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Testing alternative vicariance scenarios in Western Mediterranean discoglossid frogs

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Abstract

Dated molecular phylogenies are often used to interpret evolutionary history with respect to paleogeographic events. Where more than one interpretation is possible, it is desirable but difficult to assess the alternatives in an objective manner. The present work demonstrates a formalized method for testing molecular clock calibrations and biogeographic scenarios based on them. We assessed the plausibility of several previously published biogeographic hypotheses, using the frog genera *Alytes*, *Discoglossus*, and *Bombina* as model groups. Our data set comprised ca. 900 bp of partial mitochondrial 16S and 12S rRNA gene sequences (both genes evolved in a clock-like manner across genera) from nearly all the species and subspecies in the three genera. We tested several calibrations of a molecular clock, which resulted in competing temporal settings for the evolution of taxa. Although only one scenario was in complete accordance with paleogeographic data, statistical testing did not reject the alternatives. Limitations encountered with the present approach may be overcome by more comprehensive analyses in future.

Keywords: Phylogeny; Molecular clock; 16S; 12S; Amphibia; Discoglossidae; Bombinatoridae; Paleobiogeography; Fisher's exact test

1. Introduction

Estimating dates of lineage divergence is a common aim in molecular phylogenetic studies (e.g., Heckman et al., 2001; Hedges et al., 1996; Wray et al., 1996). If molecular distances accumulate at a uniform rate across different taxa, a known date of divergence for a given pair of taxa can be used to estimate divergence times for other nodes within a molecular phylogeny (Zuckerkandl and Pauling, 1962, 1965). To account for uncertainties associated with the assumption of rate constancy, Hillis et al. (1996) recommended that calibrations of such a "molecular clock" should refer to a regression function based on multiple calibration points. More recently, methods have been developed in a framework of likelihood and Bayesian theory that aim to relax the assumption of rate constancy by modeling the evolution of rates between multiple calibration points (Rambaut and Bromham, 1998; Sanderson, 1997; Thorne et al., 1998).

All of these methods can be expected to work well where reliable a priori information on divergence times is available. Unfortunately, this criterion will rarely be fulfilled where historical processes are concerned. Potential calibration points often refer to hypotheses, which may or may not be true. If both true and flawed calibration points are incorporated into one calibration, severely biased or confounded results will be the likely outcome. In the present study, we tried to account for this problem by using individual calibration points separately to establish independent calibrations. Based on the assumption that a correct calibration should yield plausible divergence time estimates, we developed a biogeographic scenario for each calibration and assessed its fit to the paleogeographic record. We applied a formalized procedure to evaluate the plausibility of each biogeographic scenario, thereby assessing the degree of confidence that should be placed in the respective cali-

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brations. Our molecular data set from three clades of frogs showed no significant signal of rate heterogeneity among lineages, which was an important prerequisite for this approach.

The Mediterranean region is well suited for testing biogeographic hypotheses. It harbors a rich endemic fauna and flora that has evolved through a complex interplay of geological (e.g., orogenic) and paleoclimatic vicariance events (Blondel and Aronson, 1999). The emergence of mountain chains and sea straits directly isolated populations, while islands and peninsulas provided important refugia during phases of habitat retraction (see Appendix A for a compilation of relevant paleogeographic events).

Dispersal and vicariance have been identified as the principal mechanisms responsible for the formation of biogeographic patterns (e.g., Stace, 1989). Amphibians are favorable model organisms to study the effects of vicariance, since their dispersal across marine barriers is limited due to a low salinity tolerance (Stebbins and Cohen, 1995). The discoglossid frog genera *Discoglossus* and *Alytes* are distributed throughout the Western Mediterranean, including the European and African continents and several islands (Fig. 1). Both genera comprise a set of endemic species and subspecies, and several independent biogeographic explanations for their evolution have been published.

Lanza (1973, cited in Lanza et al., 1987) explained the differentiation of all *Discoglossus* taxa by isolation of their ancestor in separate glacial refuges (3.4–0.01 million years ago = MYA; see Appendix A for paleogeographic references), a view that was contradicted by later studies when genetic data became available. The hypotheses of Capula et al. (1985) referred to much older vicariance events. They explained the basal split within *Discoglossus* by the isolation of Corsica from continental Europe at the end of the Messinian Salinity Crisis (5.33 MYA) or, alternatively, by the separation of the Corsica–Sardinia microplate from the continent (29 MYA). Furthermore, Busack (1986) suggested a Messinian (5.33 MYA) separation of Iberian and African *Discoglossus*, while García-París and Jockusch (1999) explained the divergence of the two Iberian taxa *Discoglossus galganoi* and *Discoglossus jeanneae* by the reopening of the Betic sea strait (ca. 7 MYA). In their scenario of *Alytes* evolution, Arntzen and García-París (1995, 1997) used the Messinian Salinity Crisis (5.33 MYA) to explain a split of Balearic and Iberian lineages. Alternative hypotheses concerning *Discoglossus* and *Alytes* evolution were given by Vences and Glaw (1996) and Altaba (1997). According to Szymura (1993), the differentiation of European *Bombina*, the third genus in this study, was accounted for by its isolation in glacial refuges (3.4–0.01 MYA) in south and south-east Europe (also see Arntzen, 1978; Mertens, 1928). No consensus has yet been reached regarding the relationships between

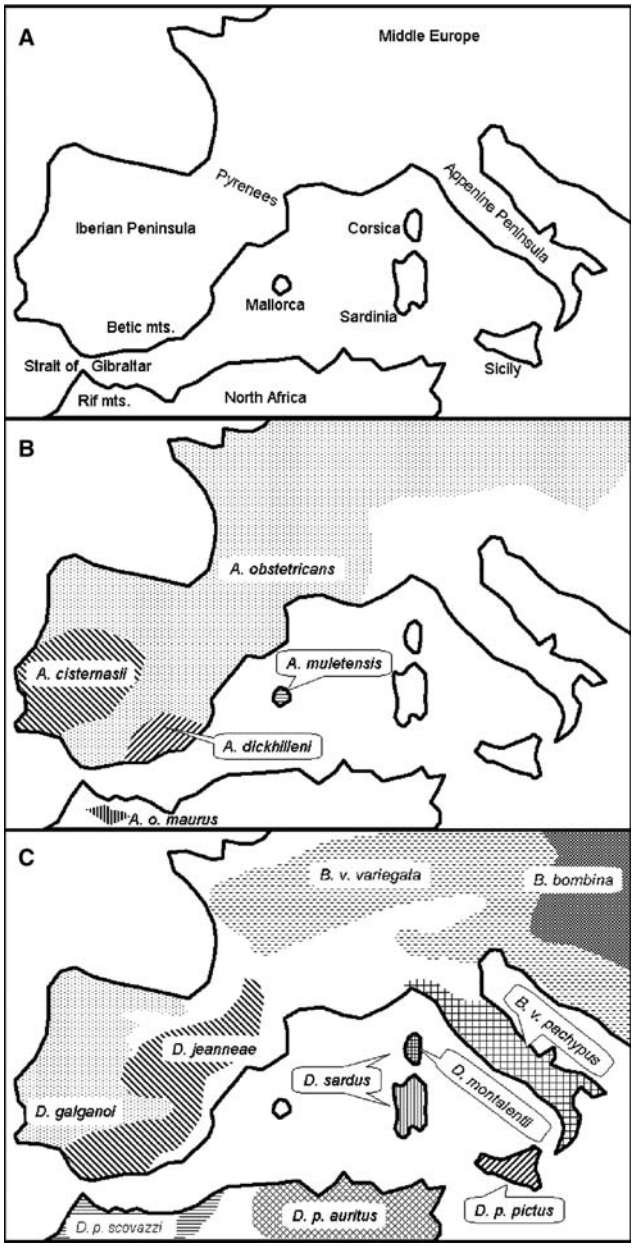

Fig. 1. Present distribution of discoglossid frogs in the Western Mediterranean: (A) important landmarks; (B) distribution of *Alytes* (simplified after Arntzen and García-París, 1995); (C) distribution of *Discoglossus* and *Bombina* (simplified after García-París and Jockusch, 1999; Gasc et al., 1997).

