*, clusters outside a (Megophryidae, Pelobatidae, Pelodytidae, Scaphiopodidae) clade. While the monophyly of each included family receives moderate to high support, and relationships within *Pelodytes* are well supported, the topology within *Pelobates* was not consistent among tree building approaches, resulting in only weak support for several of the clades shown in Fig. 1.

Reliability of the molecular clocks

In our dataset, a clocklike behaviour of the DNA sequence evolution was not rejected. This is a privileged situation in amphibians, where large differences in substitution rates among clades are common, especially in mitochondrial genes (Hoegg *et al.* 2004). This pattern also allows for the application of a regression-based molecular clock approach, in which calibration times are plotted against pairwise distances among taxa, and the age of unknown splits is deduced from the regression slope (e.g., Hillis *et al.* 1996). It is remarkable that this method, in the present dataset, produces results largely inconsistent with those of the Bayesian approach that is able to handle differences in substitution rates among lineages (Thorne *et al.* 1998). It needs further exploration how good this method is able to handle datasets of low to very low divergences among taxa, as in pelobatid and pelodytid frogs. Similar cautions may apply to likelihood methods of phylogeny reconstruction; Fromhage *et al.* (2004) were unable to recover plausible phylogenetic topologies for closely related discoglossid frogs using ML methods. Overparametrization and, consequently, usage of too complex nucleotide substitution models may explain the failure of likelihood-based methods in these examples, which is why we base the following discussion on

the regression-based molecular clock estimates using pairwise distances only.

Which paleogeographic scenario can best explain Pelobates and Pelodytes evolution?

Estimated times of divergence of our calibrations differ by a factor of ca. 2.6. In a simplistic approach to molecular clock calculations, and considering the uncorrected pairwise divergences between *Pelobates cultripes* and *Pb. varaldii* of 0.9%, calibration I would result in a rate of pairwise sequence divergence of approximately 0.17%/million years (my), whereas calibration II would result in a rate of 0.06%/my. The latter rate is much lower than rates usually assumed for mitochondrial rRNA genes in vertebrates (e.g., Veith *et al.* 1998: 0.7%/my in salamanders; Vences *et al.* 2001: between 0.100 and 0.500%/my in fish and mammals). This would argue in favour of calibration I, however, from biogeographical and geological points of view, there is support for and contradiction to both scenarios.

Assuming a Pliocene divergence of *Pb. varaldii* and *Pb. cultripes* 5.33 mya, calibration I in the regression approach dates the major splits among *Pelobates* to the Miocene. A separation of an Ibero-African lineage (*Pb. cultripes* and *Pb. varaldii*) from an E European-W Asian lineage (*Pb. fuscus* and *Pb. syriacus*) can be explained by the middle Miocene disjunction of land masses that formerly separated the Tethys and Paratethys ocean (biogeographic characteristic no. 4 of Oosterbroek and Arntzen 1992). However, assuming this we have to invoke the upper 95% confidence limits of our time estimates. Separation within the E European-W Asian lineage (split 2 in Tabs. 1 and 2) fits the onset of the first glaciation cycles at ca. 3.4-2.5 mya (Wilson *et al.* 2000) only if we invoke the respective lower 95% confidence limit (see also Veith *et al.* 2003a,b for the effect of Pleistocene glaciations on amphibian speciation in the Western Palearctic). Problems occur with the explanation of the intra-Iberian speciation of *Pelodytes* (split 5) since the end of the Messinian Salinity Crisis was not linked to any known Intra-Iberian disjunction. The current distribution of *Pd. punctatus* and *Pd. ibericus* instead corresponds to a pattern found in other pairs of vicariant amphibian taxa (e.g. in *Alytes*, *Discoglossus*, and *Salamandra*; Arntzen and Garcia-Paris 1997, García-Paris *et al.* 1998, García-Paris and Jockusch 1999), which are often explained by the disjunction of the Betic Cordillera from the Iberian mainland through the formation of the Betic Sea Strait ca. 14 mya. However, a second reopening of the Betic Strait ca. 10-6 mya (López Martínez 1989 in Martínez-Solano *et al.* 2004) falls well into the 95% CI of split 5. In addition, it needs to be taken into account that data currently being assembled by other research groups support the presence of a further *Pelodytes* lineage, probably an undescribed species, in the Iberian Peninsula and the available distribution data for *Pd. ibericus* probably subsume two lineages of yet un-

Table 1. Regression-based time estimates of divergence among lineages of *Pelobates* and *Pelodytes*, based on two molecular clock calibrations for the split *Pelobates cultripes* - *Pb. varaldii* and applying molecular distances; standard errors of TrN distances were calculated via 1000 bootstrap replicates; mean±1.96 S.E. resulted in lower and upper 95% confidence limits.

