1 **Multilocus assessment of phylogenetic relationships in *Alytes* (Anura, Alytidae)**

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20 **Abstract**

21 With the advent of large multilocus datasets, molecular systematics is experiencing very rapid
22 progress, but important challenges remain regarding data analysis and interpretation. Midwife toads
23 (genus *Alytes*) exemplify two of the most widespread problems for accurate phylogenetic
24 reconstruction: discerning the causes of discordance between gene trees, and resolving short
25 internodes produced during rapid, successive splitting events. The three species in subgenus
26 *Baleaphryne* (*A. maurus*, *A. dickhilleni* and *A. muletensis*), the sister group to *A. obstetricans*, have
27 disjunct and highly restricted geographical ranges, which are thought to result from old vicariant
28 events affecting their common ancestor, but their phylogenetic relationships are still unresolved. In
29 this study we re-address the phylogeny of *Alytes* with a special focus on the relationships in
30 *Baleaphryne* with a multilocus dataset including >9,000 base pairs of mitochondrial DNA and four
31 nuclear markers (3,490 bp) in all recognized taxa, including all subspecies of *A. obstetricans*. Both
32 concatenation and species tree analyses suggest that *A. muletensis*, endemic to the Balearic island of
33 Mallorca, is the sister taxon to a clade comprising the southeastern Iberian endemic *A. dickhilleni*
34 and the north African *A. maurus*. This scenario is consistent with palaeogeological evidence
35 associated with the fragmentation of the Betic-Rifean Massif, followed by the opening of the Strait
36 of Gibraltar. On the other hand, analyses of intraspecific variation in *A. obstetricans* are
37 inconclusive regarding relationships between major clades and conflict with current subspecific
38 taxonomy.

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40 **Keywords:** *Alytes*, *Baleaphryne*, mtDNA, Multispecies coalescent, species trees, concatenation

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44 1. Introduction

45 Molecular systematics is experiencing rapid advances in the reconstruction of historical
46 relationships among organisms. New developments are the consequence of the increasing feasibility
47 of compiling large multilocus datasets and the incorporation of new analytical tools in phylogenetic
48 inference (O'Meara, 2012; Yang and Rannala, 2012). This has led to a shift in interest from gene
49 tree to species tree inference (Edwards, 2009; Corl and Ellegren, 2013; Reid et al., 2014). However,
50 multilocus datasets present new theoretical and computational challenges for the inference of
51 species trees. For instance, events of gene tree incongruence have been widely reported in many
52 taxa, and their alternative interpretations in terms of evolutionary processes complicate
53 phylogenetic reconstruction (Degnan and Rosenberg, 2009; Nakhleh, 2013). Among the most
54 common causes of discordance between gene trees are incomplete lineage sorting and ancient or
55 recent hybridization or gene flow (Belfiore et al., 2008; Corl and Ellegren, 2013). These processes
56 are usually hard to discriminate because they produce similar molecular signatures, but their
57 consequences for the interpretation of speciation processes are very different (Leaché et al., 2014).
58 Another challenge is the difficulty in distinguishing "soft" (where lack of support is typically related
59 to lack of informative characters) *versus* "hard" polytomies, (in which population lineages are not
60 strictly bifurcating, as in rapid radiations, see Kubatko et al., 2011; Stanley et al., 2011).

61 In order to solve or minimize the impact of these problems, different alternatives have been
62 proposed (Camargo et al., 2012). For instance, enlarged taxon and data sampling (genes or alleles –
63 Belfiore et al., 2008; Chung and Ané, 2011-, as well as the use of larger gene fragments, e.g.
64 mitogenomes – Pabijan et al., 2013; Zhang et al., 2013) have been used to resolve conflicting nodes
65 in many groups of organisms (Whitfield and Lockhart, 2007; Pacheco et al., 2011; Kapralov et al.,
66 2013; Sanders et al., 2013), including amphibians (Esprequeira Themudo et al., 2009; Garcia-Porta
67 et al., 2012; Recuero et al., 2012; Williams et al., 2013). While amphibians are excellent models for
68 studies of speciation due to their low vagility and geographically structured patterns of genetic
69 variation (Vences and Wake, 2007; San Mauro, 2010), inference of phylogenetic relationships has