Discoglossus, *Alytes*, and *Bombina*, with conflicting evidence from morphological (Ford and Cannatella, 1993) and molecular analyses (Hay et al., 1995).

Here, we used several current biogeographic hypotheses for independent calibrations of a molecular clock. This way, we were able to (i) test for congruence between hypotheses that have been independently put forward for individual genera, (ii) find a unifying hypothesis that includes all genera, and most importantly, (iii) integrate information across genera to assess the consistence of

calibration attempts and scenarios with the paleogeographic record. The latter has implications for biogeographic hypothesizing and molecular clock issues.

2. Materials and methods

2.1. Samples

We studied 51 specimens that comprise almost all currently acknowledged species and subspecies of *Alytes*, *Discoglossus*, and Western Palearctic *Bombina* (Appendix B). Only the eastern Iberian *Alytes obstetricans almogavarii* was not represented. Based on an interfamilial anuran phylogeny (Hay et al., 1995), we chose several taxa for hierarchical outgroup comparison: *Pelobates cultripes*, *Leiopelma hamiltoni*, *Pipa parva*, *Rana temporaria*, and *Bufo asper*. Sequence data of *R. temporaria* (12S: AF 124103 and 16S: AF124135), *B. asper* (12S: U52733 and 16S: AF 124109), and *L. hamiltoni* (12S: X86241 and 16S: X86309, X86275) were obtained from GenBank. DNA from other taxa was extracted from either fresh or ethanol-preserved tissue samples.

2.2. DNA sequencing

DNA was extracted using the "High Pure PCR Template Preparation Kit" (Roche Diagnostics) following standard protocols. Two mitochondrial gene fragments were amplified using the following primers. GenBank Accession Nos. are given in Appendix B.

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) were used to amplify a ca. 580 bp section of the mitochondrial 16S rRNA gene homologous to positions 3976–4554 of the *Xenopus laevis* mitochondrial genome (GenBank Accession No. M10217; 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) were used to amplify a ca. 490 bp section of the mitochondrial 12S rRNA gene homologous to positions 2510–2997 of the *X. laevis* mitochondrial genome (Roe et al., 1985).

PCR products were purified using the "High Pure PCR Product Purification Kit" (Roche diagnostics). We sequenced single-stranded fragments on an ABI 377 automatic sequencer using standard protocols.

Sequences were aligned using Clustal X (Thompson et al., 1997). Some hypervariable regions were too variable to be aligned across different genera. We therefore compiled four different alignments (a–d) according to different questions: (a) all three genera, with hypervariable regions omitted, (b) genus *Discoglossus*, (c) genus *Alytes*, and (d) genus *Bombina*. We used alignment (a) to test and apply a molecular clock across genera. We used alignments (b)–(d), with greater numbers of base positions included (see Table 2), to achieve the best possible resolution within genera. All alignments are available from the authors upon request.

2.3. Phylogenetic analyses

Among identical haplotypes, we excluded all but one from phylogenetic analyses. We investigated the degree of heterogeneity between the two gene fragments using the incongruence length differential (ILD) test of (Farris et al., 1994; see also Huelsenbeck et al., 1996; Whelan et al., 2001) as implemented in PAUP*, version 4.0b10 (Swofford, 2001) with settings as follows: 100 replicates, heuristic search using the tree bisection and reconnection (TBR) branch swapping algorithm. This test is used to determine whether significantly more evolutionary steps are required when data partitions are pooled rather than analyzed separately. Prior to model assessment, we performed χ^2 tests for base distribution across sequences in order to rule out non-homogeneous base compositions. Using a hierarchical likelihood ratio test, we tested the goodness-of-fit of nested substitution models for homogeneous data partitions of ingroup taxa. Modeltest version 3.04 (Posada and Crandall, 1998) was used to calculate the test statistic $\delta = 2\log \Lambda$ with Λ being the ratio of the likelihood of the null model divided by the likelihood of the alternative model (for details see Huelsenbeck and Crandall, 1997). We used the chosen models for further analyses.

Data were subjected to three different methods of phylogenetic reconstruction: (i) neighbor-joining (NJ) (Saitou and Nei, 1987) using the selected substitution model; (ii) maximum parsimony (MP) with gaps excluded (following Swofford et al., 1996); transitions and transversions given equal weight (see Broughton et al., 2000; Rosenberg and Kumar, 2001), heuristic search with the TBR branch swapping algorithm; and (iii) maximum likelihood (ML) based on the selected substitution model. NJ and MP analyses were run with PAUP*, version 4.0b10 (Swofford, 2001). We tested the robustness of the NJ and MP tree topologies using the bootstrap method (Felsenstein, 1985), with 2000 replicates each (Hedges, 1992). Following Hillis and Bull (1993), we used bootstrap values as a measure of the probability that a recovered group represents a true clade. Due to computational constraints, the support values for ML were calculated by quartet puzzling (QP) with 2000 puzzling steps, using TREE-PUZZLE (Schmidt et al., 2000).

2.4. Test for substitution rate constancy

We performed a test for substitution rate constancy (molecular clock test) using TREE-PUZZLE (Schmidt

et al., 2000) on alignment (a) with *P. cultripes* as the outgroup. This test compares the log-likelihood of the most likely tree with and without a molecular clock enforced. We used the Tamura–Nei substitution model (Tamura and Nei, 1993) with the gamma distribution shape parameter set to $\alpha = 0.54$ according to the Modeltest result. Base frequencies ($\pi_A = 29.9\%$, $\pi_C = 26.5\%$, $\pi_G = 22.3\%$, $\pi_T = 21.4\%$) were estimated from the data.

2.5. Molecular distances and their confidence intervals

Molecular distances for the application of a molecular clock were computed between group means with standard deviations for 2000 bootstrap replications using MEGA version 2.1 (Kumar et al., 2001). Again, alignment (a) and the Tamura–Nei substitution model with $\alpha = 0.54$ were used. In this context, the bootstrap approach provides an estimate that can account for stochastic variation in distance evolution (Nei and Kumar, 2000), which is thought to be a major source of error in molecular clock applications (Hillis et al., 1996). We calculated 95% confidence intervals for genetic distances, and corresponding divergence time estimations, as the mean $\pm$ 1.96 SD. Although it is thought to be impossible to satisfy all the conditions of a perfect molecular clock model in the real world (Hillis et al., 1996), use of this method is justified in the present case because (i) there was no significant signal for rate heterogeneity among taxa (see results) and (ii) potential inaccuracy of confidence limits would affect all alternative hypotheses in our comparative approach. The comparison among hypotheses would thus still be valid.

