



Phylogenetic relationships and biogeography of midwife toads (Discoglossidae: *Alytes*)

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ABSTRACT

Aim To devise a robust phylogenetic hypothesis for midwife toads (*Alytes*: Anura: Discoglossidae) and to discuss its implications for the reconstruction of the biogeographical history of the group.

Location Western Palearctic.

Methods Analysis of sequences of mitochondrial DNA (cytochrome *b* and 16S RNA, 861 bp) and 29 characters of cranial osteology of all species and subspecies within *Alytes*.

Results Phylogenetic analyses support a sister group relationship between *Alytes dickhilleni* and *A. muletensis*, and of this clade with *A. maurus*. The monophyly of *A. obstetricans* is controversial; in particular, the phylogenetic position of *A. obstetricans almogavarii* is uncertain. The estimated dates for the cladogenetic events within *Alytes* are congruent with those derived from independent analyses (allozymes), except for the differentiation of *A. o. almogavarii* and the split between *A. dickhilleni* and *A. muletensis*.

Main conclusions The phylogeny based on the analysis of morphological and mtDNA data differs from previous hypotheses in the positions of *A. o. almogavarii* and *A. maurus*. Events associated with the radiation of *Alytes* are the formation of large inland saline lakes in Iberia *c.* 16 Ma and the 11 ° C dramatic decrease in average annual temperature during the Middle–Late Badenian transition *c.* 14–13.5 Ma (*A. cisternasii* vs. the ancestor of the remaining clades), the structuring of the Neo-Pyrenees and the reopening of the Betic Strait *c.* 10–8 Ma (*A. o. almogavarii* vs. *A. obstetricans* and vs. the subgenus *Baleaphryne*), the opening of the Gibraltar Strait at the end of the Messinian Salinity Crisis at *c.* 5.3 Ma (*A. maurus* vs. the ancestor of the *A. dickhilleni* *A. muletensis* clade) and a transmarine colonization event at *c.* 3 Ma (the ancestor of *A. muletensis* vs. *A. dickhilleni*). Following the new hypothesis, *A. maurus*, previously considered a subspecies of *A. obstetricans*, deserves species status. Second, *A. o. almogavarii* is a well-differentiated lineage that was isolated from other *A. obstetricans* more than *c.* 5 Ma, but later lost its genetic and specific identity following secondary contact, hybridization and introgression with the main stock. The presence of a marked morphological and genetic diversity within *A. obstetricans* renders reconstruction of the evolutionary history of the genus more complicated than previously appreciated.

Keywords

Alytes, Amphibia, Anura, biogeography, evolution, mitochondrial DNA, osteology, phylogeny.

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INTRODUCTION

The genus *Alytes* Wagler 1829 (midwife toads) consists of five extant species distributed in three subgenera: *Alytes* (*Alytes*) *obstetricans* (Laurenti, 1768), widely distributed in western Europe, *Alytes* (*Ammoryctis*) *cisternasii* Boscá, 1879, endemic to the centre and southwest of the Iberian Peninsula, *Alytes* (*Baleaphryne*) *muletensis* (Sanchiz & Adrover, 1979) from the island of Mallorca in the Balearic archipelago, *Alytes* (*Baleaphryne*) *dickhilleni* Arntzen & García-París 1995, endemic to the southeastern Iberian Peninsula (Crespo, 1997; Grossenbacher, 1997; García-París & Arntzen 2002; Román, 2002) and *Alytes maurus* Pasteur & Bons, 1962, a taxon inhabiting the mountains in Morocco (Bons & Geniez, 1996). *A. maurus*, which was previously considered a subspecies of *A. obstetricans*, is now considered a different species. *Alytes* (*Baleaphryne*) *talaioticus* (Sanchiz & Alcover, 1982), described from the Holocene of Menorca (Balearic Islands), is now considered a synonym of *A. muletensis* (Sanchiz, 1998).

Several studies have tried to clarify the phylogenetic relationships among the species of the genus, particularly in the 1980s after the discovery of *A. muletensis*. Clarke (1984) described the osteology of *A. muletensis* and compared it with the other species of *Alytes* known at that time (*A. cisternasii* and *A. obstetricans*). Sanchiz (1984) and Maxson & Szymura (1984) proposed phylogenetic hypotheses based on morphological characters of the skeleton and immunological data, respectively. Both studies agreed on proposing a sister-group relationship between *A. cisternasii* and the remaining taxa.

Molecular techniques (allozyme electrophoresis) helped to reveal the existence of a fourth species, the Betic midwife toad *A. dickhilleni* (Arntzen & García-París, 1995). This study also revealed a high level of genetic substructuring within *A. obstetricans*, which resulted in the recognition of *A. o. almogavarii* Arntzen & García-París, 1995, distributed from southern France to north of the Ebro river (García-París, 1995). At present, other three subspecies are recognized: the nominotypical subspecies *A. o. obstetricans* (Laurenti, 1768), distributed across western Europe and the northern Iberian Peninsula (Navarra, País Vasco and Cantabrian Mountains); *A. o. boscai* Lataste, 1879; present in northern and central Portugal, Galicia, western Castilla-León as well as along the Sistema Central; and the recently described *A. o. pertinax* García-París & Martínez-Solano, 2001; present in the central and eastern regions of the Iberian Peninsula (García-París & Martínez-Solano, 2001).

The protein electrophoretic analyses of Arntzen & García-París (1995) placed *A. dickhilleni* as the sister group of *A. muletensis*; *Alytes obstetricans* as sister of the clade *A. muletensis* + *A. dickhilleni*; and, finally, *A. cisternasii* as the sister taxon to all other species of *Alytes* (Arntzen & García-París, 1995). The molecular clock applied to these data linked the divergence among lineages with several well-documented vicariant events and biogeographic barriers, such as the opening of the Gibraltar Strait (see, for example, Busack, 1977, 1986). Accordingly, Arntzen & García-París (1995)

proposed a biogeographic scenario accounting for the successive cladogenetic events and presented a biogeographical hypothesis of *Alytes* in the Western Mediterranean. They proposed the formation of the Betic Strait *c.* 16 Ma as the vicariant event accounting for speciation in allopatry of *A. cisternasii* and proto-*A. obstetricans*. Later, the fragmentation of the Betic-Riffian Massif isolated *A. obstetricans* from the ancestor of *A. dickhilleni* and *A. muletensis* in different islands. *Alytes obstetricans* would have then colonized the Iberian Peninsula through terrestrial connections during the Messinian Salinity Crisis. At the end of this period, the refilling of the Mediterranean Basin isolated the Balearic islands and thus initiated the speciation of *A. muletensis* and *A. dickhilleni*. Altaba (1997) re-analysed the same dataset using a different coding scheme and found support for a sister-group relationship between *A. obstetricans* and *A. dickhilleni* (but see Arntzen & García-París, 1997). This finding led him to propose a different biogeographic scenario. According to this author, the speciation of *A. cisternasii* was triggered by the formation of large inland saline lakes in central Iberia at *c.* 16 Ma (Anadón *et al.*, 1989). Later, the Sub-Betic Massif (including the Balearic Promontory) was occupied by proto-*A. obstetricans*. The fragmentation of this Massif caused the isolation of *A. muletensis*. The divergence of *A. obstetricans* and *A. dickhilleni* was placed in the Upper Tortonian at *c.* 8 Ma, in association with the reopening of the Betic Strait. A third hypothesis has recently been proposed by Fromhage *et al.* (2004). These authors used mtDNA sequences to construct a phylogenetic hypothesis of *Alytes* including *A. maurus*, which was recovered as sister to *A. muletensis* or to (*A. muletensis* + *A. dickhilleni*). Their biogeographic scenario is similar to that of Arntzen & García-París (1995), be it with consideration of *A. maurus* and without *A. o. almogavarii*, the most differentiated taxon within *A. obstetricans*. Hence, no comprehensive phylogenetic hypothesis of the genus has as yet been proposed and choosing between alternative biogeographic scenarios remained ambiguous.

Both Altaba and Arntzen & García-París suggested ways to test the validity of the biogeographic scenarios that were derived from the same dataset (that is, allozyme data of Arntzen & García-París, 1995). For example, the osteology of the Betic midwife toad has not yet been described and, consequently, this information has not been included in previous phylogenetic analyses based on morphological characters (Sanchiz, 1984; Clarke, 1988). Besides, little is known about the evolutionary history of *Alytes* populations of the North African taxon *A. maurus*, which was not included in the protein electrophoresis study of Arntzen & García-París (1995) and for which morphological information is scant. The works of Arntzen & Szymura (1984) and Maxson & Szymura (1984) showed a low level of genetic differentiation between *Alytes* populations from both sides of the Gibraltar Strait. However, if the phylogenetic hypothesis of Arntzen & García-París (1995) is correct, the degree of genetic differentiation of *A. maurus* should be at least equivalent to that found for *A. muletensis* and *A. dickhilleni*, a point supported by

Fromhage *et al.* (2004). The fossil record was also suggested as an independent means of discriminating between both biogeographic hypotheses (Altaba, 1997).