70 been often complicated by findings of strong discordance across datasets, sometimes related to
71 rapid diversification (Blackburn et al., 2013). Midwife toads (*Alytes* spp.), a species-poor genus
72 including representatives of an ancient anuran clade illustrate these problems.

73 The genus *Alytes* is divided into five species, which occur throughout the Western
74 Mediterranean Basin (Figure 1). These species are classified in three subgenera, each of which is in
75 principle a monophyletic group (but see Gonçalves et al., 2007). Subgenus *Alytes* includes *Alytes*
76 *obstetricans* (Laurenti, 1768), widely distributed in western Europe, with four currently recognized
77 subspecies (Fig. 1). Subgenus *Ammoryctis* includes *Alytes cisternasii* Boscá 1879, endemic to the
78 center and southwest of the Iberian Peninsula. Finally, subgenus *Baleaphryne* comprises three
79 species: *Alytes muletensis* (Sanchiz and Adrover, 1979) from the island of Mallorca in the Balearic
80 archipelago; *Alytes dickhilleni* Arntzen and García-París, 1995, endemic to the Betic mountains in
81 southeastern Spain, and *Alytes maurus* Pasteur and Bons, 1962, which is distributed in northern
82 Africa in Morocco (Rif and Middle Atlas Mountains) and Algeria (Bons and Geniez, 1996;
83 Grossenbacher, 1997; Crespo, 1997; García-París and Arntzen, 2002; Román, 2002; Beukema et al.,
84 2013; Mateo et al., 2013; de Pous et al., 2013).

85 Phylogenetic relationships in *Alytes* have been addressed in several studies based on
86 morphology, genetic data, and both types of data combined (Arntzen and García-París, 1995;
87 Fromhage et al., 2004; Martínez-Solano et al., 2004; Gonçalves et al., 2007; Biton et al., 2013), but
88 some questions remain open. In particular, phylogenetic relationships between the three members of
89 the subgenus *Baleaphryne* are still unresolved. The origin and diversification of this clade dates
90 back to the Miocene and seems to be related to fragmentation of the Betic-Rifean Massif and the
91 Messinian Salinity crisis ending with the opening of the Strait of Gibraltar about 5.33 mya, but the
92 relative splitting order between species is apparently very close in time, precluding full resolution of
93 the trichotomy in previous studies (Martínez-Solano et al., 2004; Gonçalves et al., 2007). Moreover,
94 Gonçalves et al. (2007) questioned the monophyly of subgenus *Alytes* based on the finding of *Beta-*
95 *fibrinogen intron 7* (β -*fibint7*) haplotypes in the most divergent lineage within *A. obstetricans*

(subspecies *A. o. almogavarii*) that grouped with *Baleaphryne*, suggesting the possibility of ancient interspecific gene flow or, alternatively, the persistence of shared ancestral polymorphisms across subgenera. In this study we re-address phylogenetic relationships in *Alytes* with a special focus on the relationships in *Baleaphryne* with a multilocus dataset including >9,000 base pairs of mtDNA and four nuclear markers (3,490 bp) in all recognized taxa. Different analyses suggest a resolution of the polytomy with *A. muletensis* being the sister taxon to *A. dickhilleni* + *A. maurus* and incomplete lineage sorting as the most likely source of discordance in *β-fibint7*. We discuss the implications of this new finding for elucidation of the evolutionary history of *Alytes*.