2.6. Test for plausibility of scenarios

We used the following formalized procedure to assess the plausibility of alternative paleobiogeographic scenarios.

1. *Phylogenetic splits*. Identify splits that are suitable for biogeographic interpretation. These are well-supported clades that (i) split into two well-supported subclades or (ii) represent polytomies that can be interpreted as subsequent, temporally close splits.
   We considered nodes with NJ, MP, and QP support values of $> 70\%$ as well supported (Hillis and Bull, 1993; but see limitations listed therein). Several splits narrowly missed the 70% criterion, but were still considered suitable for interpretation as they were in accordance with previously published and well-supported allozyme and immunological data (split 4 in Table 3: Capula et al., 1985; splits 7 and 8: Arntzen and García-París, 1995, 1997; split 9: Maxson and Szymura, 1979, 1984; Szymura, 1988).
2. *Paleogeographic events*. Identify paleogeographic events that may have caused vicariance of the taxa in question. Such events are the flooding of land bridges, disruption of geologic formations, the rise of mountain chains, or climatic deterioration.
   See Appendix A for a compilation of relevant events in the context of this study.
3. *Molecular clock*. Select events that are suitable for calibration of a molecular clock. These are well-dated paleogeographic events that can explain phylogenetic splits in a spatially plausible way (see below for the assessment of spatial plausibility). Date these splits a priori and use each of them to establish an independent calibration of a molecular clock. For each calibration, calculate divergence times and confidence intervals across the remaining splits. We used four alternative calibrations with reference to previously published biogeographic hypotheses (Table 1).
4. *Temporal compatibility*. For each estimated divergence time derived from step (3), identify temporally matching paleogeographic events within its confidence limits.
5. *Spatial plausibility*. Where estimated divergence times are matched by paleogeographic events, consider the vicariant areas and compare them to the present-day distribution of the lineages in question. Accept plausibility where (i) representatives of those lineages live near or within the vicariant ranges and (ii) dispersal events that have to be assumed to explain present species ranges are deemed possible in the light of paleogeographic data.
6. *Internal consistency*. While each split was considered separately under (4) and (5), interpretations for

Table 1
Paleogeographic events and phylogenetic splits used for molecular clock calibrations

No.	Paleogeographic event	Time (MYA)	Phylogenetic split with reference
I	End of the Messinian Salinity Crisis	5.33 (Krijgsman et al., 1999)	*D. p. scovazzi–D. galganoi*/*D. jeanneae* (see Busack, 1986)
II	End of the Messinian Salinity Crisis	5.33 (Krijgsman et al., 1999)	*D. montalentii*–other *Discoglossus* (Capula et al., 1985)
III	End of the Messinian Salinity Crisis	5.33 (Krijgsman et al., 1999)	*A. dickhilleni–A. muletensis–A. o. maurus* (see Arntzen and García-París, 1995)
IV	Separation of the Corsica-Sardinia microplate from the continent	29 (Bellon et al., 1977; Orsini et al., 1980)	*D. montalentii*–other *Discoglossus* (Capula et al., 1985)

Dates are given in million years ago (MYA).

different splits must be mutually compatible to be included in one biogeographic scenario. For each calibration, identify the maximum number of vicariance explanations that can be included in a scenario without internal contradictions. Ensure that (i) the temporal sequence of proposed vicariance events matches the cladogenetic sequence of splits and (ii) spatial interpretations of individual splits do not contradict each other. If potential explanations for two splits are mutually exclusive, one split has to be declared "unexplained." It makes no difference which explanation is dismissed, since the statistical test used in step (7) considers only the total number of explained and unexplained splits.

7. *Statistical testing*. Use Fisher's exact test to determine in pairwise comparisons whether the best-fitting scenario explains significantly more splits than its alternatives. When comparing two scenarios, consider the null hypothesis (H_0) that neither alternative is true. In this case split events may be explained by chance, which leads to the prediction that explained split events should be randomly distributed across scenarios. According to the alternative hypothesis, one scenario does at least partly correspond to the historical truth. Hence an increased number of explained split events can be predicted in this scenario. Fisher's two-tailed exact test yields the exact probability P that a difference at least as extreme as the one observed between two scenarios occurs by chance, given the total numbers of explained and unexplained splits. Adjust the critical P-level for multiple testing by a sequential Bonferroni correction and reject H_0, where $P < 0.05$.

3. Results

The test for partition homogeneity revealed no conflicting phylogenetic signals between the two gene fragments (P values of alignments were as follows: $P_{(a)} = 0.57$; $P_{(b)} = 0.5$; $P_{(c)} = 0.24$; $P_{(d)} = 0.33$). Consequently, both gene fragments were combined in all alignments for further analyses. Standard sequence statistics for all alignments are given in Table 2. Transitions (ti) by far outnumbered transversions (tv), indicating only weak saturation of transitions in the chosen genes, which was confirmed by visual inspection of a plot of uncorrected distances for transitions as a function of TrN distances (data not shown). We accepted this minor degree of saturation as background noise in all analyses. Nucleotide frequencies did not differ significantly from a homogeneous distribution. Substitution models as chosen by hierarchical likelihood ratio tests are given in Table 2.

3.1. Phylogenetic relationships

Monophyly of genera was confirmed by support values of >95% each (Figs. 2–5), with lack of well-supported resolution of their inter-generic relations. The genus *Discoglossus* showed a well-resolved position of *Discoglossus montalentii* standing basal to all other species (Fig. 3). Sister-group relations appeared between *Discoglossus pictus scovazzi* and a *D. galganoi*/*D. jeanneae* clade, and between *Discoglossus sardus* and an unresolved *Discoglossus pictus pictus*/*Discoglossus pictus auritus* clade. Thus, *Discoglossus pictus* was non-monophyletic. Within *Alytes*, a basal split separated *Alytes*

Table 2
Standard sequence statistics, tests for homogeneity of base frequencies among ingroup taxa (χ^2-test), and substitution models as chosen using hierarchical likelihood ratio tests for four different alignments (a = all three genera, b = *Discoglossus*, c = *Alytes*, d = *Bombina*); bp, number of analyzed base pairs (including gaps); VS, variable sites; PI, parsimony-informative sites; π_A, π_G, π_C, π_T = empirical base frequencies; ti/tv ratios are given for ingroup taxa only; I = proportion of invariable sites, α = gamma shape parameter

	Alignment (a)	Alignment (b)	Alignment (c)	Alignment (d)
Standard sequence statistics				
bp	868	909	879	927
VS	343	217	206	179
PI	233	105	73	78
π_A	0.300	0.299	0.296	0.304
π_G	0.222	0.218	0.220	0.218
π_C	0.262	0.264	0.273	0.262
π_T	0.216	0.219	0.211	0.216
ti/tv ratio	3.142	4.426	3.349	3.132
Homogeneity test				
χ^2-value	11.21	0.95	1.12	3.15
Degrees of freedom	102	36	21	42
p value	1	1	1	1
Substitution model test				
Chosen model	TrN + *I* + *G* (Tamura and Nei, 1993)	HKY + *I* (Hasegawa et al., 1985)	HKY + *I* (Hasegawa et al., 1985)	HKY + *I* + *G* (Hasegawa et al., 1985)
I	0.5457	0.8293	0.8506	0.8105
α	0.5394	—	—	0.7425