We have compiled new morphological and molecular data for all recognized species and subspecies of *Alytes*. We analysed 861 base pairs (bp) corresponding to partial sequences of the 16S rDNA (16S) and cytochrome *b* (*cyt-b*) mitochondrial genes. We also studied morphological variation in the cranial bones of all species and examined intraspecific variability in *A. obstetricans* in order to produce a robust hypothesis of the evolutionary history of the genus. As Arntzen & García-París (1997: 266) stated, 'the best reason to reject any biogeographical reconstruction is the lack of support for the phylogeny on which it is based'. Consequently, only a robust phylogeny will provide an adequate background to rigorously test and choose among the possible alternatives.

MATERIALS AND METHODS

Molecular data

We obtained partial sequences of the mitochondrial genes 16S (*c.* 530 bp) and *cyt-b* (349–385 bp) for representatives of all species and subspecies of *Alytes* (Table 1). *Discoglossus jeanneae* Busack, 1986 was used as outgroup.

Amplification and sequencing

Tissue samples were obtained from specimens that were already used in the electrophoretic analyses of Arntzen & García-París (1995) (see Table 1), or from live animals that

were released at their place of capture. Whole genomic DNA was extracted from small amounts of frozen or ethanol preserved tissue using NaCl following a protocol modified from Miller *et al.* (1988). We sequenced 530 bp of the large 16S subunit ribosomal mtDNA gene and 349–385 bp of the cytochrome *b* gene. Amplifications were done by the polymerase chain reaction (PCR) (Saiki *et al.*, 1988), using the primers 'MVZ15' (Moritz *et al.*, 1992) and 'cyt b2' (Kocher *et al.*, 1989) for *cyt-b*, and the primers '16Sar' and '16Sbr' (Palumbi *et al.*, 1991) for 16S. PCR reactions were performed as indicated in García-París *et al.* (2003). The amplified fragments were sequenced in an automated DNA sequencer (ABI PRISM 3100) using the PCR primers and following the manufacturer's instructions. GenBank accession numbers for sequences obtained are AY442019–AY442026 and AY514027–AY514035 for cytochrome *b* and AY442027–AY442036 for 16S.

Sequence alignment and analyses

All sequences were compiled using Sequence Navigator™ version 1.0.1 (Applied Biosystems), and 16S sequences were manually aligned and adjusted by comparison with published models of secondary structure for 16S (Ortí & Meyer, 1997). Pairwise comparisons of observed proportional sequence divergences (p-distances) and transition—transversion ratios were calculated with PAUP*4.0b10 (Swofford, 2002).

Osteological data

We studied 25 disarticulated skeletons and 13 cleared, double-stained specimens of all described species and subspecies of

Table 1 Sample localities for the specimens used in the molecular study and GenBank accession numbers. MNCN: Museo Nacional de Ciencias Naturales, CSIC, Madrid, Spain. — indicates sequence not obtained

Taxa	Locality	Voucher	GenBank Number (Cyt-b; 16S)
<i>Alytes cisternasii</i>	Cadalso de los Vidrios, Madrid, Spain	No voucher	AY442019; —
	Alcaracejos, Córdoba, Spain	No voucher	—; AY442027
<i>Alytes dickhilleni</i>	Sierra de Gádor, Almería	No voucher	AY442020; AY442028
<i>Alytes maurus</i>	Chefchaouen, Morocco	MNCN 40768	AY442021; AY442029
	Ketama, Morocco	MNCN 20917	AY442022; AY442030
<i>Alytes muletensis</i>	Sierra de Tramuntana, Mallorca, Spain	No voucher	AF128915; AY442031
<i>A. obstetricans almogavarii</i>	Berga, Barcelona, Spain	MNCN 16760	AY442023; AY442032
	Ibón de Piedrafita, Huesca, Spain	MNCN 16755	AY514027; —
<i>A. obstetricans boscai</i>	Peñalara, Madrid, Spain	No voucher	AY402024; AY442033
	Vinuesa, Soria, Spain	MNCN 19901	AY514028; —
	A Coruña, Spain	MNCN 20848	AY514029; —
<i>A. obstetricans obstetricans</i>	Puerto de San Isidro, Asturias, Spain	MNCN 20838	AY402025; AY442034
	Puebla de Lillo, León, Spain	MNCN 20919	AY514030; —
	Bochum, Westfalia, Germany	MNCN 19893	AY514031; —
<i>A. obstetricans pertinax</i>	Valdelaguna, Madrid, Spain	No voucher	AY402026; AY442035
	Benicassim, Castellón, Spain	MNCN 20038–20039	AY514032–33, —
	Ciruelas, Guadalajara, Spain	MNCN 20942	AY514034; —
	Villanueva de la Torre, Guadalajara, Spain	No voucher	AY514035; —
<i>Discoglossus jeanneae</i>	Riópar, Albacete, Spain	MGP 2241	AF128906; AY442036

Alytes (Table 2). Most of these specimens were already used in the electrophoretic studies of Arntzen & García-París (1995), see material listed *op. cit.* Specimens of *Discoglossus galganoi* Capula, Nascetti, Lanza, Bullini & Crespo, 1985 and *D. jeannea* were used as outgroup. Variation in the following cranial bones was examined: premaxillar, nasal, frontoparietal, prootic-exoccipital, maxillar, squamosal, pterygoid, sphenethmoid, vomer and parasphenoid (Fig. 1). The anatomical

nomenclature is that of Roček (1980), Clarke (1984, 1988) and Sanchiz (1998). The material examined is deposited at the Museo Nacional de Ciencias Naturales (MNCN) (Spain). The definition of characters is presented in Appendix 1.

The geographic origin of the material used for the comparative analysis within *A. obstetricans* was selected as to confidently assign specimens to subspecies. This is relevant in view of the extended reticulation between subspecies along

Taxa	Locality	N	Voucher
<i>Alytes cisternasii</i>	San Agustín de Guadalix, Madrid, Spain	2	MNCN 15505–15506
	Valdemorillo, Madrid, Spain	1	MNCN 15515
	Piñuécar, Madrid, Spain	1	MNCN 15517
	Pelahustán, Toledo, Spain	1	MNCN 40396
<i>Alytes dickhilleni</i>	Ventas de Zafarraya, Granada, Spain	2	MNCN 16757, 20950
	Paterna del Madera, Albacete, Spain	4	MNCN 16782, 19881–19882, 19886
<i>Alytes maurus</i>	Chefchaouen, Morocco	1	MNCN 40768
	Ketama, Morocco	1	MNCN 20917
<i>Alytes muletensis</i>	Sierra de Tramuntana, Mallorca, Spain	5	MNCN 15373, 15410–15413
<i>A. obstetricans almogavarii</i>	Berga, Barcelona, Spain	5	MNCN 16750, 16774–16777
	Ibón de Piedrafita, Huesca, Spain	1	MNCN 20886
<i>A. obstetricans boscai</i>	Tuy, Pontevedra, Spain	2	MNCN 19896, 19899
	Venta del Obispo, Ávila, Spain	1	MNCN 20871
<i>A. obstetricans obstetricans</i>	A Coruña, Spain	1	MNCN 20852
	Rhür Basin, Germany	2	MNCN 19890–19891
<i>A. obstetricans pertinax</i>	Puerto de San Isidro, Asturias, Spain	2	MNCN 19857, 19863
	Benicassim, Castellón, Spain	3	MNCN 16767, 16773, 20039
<i>Discoglossus galganoi</i>	Caudiel, Castellón, Spain	1	MNCN 20865
	Alfara de Algimia, Valencia, Spain	2	MNCN 20882, 20885
	Los Corrales, Huelva, Spain	1	MNCN 13877
<i>Discoglossus jeannea</i>	El Berruoco, Madrid, Spain	1	MNCN 15130
	Porto Covo, Baixo Alentejo, Portugal	1	MNCN 15136
	San Agustín de Guadalix, Madrid, Spain	1	MNCN 15145

Table 2 Sample localities for the specimens used in the morphological study, sample size (*N*) and voucher numbers. MNCN: Museo Nacional de Ciencias Naturales, CSIC, Madrid, Spain

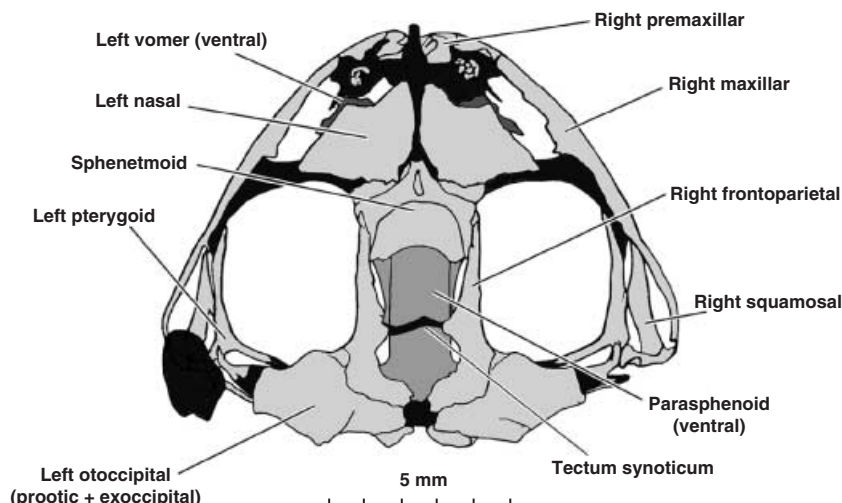


Figure 1 Dorsal view of the skull of an adult specimen of *Alytes dickhilleni*, showing the localization of the bones mentioned in this study. Cartilage is shown in black and bone in grey (dark grey for bones located on the ventral plane). The right tympanic ring has been removed to better visualize the bones in that region.

contact zones (García-París, 1995; Fonseca *et al.*, 2003). We selected specimens of *A. o. algomavarii* from Berga (Barcelona), type locality of the subspecies, *A. o. boscai* from Tuy (Pontevedra), type locality of the subspecies (García-París & Martínez-Solano 2001; but see also Fonseca *et al.*, 2003), and *A. o. obstetricans* from the Ruhr Basin, in Germany. We added the only available specimens of *A. o. pertinax* from Caudiel and Benicassim, in eastern Spain. Specimens of *A. dickhilleni* were selected from the geographically most distant populations: Paterna del Madera (Albacete) and Ventas de Zafarraya (Granada), as preliminary data had suggested the presence of geographic variation.