No.	split	*TrN*	*SD*	*Calibration I (Messinian Salinity Crisis, 5.33 mya)*			*Calibration II (Betic Crisis, 15 mya)*		
				low 95%CI	mean	up 95%CI	95% CI_{low}	mean	95% CI_{up}
0	*Pb. cultripes* from *Pb. varaldii*	0.00760	0.00396		5.33			15	
1	*Pb. cultripes/varaldii* from *Pb. fuscus/syriacus*	0.01728	0.00406	6.54	12.12	17.70	17.17	31.83	46.49
2	*Pb. s. transcaucasicus* from *Pb. s. balcanicus/Pb. fuscus*	0.01060	0.00308	3.20	7.43	11.67	8.41	19.53	30.65
3	*Pb. s. balcanicus* from *Pb. fuscus*	0.01216	0.00403	2.99	8.53	14.07	7.85	22.40	36.95
4	*Pb. fuscus*-West from *Pb. uscus*-East	0.00241	0.00152	0.00	1.69	3.78	0.00	4.44	9.93
5	*Pd. punctatus* from *Pd. ibericus*	0.00760	0.00315	1.00	5.33	9.66	2.63	14.00	25.37
6	*Pd. ibericus/punctatus* from *Pd. caucasicus*	0.03176	0.00671	13.05	22.27	31.49	34.27	58.50	82.72
7	*S. holbrooki* from *Pelodytes*	0.12726	0.01668	66.32	89.25	112.18	174.20	234.43	294.65

known relationships to each other. The time estimate for the Iberian-Caucasian vicariance within *Pelodytes*, according to our data, was estimated to the Oligocene/Miocene boundary. It matches well the time period of 15-20 mya when *Mertensiella caucasica* and *Chioglossa lusitanica* were assumed to have diverged (Veith *et al.* 1998; but see Steinfartz *et al.* 2000). Although also this estimate was deduced from molecular data (16S rDNA), it is plausible to attribute the evolution of a pair of vicariant amphibian species with such a strange but identical disjunct distribution to the same vicariance event.

Calibration II may easily explain split 5. Separation of the former Betic region from the Iberian mainland 14 mya ago may have left behind ancestors of both *Pb. varaldii* and *Pd. ibericus* on the Betic Cordillera. The southern part, today's Rif mountains, that carried *Pb. varaldii,* drifted southwards and subsequently connected to the African continent, while the north-eastern part, harbouring *Pd. ibericus*, reconnected to the Iberian mainland after the Betic Sea Strait closed again. However, if this scenario holds, two extinction events on both parts of the formerly isolated Betic mountain chain have to be assumed: extinction of the ancestral *Pb. varaldii* on its north-eastern part, and extinction of *Pd. ibericus* on its southern part. Evolution of major *Pelobates* lineages may be explained by fragmentation of today's E European/Asian Minor landmasses in the course of the restoration of marine conditions between the Tethys and Paratethys during the early Oligocene (see Oosterbroek and Arntzen 1992). Split 4 can easily be explained by any appropriate event during the last ten million years (see its broad CI ranging from 0 to almost 10 my), including the onsets of Pliocene and Pleistocene glaciation cycles; in a strict sense this makes it almost entirely non-informative. Problems arise with the Iberian-Caucasian separation within *Pelodytes* more than 60 mya. Even its lower 95% CI by far predates the above mentioned time of divergence of 15-20 mya between the Iberian-Caucasian sister taxa *Mertensiella caucasica* and *Chioglossa lusitanica* and is not supported by paleontological data (Sanchiz 1998, Rage & Rocek 2000) according to which *Pelodytes* are not known from periods earlier than the Eocene. An extinct species of *Pelodytes* (*P. arevacus*) is known from Miocene deposits of Spain and belongs to a clade with *P. punctatus* and *P. ibericus* (Sanchiz 1998; Sanchiz *et al.* 2002) or may even be conspecific with *P. punctatus* (Rage & Rocek, 2000). One may also assume a pre-Miocene extinction of intermediate linking *Pelodytes* populations between the Caucasus and Iberia. Alternatively, we could invoke an Early Paleocene disjunction of landmasses in the Tethys Ocean (Oosterbroek and Arntzen 1992) to explain the evolution of eastern and western *Pelodytes* lineages.