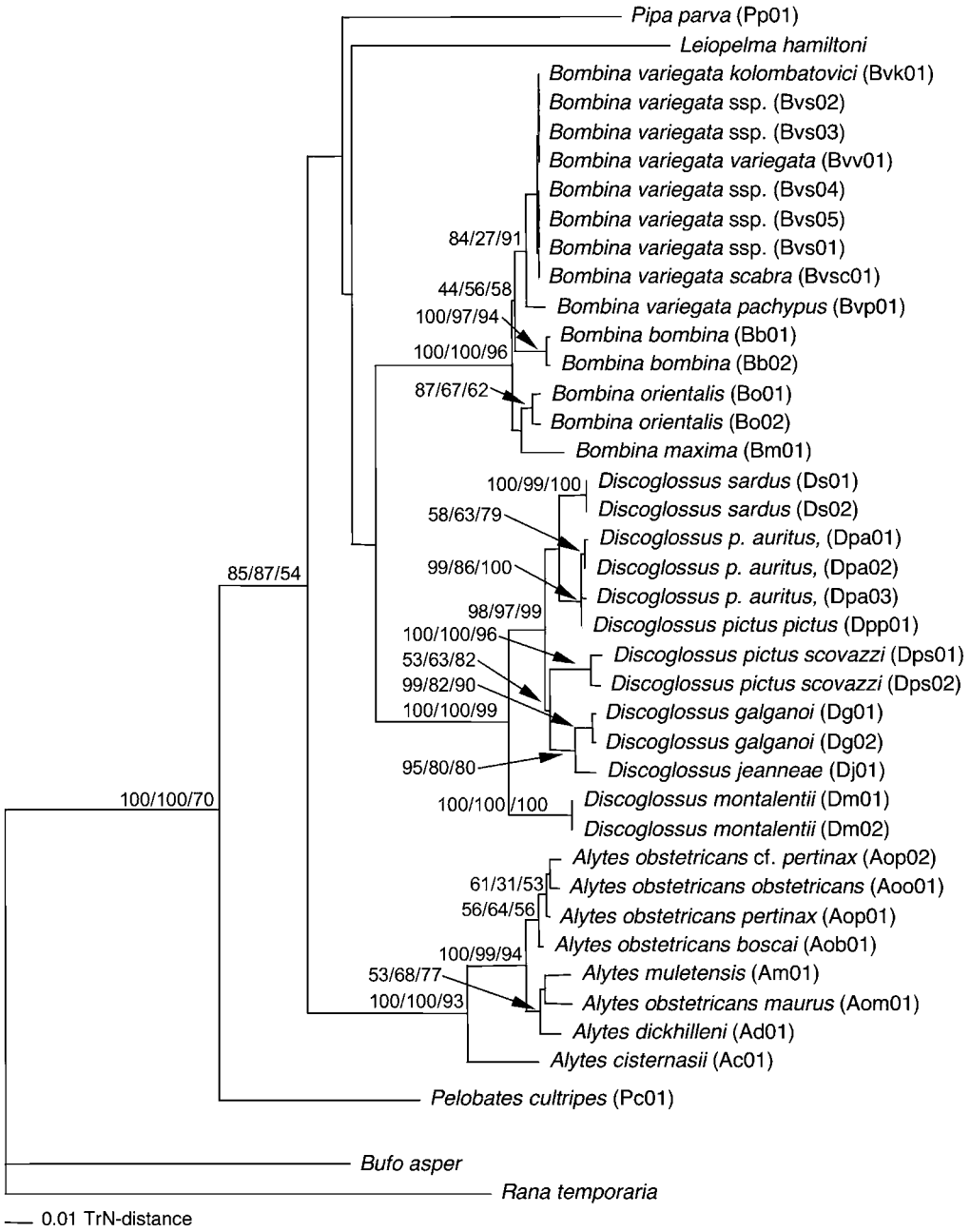

Fig. 2. NJ tree based on 868 bp of 16S and 12S (alignment a) with *Rana temporaria* defined as the outgroup; NJ/MP/QP support values (%) for 2000 bootstrap replicates, respectively, puzzling steps, are given for nodes that occurred in all applied methods of tree reconstruction.

cisternasii from all other taxa (Fig. 4), which again split into two sub-clades. One comprised *Alytes muletensis*, *Alytes obstetricans maurus*, and *Alytes dickhilleni*, the other comprised all other *A. obstetricans* subspecies. Consequently, *A. obstetricans* appeared to be non-monophyletic. Within *Bombina*, NJ and QP results showed grouping of the Asian taxa (*Bombina orientalis* and *Bombina maxima*, Fig. 5). Moreover, there was a tendency for sister-group relations between *Bombina bombina* and *Bombina variegata*. Within the latter, *Bombina variegata pachypus* was placed basal to the remaining subspecies.

3.2. Calibration and application of a molecular clock

The hypothesis of rate constancy within alignment (a) was not rejected by the molecular clock test. Under the Tamura–Nei model of evolution with no molecular clock enforced, the likelihood ($\log L = -3401.06$) was not significantly better at the 5% level than with the molecular clock enforced ($\log L = -3416.86$). Therefore, calculation of divergence times from genetic distances across genera was justified.

Calibrations based on different splits yielded alternative temporal settings for the evolution of taxa.

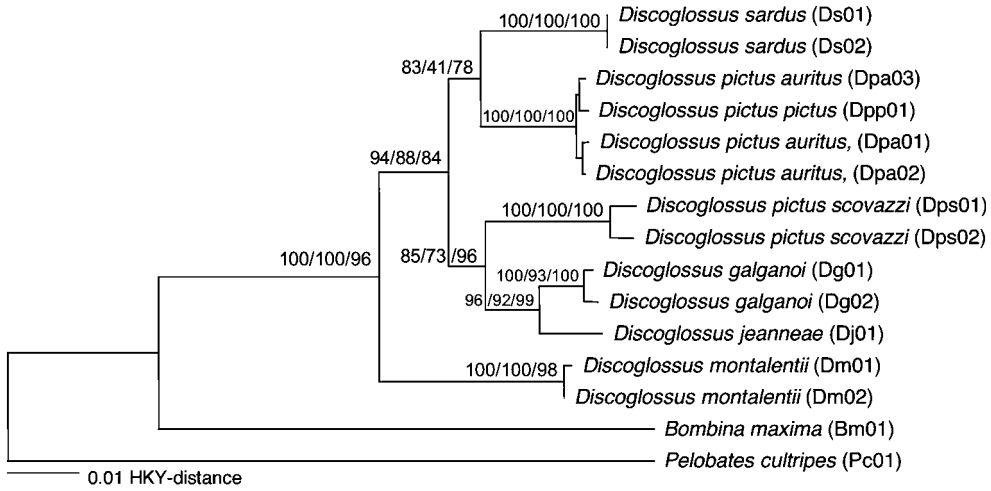

Fig. 3. NJ tree of *Discoglossus*, based on 909 bp of 16S and 12S (alignment b) with *Pelobates cultripes* defined as the outgroup; NJ/MP/QP support values (%) for 2000 bootstrap replicates, respectively, puzzling steps, are given for nodes that occurred in all applied methods of tree reconstruction.