Phylogenetic analyses

Phylogenetic inference was based on maximum parsimony (MP), maximum likelihood (ML) and Bayesian analyses. MP phylogenies were estimated with PAUP*4.0b10 using the exhaustive search algorithm. We used non-parametric bootstrapping (1000 pseudoreplicates) to assess the stability of internal branches in the cladograms (Felsenstein, 1985). Each base position was treated as an unordered character with four alternative states and gaps were treated as missing data.

We used MODELTEST 3.06 (Posada & Crandall, 1998) to find the best model of molecular evolution that fit the data for subsequent ML analyses. The model of evolution obtained when the outgroup is excluded is TrN + I. The plotting of corrected vs. uncorrected genetic distances suggested the existence of saturation when the outgroup was included in the analysis (Fig. 2).

We also conducted a phylogenetic analysis on the basis of 29 morphological characters (see Appendices 2 and 3) with PAUP*4.0b10, using the exhaustive search algorithm. All characters were considered unordered. Branch support was assessed with non-parametric bootstrapping (1000 replicates) and decay indices (Bremer, 1994).

Bayesian analyses were conducted with MR.BAYES 3.0 (Huelsenbeck & Ronquist, 2001). Analyses were initiated with random starting trees and run for 100,000 generations. Bayesian analyses were carried out for molecular data (*cyt-b* + 16S) with and without the addition of morphological characters.

We tested for substitution rate constancy between taxa using a molecular clock likelihood ratio test (Felsenstein, 1988) as implemented in TREE-PUZZLE 5.0 (Strimmer & von Haeseler, 1996). The null hypothesis of clocklike behaviour was rejected ($\Delta = 32.91$, d.f. = 8, $P < 0.01$). Consequently, in order to estimate the ages of the clades recovered in the phylogenetic analyses, branch lengths of an ML tree were transformed according to the non-parametric rate smoothing method (Sanderson, 1997). We used two different molecular clock calibrations for the estimation of transformed branch lengths: one based on immunological distances (IDU) and another one based on proteins. Maxson (1984) calculated a distance of 12 IDU between *A. obstetricans* and *A. muletensis*, which corresponds to 7.5–6.5 Myr of lineage separation (García-París and Jockusch, 1999). On the other hand, Arntzen and García-París (1995), based on allozymes, estimated the genetic

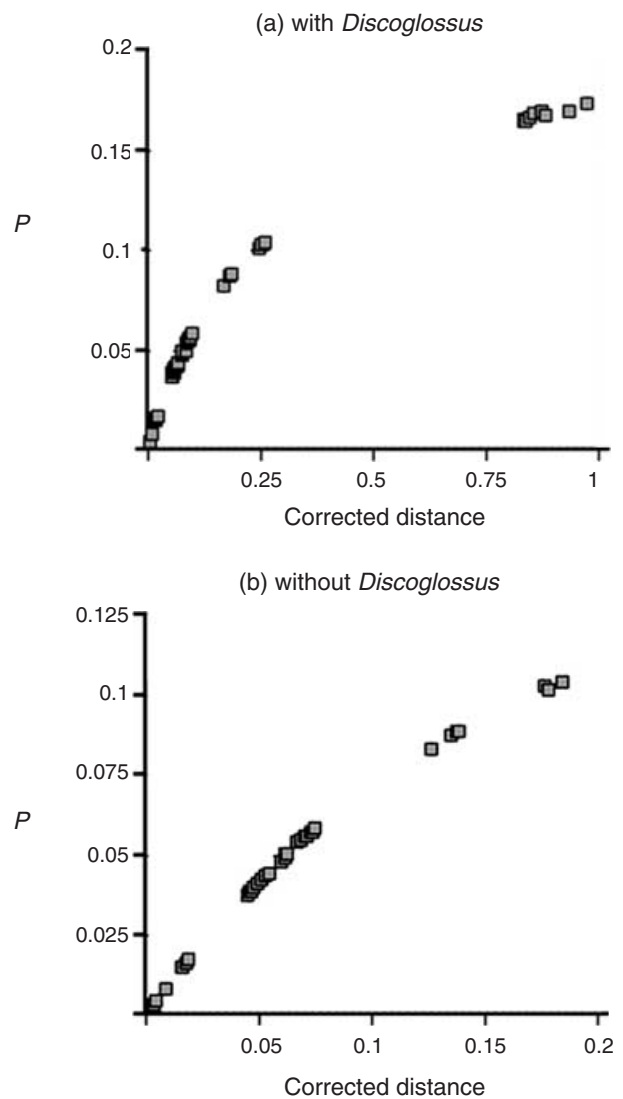


Figure 2 Scatter plot of corrected vs. uncorrected genetic distances in the *cyt-b* + 16S dataset. Above (a) with outgroup *Discoglossus*; below (b) without *Discoglossus*.

distance between *A. muletensis* and *A. dickhilleni* to be 0.27–0.31 IDU, which, following the calibration of Beerli *et al.* (1996) of 0.08–0.10 D_{Nei} Myr⁻¹, corresponds to 3.9–2.7 Myr of independent evolutionary history.

Phylogeographic analyses

Evolutionary relationships among populations within species as inferred from the analysis of gene genealogies are represented better by networks of interconnected haplotypes than by the bifurcating patterns usually recovered by methods of phylogenetic inference (Posada & Crandall, 2001). Consequently, in order to study intraspecific variation in *A. obstetricans*, we constructed a maximum parsimony haplotype network with the software TCS 1.13 (Clement *et al.*, 2000) with cytochrome

Table 3 Genetic distances (*P*-uncorrected) between ingroup taxa (above diagonal: 16S; below diagonal: cytochrome *b*)

Taxon	1	2	3	4	5	6	7	8	9
<i>Alytes cisternasii</i>	–	0.0692	0.0672	0.0673	0.0672	0.0573	0.0613	0.0632	0.0593
<i>Alytes dickhilleni</i>	0.1547	–	0.0275	0.0275	0.0256	0.0314	0.0315	0.0334	0.0295
<i>Alytes maurus</i> (Chefchaouen)	0.1519	0.0774	–	0.0040	0.0197	0.0197	0.0197	0.0217	0.0177
<i>Alytes maurus</i> (Ketama)	0.1519	0.0802	0.0029	–	0.0197	0.0197	0.0197	0.0217	0.0178
<i>Alytes muletensis</i>	0.1547	0.0860	0.0774	0.0802	–	0.0276	0.0276	0.0296	0.0256
<i>A. o. almogavarii</i>	0.1203	0.0917	0.0630	0.0659	0.0831	–	0.0118	0.0138	0.0099
<i>A. o. boscai</i>	0.1289	0.0946	0.0659	0.0688	0.0917	0.0201	–	0.0020	0.0020
<i>A. o. obstetricans</i>	0.1232	0.0946	0.0659	0.0688	0.0917	0.0201	0.0057	–	0.0040
<i>A. o. pertinax</i>	0.1318	0.0974	0.0745	0.0774	0.1003	0.0286	0.0086	0.0143	–

b sequences (385 bp) from 13 individuals representing all subspecies (Table 1).

RESULTS

Phylogenetic relationships

Of the 861 nucleotide positions studied, 114 were variable and 79 of these were phylogenetically informative. Sequence divergence (uncorrected *p*-distances) within the ingroup ranged from 0.2% to 6.9% for 16S and from 0.29% to 15.5% for *cyt-b* (Table 3). Substitutions in the *cyt-b* sequences of the ingroup involved two amino acid replacements. The divergence found between the ingroup taxa and the outgroup (*Discoglossus*) ranged from 12.1% to 14.0% for 16S and from 20.3% to 24.0% for *cyt-b*. Changes in the *cyt-b* sequences involved seven amino acid substitutions between the ingroup and the outgroup. The empirical ratio of transitions to transversions was 3.8 : 1 in the combined dataset.