Taxonomic implications

Based on an evolutionary species concept several taxonomic implications emerge from our analyses:

Table 2. Bayesian molecular dating of times of divergence among lineages of *Pelobates* and *Pelodytes*, based on two molecular clock calibrations for the split *Pelobates cultripes* - *Pb. varaldii*. Much older estimates of divergence times result if calibration II is invoked. If *Pb. varaldii* and *Pb. cultripes* are of early Miocene origin (14 mya), most other lineages should have formed in the middle and late Miocene ca. 20 and 35 mya (distance based estimates). The split between eastern and western *Pb. fuscus* lineages would be of pre-Pleistocene origin (4.4 mya). Caucasian and Iberian *Pelodytes* would have diverged ca. 60 mya. Again, Bayesian time estimates largely disagree with distance based ones.

No.	split	*Calibration I (Messinian Salinity Crisis, 5.33±0.02 mya)*			*Calibration II (Betic Crisis, 16-14 mya)*		
		low 95%CI1	mean	up 95%CI	95% CI_{low}	mean	95% CI_{up}
1	*Pb. cultripes/varaldii* from *Pb. fuscus/syriacus*	5.55	6.79	9.11	14.60	16.95	20.27
2	*Pb. s. transcaucasicus* from *Pb. s. balcanicus/Pb. fuscus*	3.83	5.60	7.84	10.09	14.44	18.12
3	*Pb. s. balcanicus* from *Pb. fuscus*	1.94	4.56	6.82	5.23	11.94	16.69
4	*Pb. fuscus*-West from *Pb. fuscus*-East	0.34	2.30	4.96	1.21	6.43	12.63
5	*Pd. punctatus* from *Pd. ibericus*	0.07	1.15	3.60	0.20	2.92	7.89
6	*Pd. ibericus/punctatus* from *Pd. caucasicus*	0.78	2.87	5.89	2.04	6.66	11.86
7	*S. holbrooki* from *Pelodytes*	2.79	5.78	9.88	6.85	12.36	19.58

(1) The monophyly of *Pb. syriacus* is not unambiguously supported. Non-monophyly would be a possible indication for the existence of two species. The degree of differentiation of *Pb. syriacus* clade 1 (*Pb. s. transcaucasicus*) and *Pb. syriacus* clade 2 (*Pb. s. balcanicus*) would justify assignment of species rank to both of them. This is corroborated by the sympatric occurrence without hybridisation of other *Pelobates* taxa that are differentiated at a similar level: *Pb. cultripes* and *Pb. f. fuscus* in France (Lescure 1984), and *Pb. f. fuscus* and *Pb. syriacus* in different areas (Eiselt 1988, Eggert *et al.* 2006).

(2) The eastern and western genome size types of *Pb. f. fuscus* are not differentiated on a level typical for other *Pelobates* species. Consequently, our molecular data do not support species rank assignment to them as was suggested by Borkin *et al.* (2001, 2003). A final conclusion must await data on the importance of the karyotypical characters for preventing viable and fertile hybridization between these forms. Final taxonomic conclusions in this complex, however, need to await a comprehensive phylogeographic sampling of *Pd. syriacus,* including all four currently accepted subspecies.

Acknowledgements

We are grateful to T. Amann, F. Andreone, W. Bischoff, L. Borkin, M. Franzen, F, Glaw, S. Litvinchuk, U. Joger, A. Kiefer, K.-D. Kühnel, R. Malkmus, B. Samraoui and F.J. Schmidtler for providing tissue samples.

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Received: 13 December 2005
Accepted: 8 September 2006

Appendix. Sample localities, sample sizes (n), haplotypes and voucher numbers; CS = private collection of F.J. Schmidtler, HLMD = Hessisches Landesmuseum Darmstadt, LM = private tissue collection of L. Maxson, UIMNH = University of Illinois Museum of Natural History.