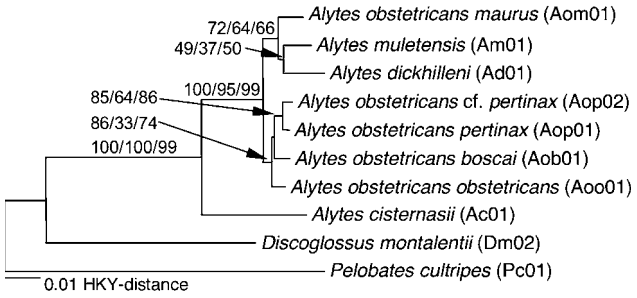

Fig. 4. NJ tree of *Alytes*, based on 879 bp of 16S and 12S (alignment c) with *Pelobates cultripes* defined as the outgroup; NJ/MP/QP support values (%) for 2000 bootstrap replicates, respectively, puzzling steps, are given for nodes that occurred in all applied methods of tree reconstruction.

Tamura–Nei distances (according to the chosen substitution model) and estimated divergence times are given in Table 3 for selected taxa.

3.3. Plausibility of scenarios

Regarding alternative calibrations, varying numbers of estimated divergence times could be explained by known paleogeographic events (Table 3). The scenario based on calibration III showed the maximum degree of plausibility with no unexplained divergence times.

Fisher's two-tailed exact test yielded but one significant result for pairwise comparisons between the best-fitting scenario and its alternatives (III vs. I, $P = 0.2$;

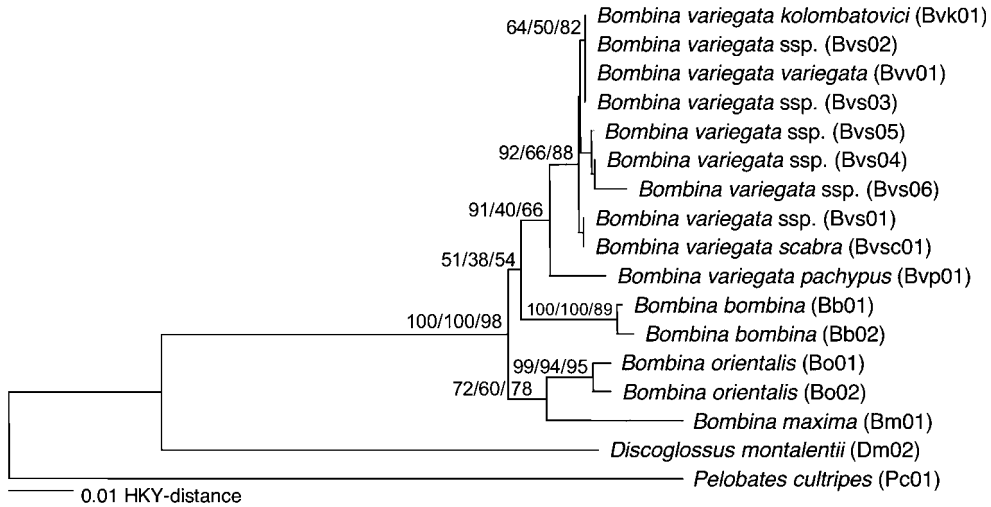

Fig. 5. NJ tree of *Bombina*, based on 927 bp of 16S and 12S (alignment d) with *Pelobates cultripes* defined as the outgroup; NJ/MP/QP support values (%) for 2000 bootstrap replicates, respectively, puzzling steps, are given for nodes that occurred in all applied methods of tree reconstruction.

Table 3
Alternative calibrations of a molecular clock based on alignment (a); molecular distances and estimated divergence times are given ±1.96 SD; splits used for calibration are highlighted in gray; spatio-temporally matching paleogeographic events (PGE = numbers according to Appendix A) that may have caused the respective vicariance are assigned to phylogenetic splits

No.	Phylogenetic split	TrN gamma distance	Time since divergence (MY)			
			Calibration I (rate: 0.0064 /MY)	Calibration II (rate: 0.0099 /MY)	Calibration III (rate: 0.0036 /MY)	Calibration IV (rate: 0.0018 /MY)
1.	*D. montalentii*–other *Discoglossus*	0.0530 ± 0.0157	8.31 ± 2.46 PGE = 7	5.33 PGE = 11	14.87 ± 4.40 PGE = 2	29.00 PGE = 1
2.	*D. galganoi*/*D. jeanneae*/ *D. p. scovazzi*–*D. sardus*/*pictus*	0.0350 ± 0.0098	5.49 ± 1.54 PGE = 11	3.52 ± 0.99 PGE = ?[a]	9.82 ± 2.75 PGE = 8	19.15 ± 5.36 PGE = ?[b]
3.	*D. pictus scovazzi*/*D. galganoi*/ *D. jeanneae*	0.0340 ± 0.0118	5.33 PGE = 11	3.42 ± 1.18 PGE = ?[a]	9.54 ± 3.30 PGE = 6, 9	18.60 ± 6.43 PGE = 3
4.	*D. p. pictus*/*auritus*–*D. sardus*	0.0200 ± 0.0098	3.14 ± 1.54 PGE = ?[a]	2.01 ± 0.99 PGE = ?[a]	5.61 ± 2.75 PGE = 10	10.94 ± 5.36 PGE = 10
5.	*D. jeanneae*–*D. galganoi*	0.0150 ± 0.0078	2.35 ± 1.23 PGE = 12	1.51 ± 0.79 PGE = 12	4.21 ± 2.20 PGE = 12[c]	8.21 ± 4.29 PGE = 6, 9
6.	*A. cisternasii* other *Alytes*	0.0580 ± 0.0176	9.09 ± 2.77 PGE = ?[d]	5.83 ± 1.77 PGE = ?[e]	16.27 ± 4.95 PGE = 3 or 4	31.74 ± 9.65 PGE = ?[f]
7.	*A. muletensis*/*A. o. maurus*/ *A. dickhilleni* other *A. obstetricans* ssp.	0.0220 ± 0.0078	3.45 ± 1.23 PGE = 12[g]	2.21 ± 0.79 PGE = 12[g]	6.17 ± 2.20 PGE = 6	12.04 ± 4.29 PGE = 3[h]
8.	*A. muletensis*–*A. dickhilleni*–*A. o. maurus*	0.0190 ± 0.0098	2.98 ± 1.54 PGE = ?[a]	1.91 ± 0.99 PGE = ?[a]	5.33 PGE = 11	10.40 ± 5.36 PGE = 5, 6
9.	*B. variegata*–*B. bombina*	0.0210 ± 0.0098	3.29 ± 1.54 PGE = 12	2.11 ± 0.99 PGE = 12	5.89 ± 2.75 PGE = 12	11.49 ± 5.36 PGE = ?[i]
Explained versus unexplained time estimations			5:3	3:5	8:0	5:3

[a] PGE = 12 fits temporally but not spatially. While sea-level subsidence up to 150 m below the present level occurred during ice ages (Andersen and Borns, 1994; Wilson et al., 1999), marine barriers between the ranges in question are presently more than 200 m deep.

[b] PGE = 2, 3, 4, and 5 fit temporally but not spatially.

[c] PGE = 6 fits, but is not consistent with the explanation given for split 3, calibration III (see discussion). PGE = 7 and 11 fit temporally but not spatially.

[d] PGE = 6, 7, 8, and 10 fit temporally but not spatially.