An exhaustive maximum parsimony analysis produced two equally parsimonious trees [286 steps; consistency index (CI) = 0.846, retention index (RI) = 0.679]. The consensus tree is shown in Fig. 3. *Alytes cisternasii* is the sister taxon to the remaining species of *Alytes*, rendering a monotypic *Ammoryctis*. *Alytes dickhilleni* and *A. muletensis* to form a sister group and *A. maurus* is sister to *A. dickhilleni* and *A. muletensis*, constituting the *Baleaphryne* clade. *Alytes obstetricans* is a monophyletic group, conforming the subgenus *Alytes*, with *A. o. almogavarii* sister to the other taxa.

The topologies resulting from the ML non-parametric bootstrap analysis (best tree $\ln L = 2465.69$) without enforcing a molecular clock and the majority rule consensus tree from the Bayesian analysis differ in the position of *A. o. obstetricans* relative to the other subspecies of *A. obstetricans*. The topology obtained in the MP consensus tree differs from these in the position of *A. o. almogavarii* only (Fig. 4). ML and Bayesian analyses recover three clades forming a polytomy (*A. o. almogavarii*, the clade formed by the other subspecies of *A. obstetricans* and the clade formed by *A. maurus*, *A. dickhilleni* and *A. muletensis*) to which *A. cisternasii* is the sister group; while MP results in

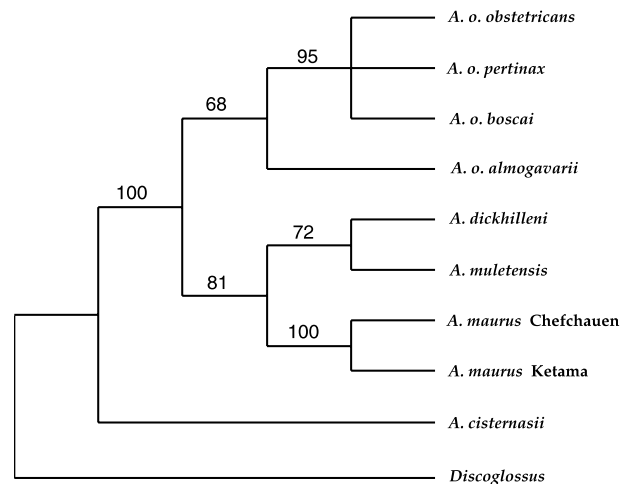


Figure 3 Phylogenetic relationships within *Alytes*. Strict consensus of two equally parsimonious trees ($L = 286$ steps; consistency index (CI) = 0.846, retention index (RI) = 0.679), constructed for the combined *cyt-b* and 16S data set. Seventy-nine characters were parsimony informative. Non-parametric bootstrap values based on 1000 pseudoreplicates using the 'branch and bound' algorithm are shown above branches.

A. o. almogavarii sister to the other subspecies of *A. obstetricans*.

Specific differences in cranial osteology are described in Appendix 2. Figures 5 and 6 show the cleared and double-stained skulls of all *Alytes* taxa in dorsal view. Numbers in parentheses indicate character number in the matrix of morphological characters (presented in Appendix 3). The phylogenetic analyses of morphological characters produced 12 equally parsimonious trees (38 steps, CI = 0.816, RI = 0.611, Fig. 7). Decay indices (not shown) and bootstrap values indicate weak support for the few branches retained in the strict consensus tree. *Alytes cisternasii* is recovered as the sister group to the rest of the species of *Alytes*, among which there is a basal polytomy formed by *A. o. boscai*, *A. o. obstetricans*, *A. o. pertinax* and a clade containing *A. o. almogavarii*, *A. dickhilleni*, *A. muletensis* and *A. maurus*.

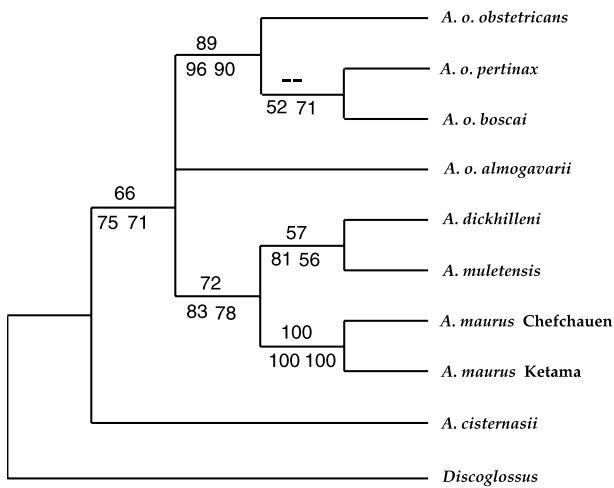


Figure 4 Bootstrap consensus tree recovered by ML and Bayesian analyses for the phylogenetic relationships within *Alytes*. Figures at nodes indicate bootstrap support in the ML analyses (above node) and posterior probabilities in the Bayesian analysis of molecular data (below nodes, left) and combined molecular and morphological dataset (below nodes, right).

Within this clade, relationships are unresolved, except that *A. muletensis* and *A. maurus* are sister taxa.

The topology recovered in the Bayesian analysis of the combined (molecular and morphological) dataset is identical to that obtained with the molecular data set exclusively, with, however, lower posterior clade probability values for some

clades, notably for the sister-group relationship of *A. muletensis* and *A. dickhilleni* (Fig. 4).

Figure 8 shows an ultrametric tree constructed with corrected ML branch lengths, with divergence times according to both calibrations shown at the nodes. Following these estimates, the earliest split within *Alytes* took place between *A. cisternasii* and the other species, between 18.5 and 12.8 Ma. The separation of *A. obstetricans* and the clade *A. maurus* + *A. dickhilleni* + *A. muletensis* took place 9.0–6.2 Ma. *Alytes obstetricans almogavarii* diverged from the other *A. obstetricans* subspecies 7.5–5.2 Ma, while divergence among *A. o. pertinax*, *A. o. boscai* and *A. o. obstetricans* took place between 3.1 and 2.1 Ma. The split between the North African *A. maurus* and the clade *A. muletensis* + *A. dickhilleni* took place 5.6–3.8 Ma, before the divergence between the Balearic *A. muletensis* and the Betic *A. dickhilleni* which occurred 3.9–2.7 Ma.

Phylogeographic analysis

We observed 10 distinct haplotypes among 13 sequences across all four subspecies of *A. obstetricans*. This produced two independent networks: one comprising the one sample of *A. o. almogavarii* from the province of Huesca; and the other connecting the other haplotypes with no more than eight mutational steps. Within the latter, four groups were established separated by one non-observed haplotype encompassing (Fig. 9): (1) Germany and the Cantabrian mountains; (2) Galicia, Sierra de Guadarrama (Sistema Central, Madrid) and

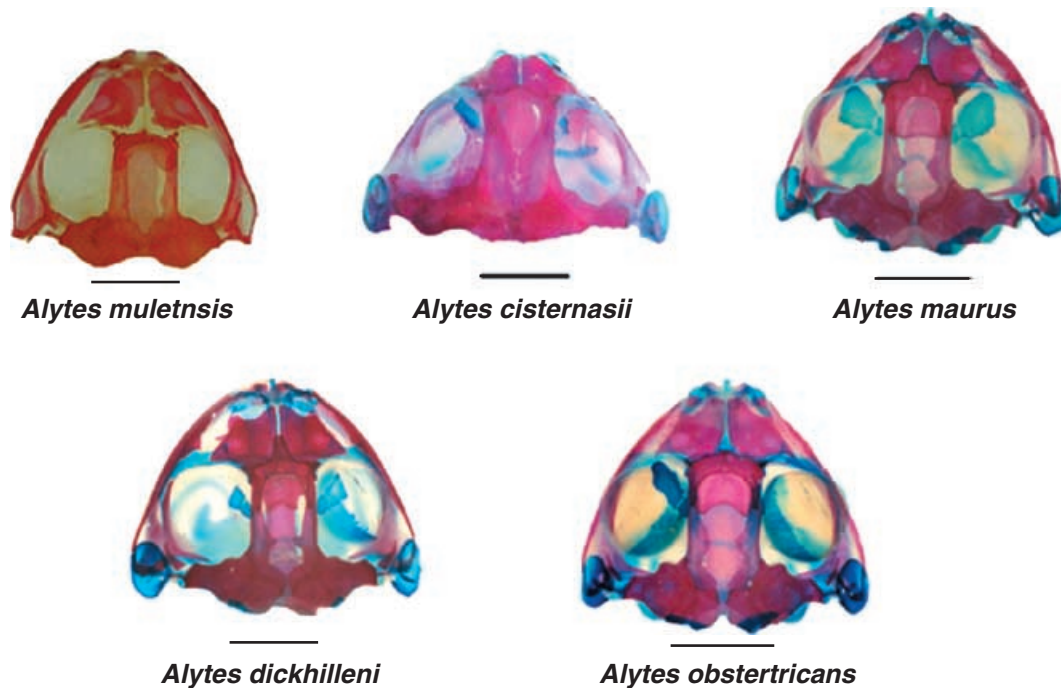


Figure 5 Dorsal views of double-stained, cleared skulls of specimens of all species of *Alytes*: *A. muletensis* (MNCN 15373), *A. cisternasii*, (MNCN 40396), *A. maurus* (MNCN 20917), *A. dickhilleni* (MNCN 19886) and *A. obstetricans* (MNCN 16750). Scale equals 5 mm.