Taxon	Haplotype	N	Locality	Voucher number	GenBank accession no.	
				16S rRNA	12S rRNA	
Pelobates cultripes	Pbc-1	2	Argeles-sur-Mer, F	Mainz	DQ642098	DQ642123
Pelobates cultripes	Pbc-2	1	Lanzada, Pontevedra, Spain	voucher not preserved	DQ642099	DQ642124
Pelobates fuscus fuscus	Pbff-1	2	Oppenheim, Germany	Mainz	DQ642100	DQ642125
Pelobates fuscus fuscus	Pbff-2	2	Oppenheim, Germany	Mainz	DQ642101	DQ642126
Pelobates fuscus fuscus	Pbff-3	1	Stavropol, Russia	voucher not preserved	DQ642102	DQ642127
Pelobates fuscus fuscus	Pbff-4	1	Samy, Ukraine	voucher not preserved	DQ642103	DQ642128
Pelobates fuscus fuscus	Pbff-5	1	Riga, Latvia	voucher not preserved	DQ642104	DQ642129
Pelobates fuscus insubricus	Pbfi	2	Torino, Italia	voucher not preserved	DQ642105	DQ642130
Pelobates syriacus transcaucasicus	Pbst1-1	5	N-Turkey	Mainz		
Pelobates syriacus transcaucasicus	Pbst1-1	1	Kahramanmaras, Turkey	CS 77 P:1		
Pelobates syriacus transcaucasicus	Pbst1-1	1	Aksaray, Turkey	CS 96 P:1	DQ642106	DQ642131
Pelobates syriacus transcaucasicus	Pbst1-1	1	Karacadag, Turkey	CS 86 P:1		
Pelobates syriacus transcaucasicus	Pbst1-1	1	Mercin, Turkey	CS 74 P:1		
Pelobates syriacus transcaucasicus	Pbst1-1	1	Perge, Turkey	voucher not preserved		
Pelobates syriacus transcaucasicus	Pbst1-2	2	Moskkan, Azerbaijan	voucher not preserved	DQ642107	DQ642132
Pelobates syriacus balcanicus	Pbsb2-1	2	Loutros river, NE-Greece	HLMD 207-255	DQ642108	DQ642133
Pelobates syriacus balcanicus	Pbsb2-2	1	Peloponnes, Greece	Mainz	DQ642109	DQ642134
Pelobates varaldii	Pbv	2	Raban, Morocco	Mainz	DQ642110	DQ642135
Pelodytes caucasicus	Pdc	2	Senyura,Viayet Rize Turkey	voucher not preserved	DQ642111	DQ642136
Pelodytes ibericus	Pdi	1	Tarifa, Spain	voucher not preserved	DQ642112	DQ642137
Pelodytes punctatus	Pdp-1	1	Argeles-sur-Mer, France	Mainz	DQ642113	DQ642138
Pelodytes punctatus	Pdp-2	1	Argeles-sur-Mer, France	Mainz	DQ642114	DQ642139
Outgroups						
Alytes cisternasii			Abela, Portugal	Mainz	DQ642115	DQ642140
Discoglossus montalentii			Porto, Corsica, France	voucher not preserved	DQ642116	DQ642141
Leptobrachium montanum			Kinabalu/Borneo	voucher not preserved	DQ642117	DQ642142
Leptobrachium gununngensis			Kinabalu/Borneo	voucher not preserved	DQ642118	DQ642143
Leptolalax arrayai			Kinabalu/Borneo	voucher not preserved	DQ642119	DQ642144
Leptolalax pictus			Kinabalu/Borneo	voucher not preserved	DQ642120	DQ642145
Megophrys baluensis			Kinabalu/Borneo	voucher not preserved	DQ642121	DQ642146
Scaphiopus holbrooki			North America	LM 3070	X86294	X86226
Pipa parva			Pet trade	voucher not preserved	DQ642122	DQ642147
Leiopelma			Maud Island, New Zealand	LM 3174	X86309	X86241
Ascaphus truei			Oregon, Wallowa Mountains	UIMNH 94103-06	X86293	X86225
Triturus vulgaris			not known	not available	U04705	U04704