[e] PGE = 6, 7, and 10 fit temporally but not spatially. PGE = 11 fits, but is inconsistent with the explanation given for split 7, calibration II.

[f] PGE = 1 and 2 fit temporally but not spatially.

[g] PGE = Glacial events may seem like a unsatisfying explanation for this split, since it remains unclear how the descendent lineages could have reached their current ranges across sea barriers. However this argument concerns split 8 and should not be used more than once to dismiss explanations for independent split events.

[h] In accordance with Altaba (1997).

[i] PGE = 2, 3, 4, 5, 6, 7, 8, 10, 11, and 12 fit temporally but not spatially.

III vs. II, $P = 0.03$; and III vs. IV, $P = 0.2$). This result became non-significant when the critical value $\alpha = 0.05$ was Bonferroni-corrected for multiple tests.

4. Discussion

Alternative molecular clock calibrations led to competing scenarios for the evolution of Western Mediterranean discoglossid frogs. Some currently accepted and plausible hypotheses for the genera *Alytes* and *Discoglossus* proved to be incompatible. One scenario, based on calibration III, completely fitted the paleogeographic data and therefore has considerable appeal. It deserves to be described in more detail below, although statistical testing did not reject the alternatives.

4.1. Scenario according to calibration III

Calibration III was based on a hypothesis of Arntzen and García-París (1995, 1997): *A. dickhilleni*, *A. muletensis* and *A. o. maurus* may have become isolated in their current ranges 5.33 MYA after dispersal of an ancestral lineage through a desiccated Mediterranean basin (Fig. 6E). The remaining divergence times within *Alytes* can also be explained in accordance with the

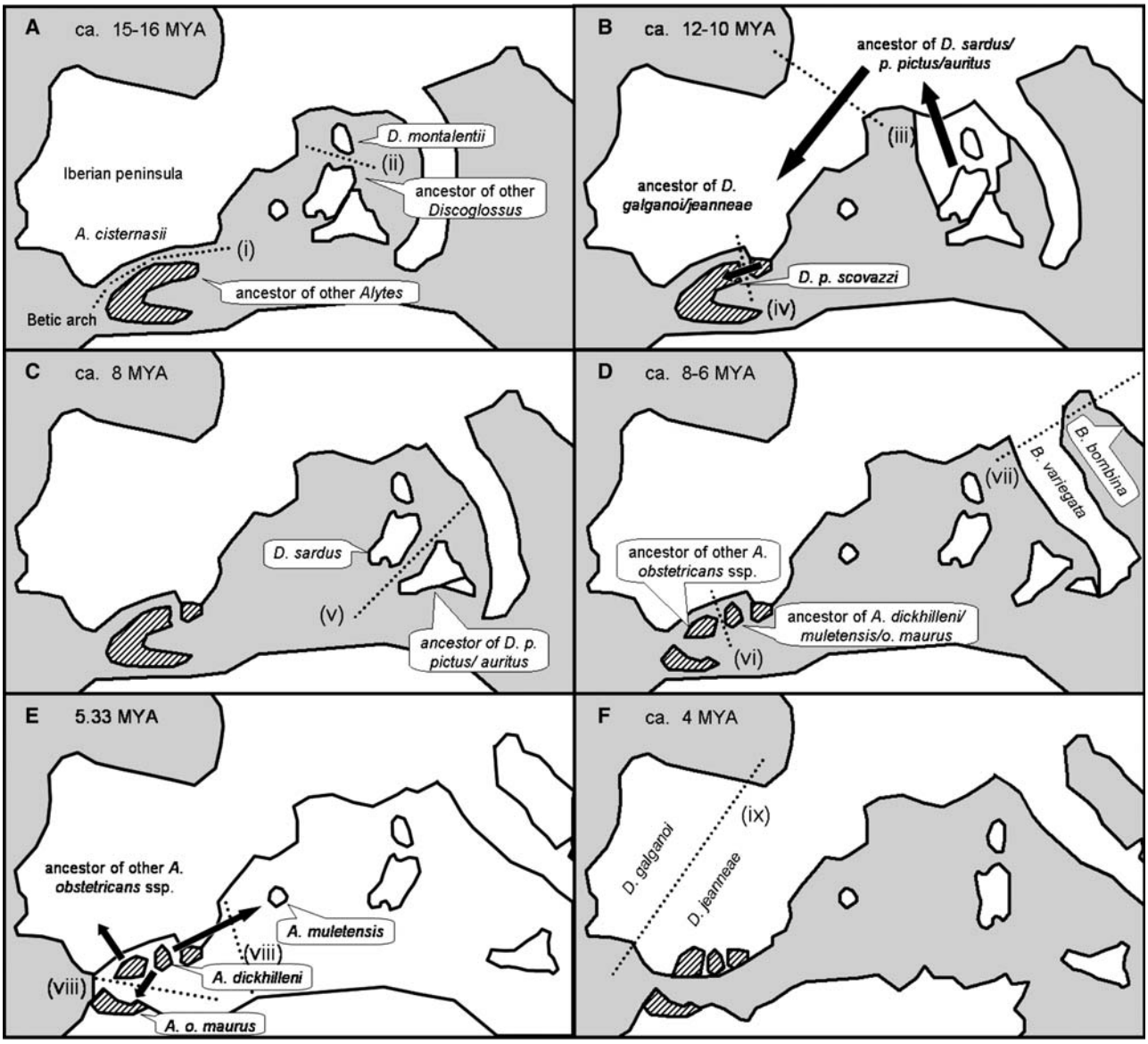

Fig. 6. Paleobiogeographic scenario based on calibration III; arrows indicate assumed dispersal routes; dotted lines denote the following vicariance events: (A) (i) opening of the Betic sea strait and (ii) Corsica-Sardinia rift; (B) (iii) structuring of the Neo-Pyrenees and (iv) fragmentation of the Betic region (hatched); (C) (v) separation of Sardinia from the Calabro-Peloritan massif; (D) (vi) further fragmentation of the Betic region and (vii) restriction to glacial refugia; (E) (viii) sea barriers occurring after the end of the Messinian Salinity Crisis; (F) (ix) restriction to glacial refugia; paleogeographic events are compiled from sources referenced in Appendix A; see text for details.

above-mentioned authors: opening of the Betic sea strait separated an ancestral lineage that inhabited the insular Betic region (Fig. 6A), and fragmentation of the Betic region isolated *A. obstetricans*, which later colonized the continent (Figs. 6D and E). Within *Discoglossus*, an estimated age of ca. 15 million years (MY) for *D. montalentii* matches the Corsica-Sardina rift 20–15 MYA (Fig. 6A; Alvarez et al., 1974; Orsini et al., 1980), rather than the separation of the Corsica-Sardinia microplate from the continent ca. 29 MYA (Bellon et al., 1977) as suggested by Capula et al. (1985). In this case, and based on the phylogenetic relationships within the genus, one has to assume that a Sardinian sister taxon of *D. montalentii* later colonized the continent (Fig. 6B) via a land bridge (Orszag-Sperber et al., 1993). Divergence of an Iberian lineage (*D. p. scovazzi*, *D. galganoi*, and *D. jeanneae*; Fig. 6B) may have been caused by the structuring of the Neo-Pyrenees around 10 MYA. The separation of the African *D. p. scovazzi* from the Iberian taxa (*D. galganoi*/*D. jeanneae*) may be explained by the fragmentation of the Betic region (Fig. 6B). In this context it has to be noted that secondary contact between the Betic region and the continent is likely to have occurred ca. 10–7 MYA due to crustal narrowing in the eastern Betic sea strait (Lonergan and White, 1997; Weijermars, 1988). Based on the previous explanation, we have to assume an Iberian range of the *D. galganoi*/*D. jeanneae* ancestor. Hence the split between *D. galganoi* and *D. jeanneae* can be accounted for by isolation in separate glacial refuges (Fig. 6F), rather than by tectonics in the Betic region (see García-París and Jockusch, 1999).