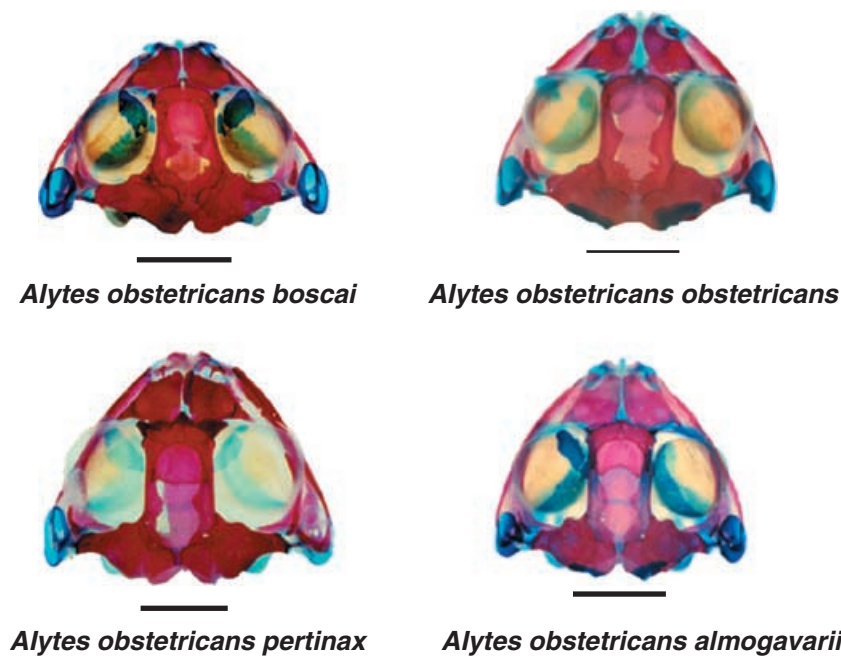


Figure 6 Dorsal views of double-stained, cleared skulls of specimens of all subspecies of *Alytes obstetricans*: *A. o. boscai* (MNCN 20871), *A. o. obstetricans* (19863); *A. o. pertinax* (MNCN 20885) and *A. o. almogavarii* (MNCN 16750). Scale equals 5 mm.

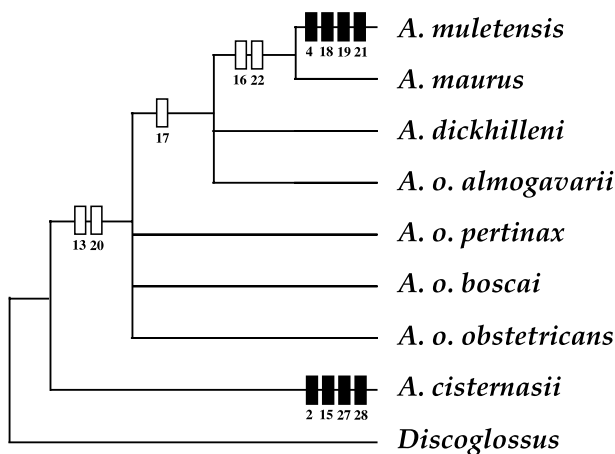


Figure 7 Strict consensus of 12 equally parsimonious trees based on 29 osteological characters in *Alytes*. Solid and open boxes indicate non-ambiguous and ambiguous change state changes, respectively (numbered as in Appendix 1).

Soria (Sistema Ibérico); (3) Castellón, Guadalajara and south-eastern Madrid; and (5) Barcelona.

DISCUSSION

Phylogenetic relationships within *Alytes*

Our results support the sister group relationship between *A. multensis* and *A. dickhilleni* proposed by Arntzen & García-París (1995) and Fromhage *et al.* (2004). A sister group relationship between *A. obstetricans* and *A. dickhilleni* (Altaba, 1997) is not supported. According to the combined morphological and molecular dataset, *A. maurus* is sister to

A. dickhilleni and *A. multensis* and not to *A. obstetricans* (see Arntzen & Szymura, 1984; Maxson & Szymura, 1984). This clarifies some of the problems of the biogeographic scenario identified by Arntzen & García-París (1995) (see below). A third, independent type of evidence, the fossil record, might also support this hypothesis, if the presence of *Baleaphryne* in the Early Pleistocene ('Villafranchian') of Morocco was to be confirmed (Hossini, 2001).

The phylogenetic position of *A. o. almogavarii* remains uncertain. MP analyses include this taxon within *A. obstetricans*. ML and Bayesian analyses, on the other hand, recover a polytomy of three clades: (1) *Baleaphryne*, including *A. maurus* + *A. multensis* + *A. dickhilleni*; (2) *A. o. almogavarii*; and (3) the remaining subspecies of *A. obstetricans* (*A. o. boscai*, *A. o. obstetricans* and *A. o. pertinax*). Both analyses suggest that *A. o. almogavarii* might have had a long independent evolutionary history. In fact, this taxon is well differentiated from other subspecies at the allozyme level, presenting several private alleles (Arntzen & García-París, 1995). However, large areas of hybridization and a high degree of gene flow between *A. o. almogavarii* and both *A. o. obstetricans* and *A. o. pertinax* have also been reported (García-París, 1995). The combination of allozyme, mtDNA and morphological data (with some character states resembling those observed in *A. multensis*) strongly suggest that *A. o. almogavarii* might have been a well-differentiated lineage that progressively lost its genetic and specific identity through extensive hybridization and introgression following secondary contact among previously isolated lineages.

From a taxonomic and phylogenetic perspective, our data confirm the species status for the Moroccan populations, a position already taken by Llorente *et al.* (1995) and Fromhage *et al.* (2004). Therefore, this taxon should be referred to as: *Alytes (Baleaphryne) maurus* Pasteur & Bons, 1962 stat. nov.

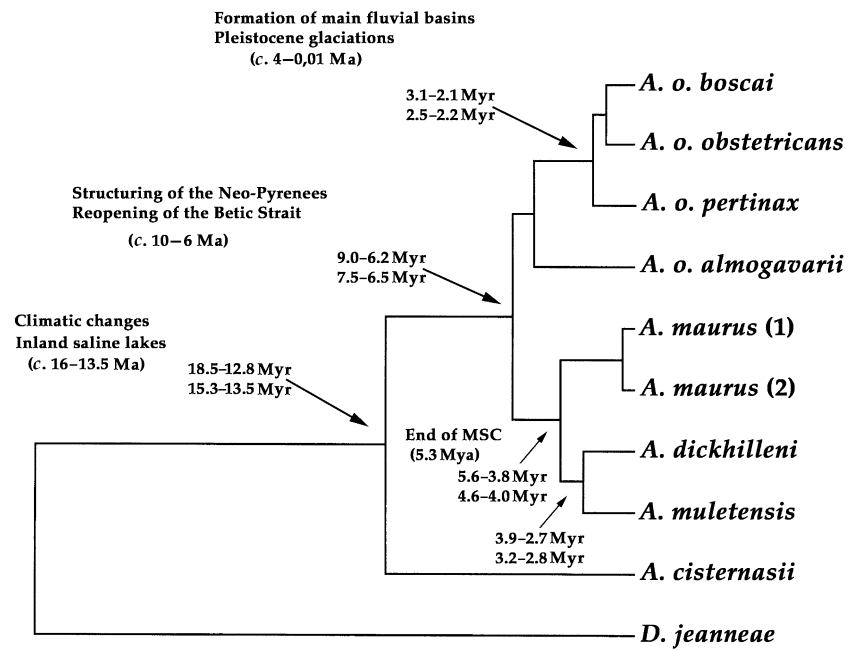


Figure 8 Ultrametric tree constructed with corrected ML branch lengths following the method of Sanderson (1997). Divergence time estimates shown at the nodes were calculated from two different calibrations: one based on immunological distances (Maxson, 1984, below), and other one based on proteins (Beerli *et al.*, 1996, above). Postulated vicariant events are shown at nodes.

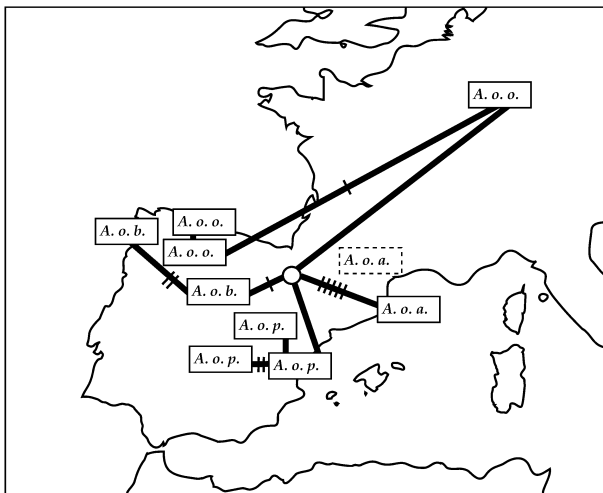


Figure 9 Haplotype network of *cyt-b* sequences of subspecies within *A. obstetricans*. The circle represents a non-observed haplotype that connects haplotypes observed in *A. o. obstetricans* (*A. o. o.*), *A. o. boscai* (*A. o. b.*), *A. o. pertinax* (*A. o. p.*) and *A. o. almogavarii* from Barcelona (*A. o. a.*). Transverse bars indicate mutational steps. Dashed lines indicate the geographic location of an independent haplotype of *A. o. almogavarii* from Huesca.