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105 **2. Material and methods**

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107 *2.1. Sample collection*

108 A total of 32 individuals was analyzed in the present study, representing all the recognized
109 species and subspecies of the genus *Alytes* (Figure 1 and Table 1). Some of these samples were
110 previously used by Gonçalves et al. (2007) and Pinho et al. (2010), and we incorporated some
111 sequences from those studies in our analyses (see Supplementary Table S1). Newly collected tissue
112 samples were obtained from toe tips of adults or tail tips of larvae, and preserved in 95% ethanol.
113 All individuals were released back in their place of capture after tissue collection.

114 Genomic DNA was extracted using EasySpin Genomic DNA Minipreps Tissue Kit (SP-
115 DT-250, Qiagen, Hilden, Germany) following the fabricant's protocol. We amplified by polymerase
116 chain reaction (PCR) several fragments of mitochondrial DNA (mtDNA) (see below) and fragments
117 of four nuclear genes: Protein phosphatase 3, catalytic subunit, alpha isoform (*PPP3CAint4*),
118 Ribosomal protein L9 intron 4 (*RPL9int4*), Cellular myelocytomatosis (*C-myc*) and Beta fibrinogen
119 intron 7 (*β-fibint7*). PCRs were performed in 10 µL reaction volumes containing 5 µL of Phusion
120 Master Mix (Thermo Scientific, Waltham, Massachusetts, USA), 0.2 mM each primer and 50 ng of
121 genomic DNA.

122 *mtDNA*. We amplified and sequenced the fragment *ND4-tRNA^{LEU}* using the primers
123 described by Arévalo et al. (1994), and following the amplification conditions of Gonçalves et al.
124 (2007). Additionally, we designed several primers along the mitochondrial genome with melting
125 temperatures (*T_m*) around 56°C to avoid nonspecific amplifications. Primers were combined in
126 different ways to amplify templates between 1,000 and 2,000 base pairs. A touchdown PCR
127 program was applied in all cases, consisting of: 98°C for 3 min (pre-denaturing step); 1st round (9
128 cycles) of 98°C for 30 s, 60°C to 56°C (decreasing 0.5°C in each cycle) for 40 s, and 72°C for 40 s;
129 2nd round (31 cycles) of 98°C for 30 s, 56°C for 30 s, and 72°C for 40 s, with a final extension at
130 72°C for 5 min. Successful amplification products of the same fragment for all taxa (Table 2) were
131 sequenced in both forward and reverse directions.

132 *nDNA*. For *PPP3CAint4* and *RPL9int4*, amplification conditions followed Pinho et al.
133 (2010), with some modifications: 98°C for 3 min (pre-denaturing step); 40 cycles of 98°C for 30 s,
134 60°C for 20 s, and 72°C for 20 s; and a final extension at 72°C for 5 min. For *C-myc*, we used
135 primers Cmyc1U (Crawford, 2003) and Cmyc3cat (Brunes et al., 2010). Amplification was
136 performed as follows: 98°C for 3 min (pre-denaturing step); 40 cycles of 98°C for 30 s, 62,5°C for
137 40 s, and 72°C for 40 s; and a final extension at 72°C for 5 min. For *β-fibint7* a two-step
138 amplification procedure, with the primers described by Sequeira et al. (2006), was followed.
139 Amplification conditions were performed as described in Gonçalves et al. (2007).

140 Purified products of each reaction were sequenced with the ABI Prism BigDye Terminator
141 v3.1 Sequencing Kit protocol on an ABI3130xl DNA analyzer (Applied Biosystems, Foster City,
142 California, USA). GenBank Accession Numbers of the new sequences are: KJ858769-KJ859062
143 (Table S1).

144 Some DNA fragments (*mtDNA* and *nDNA*) could not be obtained for a small group of
145 samples. In these cases, to avoid biases in the analyses, we replaced the sample in question by
146 another one of the same taxon and, when possible, of the same population of origin, as indicated in
147 Table S1. This is equivalent to the concept of “composite taxa” (Campbell and Lapointe, 2009;

148 Alonso et al., 2012); we used this approach to increase phylogenetic accuracy. As mentioned above,
149 some sequences were available from GenBank (*16S* and *ND4* mtDNA sequences and some
150 *PPP3CAint4*, *RPL9int4*, *β -fibint7* nuclear sequences, see Table S1).