The divergence of *D. p. pictus*/*D. p. auritus* and *D. sardus* matches the separation of the Calabro-Peloritan massif (presently Sicily and south Italy) from Sardinia (Duermeijer et al., 1998; Fig. 6C). The split between *B. bombina* and *B. variegata* can be explained in accordance with Szymura (1993) by glaciations and their effect on the overall climate in the Northern Hemisphere (Fig. 6D).

4.2. Comparison with documented rates of protein evolution

To further consider the plausibility of alternative scenarios, an independent criterion comes from the comparison of estimated rates of protein evolution based on calibrations I–IV with previously published rates.

According to Avise and Aquadro (1982), vertebrate protein evolution rates range from 0.7 MY/1 D_{Nei} (mammalians) up to 18 MY/1 D_{Nei} (reptiles). For amphibians, rates between 8 and 10 MY/1 $D*_{Nei}$ (Beerli et al., 1996) and 14 MY/1 D_{Nei} (Maxson and Maxson, 1979) have been reported ($D*_{Nei}$ represents Nei's, 1972 genetic distance, D_{Nei}, as modified by Hillis, 1984), which makes no relevant difference in this case; Beerli et al., 1996). Using documented D_{Nei} distances within *Discoglossus* (Lanza et al., 1987), *Alytes* (Arntzen and García-París, 1995), and *Bombina* (Szymura, 1983), rates of allozyme evolution can be estimated based on calibrations I–IV (Table 4).

Differences between genera may indicate that (i) D_{Nei} distances did not evolve at a constant rate, or (ii) methodological differences existed between laboratories (Veith, 1996). In any case, rates of allozyme evolution based on calibration IV are outside the range of documented values, which argues against calibration IV.

4.3. Evidence from the fossil record

Despite numerous fossil records of *Alytes*, *Discoglossus* and *Bombina* (Rocek and Rage, 2000; Sanchíz, 1998), there is little evidence relevant to the present study. Morphological differentiation within genera is subtle, so that most fossil remains cannot be assigned to species or lineages with sufficient confidence. According to Sanchíz (1998), fossil specimens of *B. bombina* and questionable *B. variegata* remains are known from the Pliocene (5.3–1.8 MYA). Depending on our calibrations, the divergence time of these species was dated between 2.1 and 11.5 MYA. Since fossils can only indicate the minimum age of lineages, none of the scenarios discussed above can be ruled out on these grounds.

4.4. Constraints and perspectives of the presented method

In this study, we applied a formalized procedure for comparing biogeographic hypotheses, which did not allow us to reject alternative interpretations in the present case. While this conservatism may be perceived as a drawback of the method, we are inclined to see it

Table 4
Rates of allozyme evolution estimated from calibrations I–IV (±SD)

Taxa	D_{Nei} distance (reference)	Estimated rates of allozyme evolution (MY/1 D_{Nei}) based on calibration			
		I	II	III	IV
A. cisternasii–A. obstetricans	0.72 (Arntzen and García-París, 1995)	12.6 ± 2.0	8.1 ± 1.3	22.6 ± 3.5	44.1 ± 6.8
B. bombina–B. variegata	0.49 (Szymura, 1983)	6.7 ± 1.6	4.3 ± 1.0	12.0 ± 2.9	23.5 ± 5.6
D. montalentii–D. p. pictus	0.99 (Lanza et al., 1987)	8.4 ± 1.3	5.4	15.0 ± 2.3	29.3

as the affirmation of a pre-existing problem: a high degree of uncertainty is commonly associated with the timing of paleobiogeographic hypotheses. In the absence of an appropriate test, however, one may be inclined to accept any hypothesis that receives some limited support.

On the positive side, identifying a problem is usually a step toward developing a solution. The statistical power of our test, and thus the probability of achieving a significant result, can be improved by including more phylogenetic splits into the analysis. This may be accomplished by using a larger taxa set that includes additional clades, and by using more sequence data to resolve more splits.

When interpreting the results of the test introduced here, one has to acknowledge the possibility that splits may not be explained by chance with equal probability across all time frames. If, for example, the probability of paleogeographic events becoming known to science decreases with increasing age, a bias towards a lower plausibility of older scenarios will result. On the other hand, a temporal fit with paleogeographic events is harder to reject for older estimates of divergence times, since confidence limits broaden temporally when a low rate of sequence evolution is assumed. We cannot assess quantitatively to which extent these opposed biases may balance each other.

5. Conclusions

Estimating dates of lineage divergence remains a difficult task in phylogenetic studies. Where potential calibration points for a molecular clock are not trustworthy a priori, we recommend using them separately for independent calibrations and comparing the resulting scenarios with an appropriate test.

Our results demonstrate that it is not necessarily justified to regard a paleogeographic scenario as well-confirmed if estimated divergence times can be explained with known paleogeographic events. This may reflect some major difficulties that paleobiogeography has to face to move beyond the stage of hypothesizing. More comprehensive analyses including numerous split events may provide a perspective on how to tackle this problem in future.

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Appendix A. Dated paleogeographic events (PGE) in the Western Mediterranean; MYA = million years ago

No.	MYA	Description of event (references)
1	29	Separation of the Corsica-Sardinia microplate from the continent (Bellon et al., 1977; Orsini et al., 1980).
2	20–15	Separation of Corsica from Sardinia (Alvarez et al., 1974; Orsini et al., 1980).
3	16–14	Opening of the Betic sea strait separated the south Iberian Betic region from the continent (Bustillo and Lópes García, 1997; Lonergan and White, 1997).
4	ca. 16	Large inland saline lakes formed on the Iberian Peninsula (Altaba, 1997).
5	ca. 15	A land bridge, which presumably connected Mallorca and the Betic region, was disrupted at the Langhian-Serravallian transition (Fontboté et al., 1990).
6	12–6	Fragmentation of the insular Betic region gave rise to several smaller islands. The southwestern part eventually joined North Africa (Rif Mountains) (Guerra-Merchán and Serrano, 1993; López Martinez, 1989; Weijermars, 1988).
7	11.5–6	A land bridge of unspecified duration connected Corsica, Sardinia and the continent during this period (Orszag-Sperber et al., 1993).
8	ca. 10	Final structuring of the Neo-Pyrenees (Oosterbroek and Arntzen, 1992).
9	10–7	Secondary contact between the Betic region and the continent occurred due to crustal shortening in the prebetic zone (eastern part of the Betic sea strait) (Lonergan and White, 1997; Weijermars, 1988).
10	8.6–7.6	The Calabro-Peloritan massif (presently Sicily and south Italy) broke off from Sardinia and started to drift eastwards (Duermeijer et al., 1998).
11	5.33	End of the Messinian Salinity Crisis: The formerly dried out Mediterranean basin refilled after the opening of the Strait of Gibraltar so that land bridges between Europe, Africa and several islands became flooded (Krijgsman et al., 1999).