Our data are congruent with the recognition of three subgenera: *Alytes*, only including *A. (A.) obstetricans*, *Ammoryctis*, composed of *A. (A.) cisternasii*, and *Baleaphryne* comprising three species *A. (B.) dickhilleni*, *A. (B.) maurus*, and *A. (B.) muletensis*.

Morphological evolution in *Alytes*

Alytes cisternasii and *A. muletensis* are so distinctive morphologically that their discovery led to the erection of new genera

for each of them: *Ammoryctis* Lataste, 1879 and *Baleaphryne* Sanchiz & Adrover, 1979, respectively. Their morphological differentiation has been related to functional adaptations (Crespo, 1982; Sanchiz, 1984). Accordingly, *A. cisternasii* is a fossorial species, specialized in using its forearms for burrowing, whereas *A. muletensis* has a locomotive morphotype associated with climbing. These adaptations are reflected in the robust and ossified skeleton of *A. cisternasii*, including markedly ossified frontoparietals with reduced dorsal fontanelles and well-developed lateral rami of the prootic (Clarke, 1984, 1988). In *A. muletensis*, the skeleton is less ossified than in the other species of *Alytes*, and the skull is the least ossified within the genus. The frontoparietals have a wide, not subdivided dorsal fontanelle, although the medial processes of the frontoparietals suggest an anterior–posterior division. Besides, the paired ossification nuclei that form the sphenethmoid do not fuse completely in adult specimens, a unique feature in *Alytes* (although the condition found in *A. maurus* is very similar to that of *A. muletensis*). This has been related to heterochronic processes in the *A. muletensis* lineage, by which development would have been truncated in its initial stages (Clarke, 1984, 1988; Sanchiz, 1984). However, the distribution of some character states is not in line with this interpretation, including the high counts of maxillary and premaxillary teeth in some specimens of *A. o. almogavarii* and *A. muletensis*. The number of maxillary teeth generally increases in the course of development of Discoglossidae, if not in most Anura (Clarke, 1988).

The uncertain phylogenetic position of *A. o. almogavarii* has implications for hypotheses on the ancestral morphology of *Alytes*. Sanchiz (1984) considered the ‘generalist’ morphotype of *A. obstetricans* as the ancestral condition within the genus. Alternatively, the ancestral condition in *Alytes* may have been a mixture of ‘generalized’ and ‘specialized’, such as exhibited by

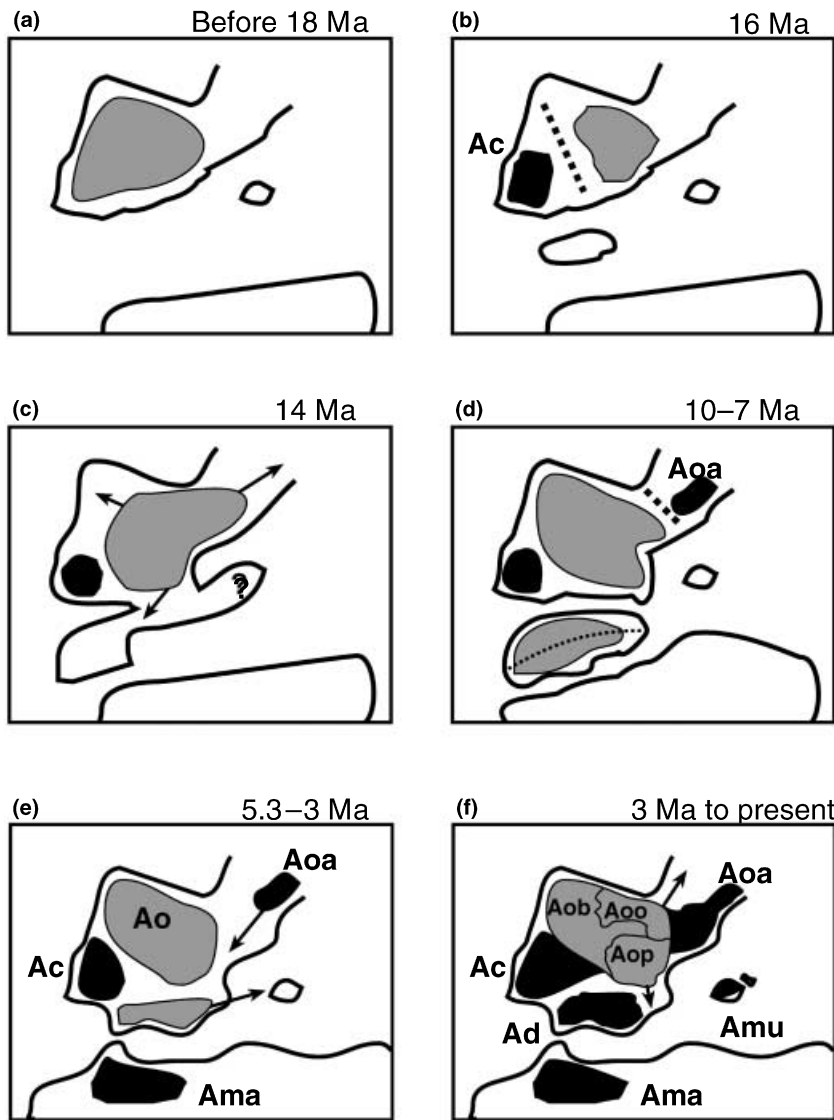


Figure 10 Historical biogeography of the genus *Alytes*. Note that for lineages subject to cladogenesis a grey shading is used instead of the black one. (a) The ancestor of extant species of *Alytes* occupied Iberia. (b) The formation of large inland saline lakes in central Iberia or drastic climatic changes promoted the differentiation of the *A. cisternasii* (Ac) clade vs. the remaining *Alytes* stock. (c) The latter taxon expanded its range, including the Sub-Betic Massif. (d) The structuring of the Neo-Pyrenees and the reopening of the Betic Strait after the marine transgression of the Upper Tortonian promoted the divergence of the *A. o. almogavarii* (Aoa) clade and *Baleaphryne*, respectively, vs. the ancestor of *A. obstetricans* (Ao). (e) The opening of the Gibraltar Strait isolated the ancestors of *A. maurus* (Ama) and [*A. dickhilleni* (Ad) plus *A. muletensis* (Amu)] on opposite shores of the incipient Mediterranean Sea. The ancestor of *A. muletensis* reached the Balearic islands. (f) Subspecies of *A. obstetricans* differentiate and expand their ranges. *Alytes o. obstetricans* contacts and deeply introgress *A. o. almogavarii* which loses its nuclear identity.

extant *A. o. almogavarii*. Such polymorphisms might have represented an important source for morphological specialization that was later exploited by some taxa within the *Baleaphryne* clade, *A. muletensis* in particular.

Biogeography

Our estimates for divergence times between lineages are largely congruent with those proposed by Arntzen & García-París (1995, see their Fig. 5), with two exceptions: *A. o. almogavarii* *A. obstetricans* (*c.* 3 vs. *c.* 6 Ma) and *A. muletensis* *A. dickhilleni* (*c.* 4.5 vs. 3 Ma). These discordances might be caused by the absence of *A. maurus* in the protein electrophoresis study, by problems with the calibration of the molecular clock, or, in the case of *A. o. almogavarii*, by differential cytoplasmic/nuclear introgression among lineages, which would render the use of a molecular clock unreliable. At any rate, some of the geological events proposed by these authors do not fit

into the time frame associated with the new phylogeny. This, and the differences between our and previous phylogenetic hypotheses (Arntzen & García-París, 1995; Altaba, 1997; Fromhage *et al.*, 2004) requires the proposal of a new biogeographic scenario.

In the Lower Miocene, the faunistic exchange between Asia, Europe and Africa may have been widespread (Barbadillo *et al.*, 1997) and the ancestral stock of *Alytes* may, in this epoch, have settled in Iberia (Fig. 10a), although it might also have been endemic to this area. A settlement of the proto-*Alytes* in the Betic–Riffian Massif, as postulated by all previous hypotheses, is not possible since the massif only just started to emerge in this period (Weijermars, 1991). The vicariant event that promoted the split between the *A. cisternasii* clade and the remaining stock of *Alytes* might have been the formation of large inland saline lakes in central Iberia *c.* 16 Ma (Anadón *et al.*, 1989), as suggested by Altaba (1997). Alternatively, it could be correlated with dramatic climatic changes in the

Middle–Late Badenian transition (Böhme, 2003). The ancestor of *Alytes cisternasii* would have remained restricted to the arid sandy soils of the southwestern Iberian Peninsula, whereas the ancestor of all other *Alytes* extended over the rest of Iberia (Fig. 10b). Later, this stock expanded and entered the Betic–Riffian Massif that emerged *c.* 14 Ma (Weijermars, 1991) (Fig. 10c). Altaba (1997) suggested that at this time, the ancestor of *A. muletensis* could have reached the Balearic Promontory through a postulated terrestrial connection, however, none of the proposed phylogenies support this hypothesis. The final structuring of the Neo-Pyrenees (Oosterbroek & Arntzen, 1992) and the reopening of the Betic Strait after the marine transgression of the Upper Tortonian that isolated the Sub-Betic region (López Martínez, 1989) fragmented *Alytes* populations into three lineages: the ancestor of *A. o. almogavarii* north of the Pyrenees, proto-*A. obstetricans* in Iberia and the ancestor of *Baleaphryne* (*A. dickhilleni*, *A. maurus* and *A. muletensis*) in the Sub-Betic region (Fig. 10d). The divergence of the lineage leading to *A. o. almogavarii* from the *A. obstetricans* lineage is thus older than previously estimated and it is almost contemporary to the separation of *A. obstetricans* from the Riffian–Betic–Balearic clade. This, together with the unresolved position of *A. o. almogavarii* in our phylogenetic hypothesis, allows rejection of the hypothesis of an early differentiation of *A. obstetricans* in the Betic–Riffian Massif (Altaba, 1997). This Massif remained occupied by the ancestors to the *Baleaphryne* clade: *A. maurus*, *A. muletensis* and *A. dickhilleni* exclusively. Although the fragmentation of the Betic–Riffian Massif at 8–6 Ma might have initiated the separation of the lineages leading to *A. maurus* and the ancestor of *A. muletensis* and *A. dickhilleni*, the split of these two lineages is also consistent with the opening of the Strait of Gibraltar at the end of the Messinian Salinity Crisis 5.3 Ma (Krijgsman *et al.*, 1999) (Fig. 10e). *Alytes muletensis* and *A. dickhilleni* diverged more recently, at *c.* 3 Ma, long after the formation of the Balearic Islands. This suggests that the ancestor of *A. muletensis* has reached the Balearic Islands through a transmarine colonization process (Fig. 10f). Events of long-distance colonization, though uncommon in amphibians, have been invoked to explain other biogeographic patterns (see, for example, Feller & Hedges, 1998; Vences *et al.*, 2003). The differentiation within *A. obstetricans* may be related to the formation of the main fluvial drainages in the Iberian Peninsula (Arntzen & García-París, 1995) or, alternatively, be associated with the glacial refugia of Pleistocene (Fig. 9).

The singular evolutionary history of *Alytes* is characterized by the existence of high levels of morphological and genetic diversity within some lineages (*A. obstetricans*) but not in others (*A. cisternasii*, *A. muletensis*). The reconstructed spatio-temporal associations render the reconstruction of the evolutionary history of the genus more complicated than previously appreciated. As for *A. obstetricans*, discordances between nuclear (García-París, 1995) and mitochondrial markers (this study) in the populations of northern Guadalajara and Sistema Ibérico suggest the existence of a past,

wide intermixing area in the northern Iberian subplateau involving populations of *A. o. almogavarii*, *A. o. boscai* and *A. o. pertinax*. Further studies focusing on the geographic distribution of genetic variability within *A. obstetricans* and the phylogenetic position of *A. o. almogavarii* are required for testing hypotheses of fragmentation and subsequent reticulation within these lineages (García-París, 1995; Fonseca *et al.*, 2003).

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APPENDIX 1 MORPHOLOGICAL CHARACTERS: DEFINITIONS AND CHARACTER STATES

Some of the studied characters have been used or modified from Clarke (1984, 1988).

1. Frontoparietal. Medial process present, well developed at the medial border, extending to the sagittal axis (0); absent or poorly developed, not reaching that level (1).
2. Frontoparietal. Paraoccipital process present (0) or absent (1).
3. Frontoparietal. Dorsal fontanelles. A single anterior fontanelle with triangular shape (0); a double fontanelle formed by an anterior and a posterior fontanelle and separated by the medial processes of the frontoparietal, with a slight constriction at that point (1); two fontanelles, anterior and posterior, separated by the medial processes of the frontoparietal, that contact at the sagittal axis (2).
4. Frontoparietal. Lateral margin in the orbitary area clearly protruding with respect to the anterior portion of the lateral margin (0) or narrower and not protruding (1).
5. Maxillar. Posterior process present (0) or absent (1).
6. Maxillar. Zygomaxillar process present (0) or absent (1).
7. Maxillar. Palatine process present (0) or absent (1).
8. Maxillar. Posterior process straight (0) or slightly convex distally (1).
9. Maxillar. Longitudinal groove in the base of the teeth row present (0) or absent (1).

10. Maxillar. Pterygoid process present (0) or absent (1).
11. >Nasals. Maxillar process elongated, extending to the nasal process of the maxillar (0) or short, not reaching the maxillar (1).
12. Nasals. Length of the rostral process roughly equivalent to one-third of the total length of the nasal (0); length of the rostral process roughly equivalent to one-fourth of the total length of the nasal (1).
13. Nasals. Ratio total length : maximum width (measured at the level of the maxillar process) is wider than long (0) or of subequal length or slightly longer than wide (1).
14. Paraoccipital. Paraoccipital crests present (0) or absent (1).
15. Parasphenoid. Posterior process long, clearly surpassing the posterior margin of the parasphenoid allae (0) or short, not surpassing that margin (1).
16. Parasphenoid. The cultriform process is narrow at the level of the allae, general shape biconvex (0) or cultriform process uniformly wide, general shape pointed (1).
17. Parasphenoid. Well-marked transverse keels in the allae (0) or transverse keels absent (1).
18. Parasphenoid. The allae are narrower in their proximal end and wider distally (0) or allae uniformly wide or narrowing distally (1).
19. Premaxillar. Number of teeth: greater than or equal to 14 (0), or between 13 and 10 (1) or less than 10 (2).
20. Prootic + exoccipital. Prootic process short and wide, does not extend over the external border of the orbital fossa (0) or elongated and narrow, extending over the external border of the orbital fossa and reaching the internal border of the pterygoid fossa (1).
21. Pterygoid. Ventral expansion absent (0) or present (1).
22. Sphenethmoid. Unpaired (0) or two paired pieces incompletely fused (1).
23. Sphenethmoid. Anterior process short, not surpassing the anterior margins of the lateral processes of the sphenethmoid (0) or elongated, clearly surpassing the anterior margins of the lateral processes (1).
24. Sphenethmoid. Lateral processes uniformly wide (0) or narrowing distally (1).
25. Sphenethmoid. In frontal view, the ventral border is clearly wider than the dorsal border (0) or the ventral and dorsal borders are approximately equally wide (1).
26. Squamosals. In a dorsal view, the zygomatic rami of the squamosals diverge caudally in anteromedial-posterolateral orientation (0) or diverge rostrally in posteromedial-anterolateral orientation (1).

27. Squamosals. Otic and interior rami well developed (one-third to half of the total length of the squamosal, measured from the apical end of the zygomatic ramus to an axis connecting the distal ends of the otic and interior rami) (0); poorly developed, less than one-third of the total length of the squamosal (1).

28. Squamosal. Zygomatic ramus elongated, more than two times the length of the otic ramus (0) or short and blunt (1).

29. Vomer. Posterior choanal process uniformly wide and bifurcated (0) or pointed (spine shape) (1).

APPENDIX 2 DESCRIPTION OF CHARACTERS AND CHARACTER STATES

Frontoparietal: The extension of the median process of the frontoparietal does not reach the sagittal axis in *A. muletensis*, *A. dickhilleni*, *A. maurus*, *A. o. almogavarii* and *A. o. obstetricans*, whereas *A. o. boscai* and *A. cisternasii* possess a well-developed medial process of the frontoparietal (1). In *A. o. pertinax*, both character states are observed.

Alytes cisternasii is characterized by the absence of a paraoccipital process, which is present in the other taxa (2). Besides, the dorsal fontanelle is in *A. cisternasii* clearly divided by the medial processes of the frontoparietals (3). The posterior fontanelles are especially small. The other species have a dorsal fontanelle with the typical 'shoe sole' shape described by Boulenger (1897).

The posterior part of the frontoparietal expands laterally in all *Alytes* species, but to a lesser extent in *A. muletensis* and some specimens of *A. o. almogavarii* (4).

Maxillar: The posterior process is curved to some degree in all *Alytes* except *A. muletensis*, which have posterior processes typically straight or slightly curved distally (8).

Nasal: The nasals of *A. cisternasii* are wider than long, while the other species present nasals of subequal length, or that clearly are longer than wide (13).