Appendix A (*continued*)

No.	MYA	Description of event (references)
12	3.4–0.01	Ice ages brought about dramatic climatic changes and considerable sea-level subsidence (up to 150 m below the present level). Glacial maxima in the Northern Hemisphere at 3.4, 2.4 and 0.7 MYA have most often been invoked to explain vicariance events in European amphibians (Andersen and Borns, 1994; Müller, 1985; Wilson et al., 1999).

Appendix B. Sample list; nomenclature after Frost (2000); ZFMK = Zool. Forschungsinstitut und Museum Alexander Koenig, Bonn

Taxon	Sample locality	*N*	Haplotype	Voucher	GenBank Accession Nos. 12S, 16S
Alytes cisternasii	Abela, Portugal	1	Ac01	ZFMK 76720	AY333670, AY333708
Alytes dickhilleni	Parejo, near Sierra Nevada, Spain	1	Ad01	Voucher not preserved	AY333672, AY333710
Alytes muletensis	Mallorca	2	Am01	ZFMK 44683	AY333671, AY333709
Alytes obstetricans pertinax	Valencia, Spain	1	Aop01	Voucher not preserved	AY333667, AY333705
Alytes obstetricans cf. *pertinax*	Rabagao near Pisoes, Portugal	1	Aop02	Voucher not preserved	AY333666, AY333704
Alytes obstetricans boscai	Sortelha, Portugal	1	Aob01	ZFMK 76721	AY333669, AY333707
Alytes obstetricans maurus	Rif-mountains, Morocco	1	Aom01	Voucher not preserved	AY333637, AY333711
Alytes obstetricans obstetricans	Argelès-sur-Mer, France	2	Aoo01	ZFMK 76716	AY333668, AY333706
Bombina bombina	Lüchow-Dannenberg, Germany	2	Bb01; Bb02	ZFMK 76717; 76718	AY333657, AY333695; AY333663, AY333701
Bombina maxima	Pet trade	2	Bm01	Vouchers not preserved	AY333659, AY333697
Bombina orientalis	Pet trade	3	Bo01; Bo02	Vouchers not preserved	AY333658, AY333696; AY333660, AY333698
*Bombina variegata*ssp.	Bjeljasnica near Sarajevo, Bosnia-Hrzg.	1	Bvk01	Private collection of J.F. Schmidtler	AY333653, AY333691
Bombina variegata ssp.	Trebistovo near Posusje, Bosnia-Hrzg.	1	Bvs01	Private collection of J.F. Schmidtler	AY333654, AY333692
Bombina variegata ssp.	Zepce/Bosna valley, Bosnia-Hrzg.	1	Bvs02	Private collection of J.F. Schmidtler	AY333655, AY333693
Bombina variegata ssp.	Mirna valley/Istrien, Croatia	1	Bvs03	Private collection of J.F. Schmidtler	AY333665, AY333703
Bombina variegata ssp.	Gornja Brela/ Biokovo/Pr. Makarska, Croatia	1	Bvk01	Private collection of J.F. Schmidtler	AY333653, AY333691

Appendix B (*continued*)

Taxon	Sample locality	*N*	Haplotype	Voucher	GenBank Accession Nos. 12S, 16S
Bombina variegata ssp.	Metsovo, Pindus-mountains, Greece	2	Bvs04; Bvs05	Private collection of J.F. Schmidtler	AY333662, AY333700; AY333687, AY333725
Bombina variegata ssp.	Smolikas mountains, Greece	1	Bvs06	Voucher not preserved	AY333688, AY333726
Bombina variegata pachypus	Italy	1	Bvp01	Voucher not preserved	AY333656, AY333694
Bombina variegata scabra	Kotor, Montenegro	1	Bvsc01	ZFMK 76719	AY333661, AY333699
Bombina variegata kolombatovici	Mosor mountain near Split, Croatia	1	Bvk01	Private collection of J.F. Schmidtler	AY333653, AY333691
Bombina variegata variegata	Mt. Baldo, Italy	2	Bvv01	Vouchers not preserved	AY333664, AY333702
Discoglossus galganoi	Carregosa, Spain	1	Dg01	Voucher not preserved	AY333680, AY333718
Discoglossus galganoi	Monchique, Spain	1	Dg02	Voucher not preserved	AY333681, AY333719
Discoglossus jeanneae	Facinas, Spain	1	Dj01	Voucher not preserved	AY333682, AY333720
Discoglossus jeanneae	Rio Verde near Marbella, Spain	1	Dj01	Voucher not preserved	AY333682, AY333720
Discoglossus montalentii	Porto, Corsica	2	Dm01	Vouchers not preserved	AY333676, AY333714
Discoglossus montalentii	Vizzavona, Corsica	1	Dm01	Voucher not preserved	AY333676, AY333714
Discoglossus montalentii	Bonifacio, Corsica	1	Dm02	Voucher not reserved	AY333677, AY333715
Discoglossus pictus auritus	Tunisia	2	Dpa01; Dpa02	Vouchers not preserved	AY333683, AY333721; AY333684, AY333722
Discoglossus pictus auritus	Argelès-sur-Mer, France[a]	2	Dpa03	ZFMK 76714; 76715	AY333685, AY333723
Discoglossus pictus pictus	Malta	1	Dpp01	Voucher not preserved	AY333686, AY333724
Discoglossus pictus scovazzi	Ceuta, Spanish enclave in North Africa	3	Dps01; Dps02	Vouchers not preserved	AY333678, AY333716; AY333679, AY333717
Discoglossus sardus	Ajaccio, Corsica	1	Ds01	Voucher not preserved	AY333674, AY333712
Discoglossus sardus	Ill Rousse, Corsica	1	Ds02	Voucher not preserved	AY333675, AY333713
Discoglossus sardus	Cap Corse, Corsica	1	Ds02	Voucher not preserved	AY333675, AY333713
Pelobates cultripes	Playa de la Lanzada, Spain	1	Pc01	Voucher not preserved	AY333651, AY333689
Pipa parva	Pet trade	1	Pp01	ZFMK 76722	AY333652, AY333690

[a] Introduced from Africa (Martens and Veith, 1987).

Appendix C. Taxonomic conclusions

Discoglossus pictus is not a monophyletic unit. The taxon *D. pictus scovazzi* turns out to be a highly differentiated genetic lineage, the sister-group of which contains the Iberian species *D. galganoi* and *D. jeanneae* (Figs. 2 and 3). Some doubt regarding monophyly of *D. pictus* has already been raised by García-París and Jockusch (1999). *D. p. scovazzi* should therefore be assigned species rank.

Alytes obstetricans appears to be a non-monophyletic unit. The taxon *A. obstetricans maurus* turns out to be as equally differentiated as the species *A. muletensis* and *A. dickhilleni*. According to a strong tendency, these three taxa form a monophyletic group (Figs. 2 and 4). *A. obstetricans maurus* is therefore a candidate for assignment of species rank.

Based on allozyme data, Nascetti et al. (1986) found no differentiation between *D. pictus auritus* and *D. pictus pictus*. This interpretation is supported by similar findings in the present study (Figs. 2 and 3), which leads to the conclusion that *D. p. auritus* is not a valid subspecies.

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