Parasphenoid: The posterior process is less developed in *A. cisternasii* than in the other species of *Alytes* (15). In the former, the posterior process does not surpass the posterior margin of the parasphenoid allae, in contrast to the other taxa. In most *Alytes* species, the cultriform process has a constriction at its base (16), which results in a general biconvex appearance. The basal constriction is absent in *A. muletensis*, *A. maurus* and some specimens of *A. o. almogavarii*, whose cultriform processes have a general pointed shape. More or less developed transverse keels are present in the parasphenoid allae of all *Alytes* taxa, except *A. muletensis*, *A. dickhilleni*, *A. maurus* and *A. o. almogavarii* (17). The width of the allae is also variable (18). In *A. muletensis* and *A. cisternasii* they are narrow in their

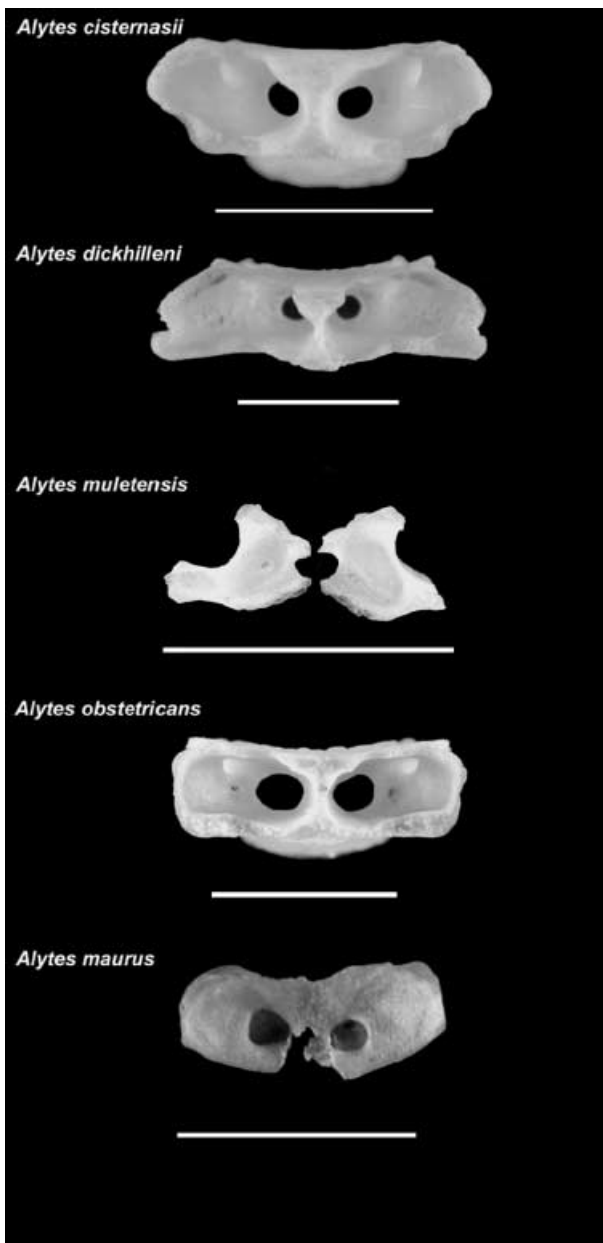


Figure A1 Variation in the sphenethmoid across species of *Alytes*. Scale equals 3 mm.

proximal portion and wider distally. In *A. dickhilleni*, *A. maurus* and the subspecies of *A. obstetricans* (with the exception of some specimens of *A. o. pertinax*), the alae are uniform in width throughout their total length or narrower distally.

Premaxillar: The number of premaxillary teeth shows variation in *Alytes* (19). *Alytes muletensis* and several specimens of *A. o. almogavarii* have high counts of premaxillary teeth (14 or more), while other species have counts between 10 and 12, except for *A. cisternasii* that has generally eight or nine premaxillary teeth.

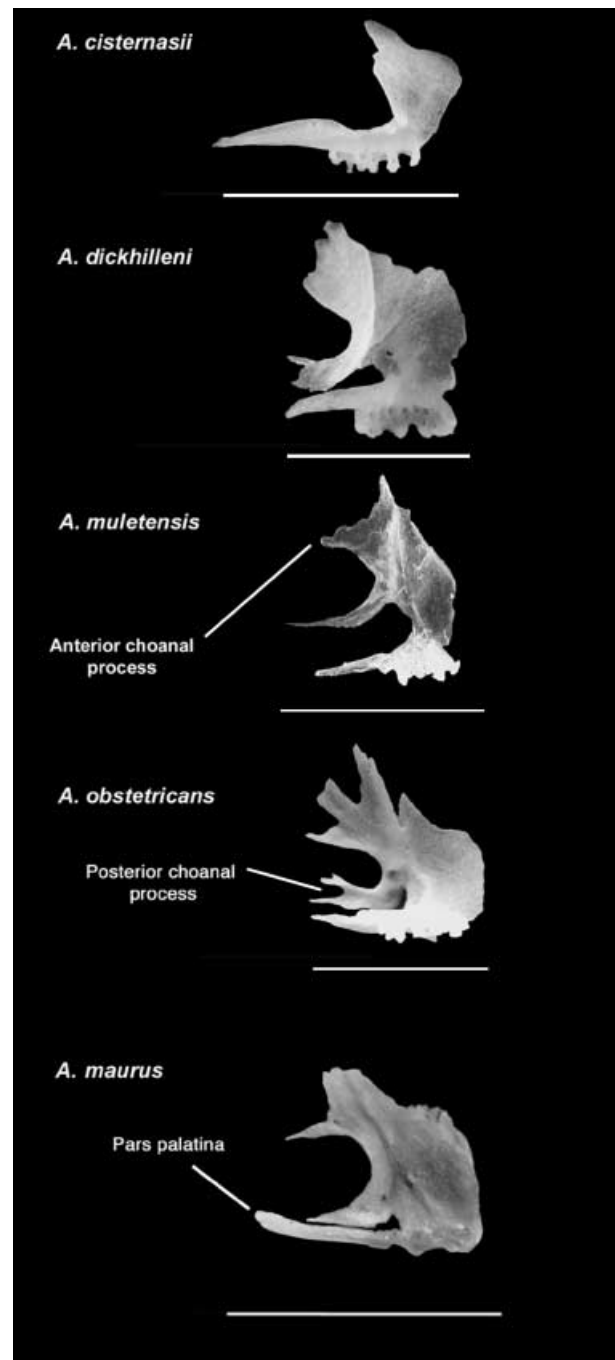


Figure A2 Anatomic terminology and variation in the vomer across species of *Alytes*. Scale equals 3 mm.

Prootic + exoccipital: In all species of *Alytes* except *A. cisternasii*, the prootic process is relatively short, not reaching the lateral border of the orbital fossa (20). In *A. cisternasii*, the prootic process reaches or surpasses the external border of the orbital fossa.

Pterygoid: Specimens of *A. muletensis* have a ventral expansion in the pterygoid, which is absent in the other species of *Alytes* (21).

Sphenethmoid (Fig. A1): In *A. muletensis*, the paired ossification nuclei that later in the ontogeny confirm the sphenethmoid do not fuse completely as in the other species of *Alytes* (22). An intermediate condition is found in *A. maurus*: the fusion of both ossification nuclei is incomplete (Fig. A1). There is also variation in the relation between the length of the anterior process of the sphenethmoid and that of the anterior border of the anterolateral processes (23). In *A. muletensis* and *A. maurus*, the anterior process is relatively short, never clearly surpassing the anterior border of the anterolateral processes as in the other species of *Alytes*. *Alytes o. pertinax* and *A. o. almogavarii* are polymorphic with respect to this character. The relative length of the dorsal and ventral borders of the sphenethmoid in frontal view is less than 1 in *A. dickhilleni* and greater than 1 in *A. cisternasii* and *A. obstetricans* (25). This character is inapplicable to

A. muletensis and *A. maurus* because of the incomplete fusion of the ossification centres of the sphenethmoid in this species (see above).

Squamosal: The degree of development of the otic and interior rami is lesser in *A. cisternasii* when compared to other *Alytes* species (27). Also, the zygomatic ramus is shorter and blunter in *A. cisternasii* than in the other taxa (28).

Vomer (Fig. A2): The posterior choanal process is distally bifurcated, not pointed and of uniform width in *A. dickhilleni*, *A. maurus* and some specimens of *A. o. almogavarii*, and pointed distally and not bifurcated in other *Alytes* species (29).

Characters 5–7, 9–12, 14, 24 and 26 show no marked variation at the level of the ingroup.

APPENDIX 3 CHARACTER MATRIX

Taxon	Characters																												
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
<i>Discoglossus</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>A. o. boscai</i>	0	0	1	0	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0	1	1	1	1	0	0	1
<i>A. o. pertinax</i>	0–1	0	1	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0–1	1	1	1	0	0–1	1	1	1	0	0	1
<i>A. o. almogavarii</i>	1	0	1	0–1	1	1	1	1	1	1	1	1	1	1	0	0–1	1	1	0–1	1	1	0	0–1	1	1	1	0	0	0–1
<i>A. o. obstetricans</i>	1	0	1	0	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0	1	1	1	1	0	0	1
<i>A. dickhilleni</i>	1	0	1	0	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	0	1	0	0	0
<i>A. cisternasii</i>	0	1	2	0	1	1	1	1	1	1	1	1	0	1	1	0	0	0	2	0	1	0	1	1	1	1	1	1	1
<i>A. muletensis</i>	1	0	1	1	1	1	1	0–1	1	1	1	1	1	1	0	1	1	0	0	1	0	1	0	1	?	1	0	0	1
<i>A. maurus</i>	1	0	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	?	1	0	0	0