151

152 2.2 Sequence analyses and genetic variation

153 Sequences were assembled with the software CHROMASPRO v1.5
154 (www.technelysium.com.au/ChromasPro.html). The assembled sequences were then edited and
155 aligned using the program BIOEDIT v7.1.3.0 (Hall, 1999); this preliminary alignment was
156 subsequently refined by eye. Since at the relatively deep phylogenetic levels investigated some
157 saturation might be expected to occur in faster-evolving markers, we performed saturation tests in
158 different mtDNA fragments with DAMBE v5.3.70 (Xia, 2013). On the other hand, single and
159 multiple-base insertions or deletions (indels) were observed at nuclear markers. These events were
160 used to solve the haplotype phase in all cases (see below). Decoding was made with the direct
161 interpretation of the mixed trace formed by the two allele peaks superimposed onto each other
162 downstream of the indel (Sousa-Neves et al., 2013). Other polymorphic sites in heterozygous
163 individuals were coded with IUPAC ambiguity codes. In these cases, the probabilistic Bayesian
164 algorithm implemented in PHASE v2.1.1 (Stephens et al., 2001; Stephens and Donnelly, 2003) was
165 used to phase haplotypes. Input files were formatted with SEQPHASE (Flot, 2010). Due to the low
166 haplotype variability in our data set, we incorporated additional sequences from a larger *A.*
167 *obstetricans* dataset (Gonçalves et al., in prep.) into each input file to increase the precision of
168 phased haplotypes. We ran PHASE three times with different random seeds to check the consistency
169 of haplotype reconstructions across runs. All ambiguous positions could be resolved with posterior
170 probabilities of 0.90 or higher. Finally, we tested for recombination in the nuclear markers through
171 calculation of the R_m statistic (minimum number of recombination events - Hudson and Kaplan,
172 1985), as implemented in DNASP v5.10 (Librado and Rozas, 2009).

173 We calculated several summary statistics for each marker to describe their levels of
174 polymorphism, using the software DNASP: number of segregating sites (S), nucleotide diversity (π)
175 (Nei, 1987), and Theta (θ per site, Watterson, 1975). We also calculated genetic distances (p -
176 uncorrected) within and between species with MEGA5 (Tamura et al., 2011).

177

178 *2.3. Phylogenetic analyses*

179 We performed phylogenetic analyses based on two optimality criteria: Maximum
180 Likelihood (ML) and Bayesian Inference (BI). Different analyses were performed on: (1) the
181 concatenated mtDNA dataset; (2) each nuclear gene analyzed separately; (3) the four nuclear genes
182 concatenated; and 4) a concatenated mtDNA + nDNA dataset. For the concatenated mtDNA and
183 nDNA datasets, the optimal partitioning strategies and the respective best-fit models of evolution
184 for each partition were estimated under the Bayesian Information Criterion (Schwarz, 1978) as
185 implemented in PARTITIONFINDER v1.1.0 (Lanfear et al., 2012) (see Supplementary Table S2). For
186 this, the mtDNA alignment was divided based on functional categories (genes, tRNAs and rRNAs)
187 and into 1st, 2nd and 3rd codon position for protein-coding genes (see Brandley et al., 2005; Pabijan
188 et al., 2013). In nDNA markers, partitioning was between intron and exon regions (see Wiens et al.,
189 2010). For estimation of individual gene trees of the four nuclear DNA markers, we selected the
190 best-fit substitution model for each gene with JMODELTEST 2.1.1, based on the Bayesian
191 Information Criterion (Darriba et al., 2012) (Table S2). For the combined mtDNA + nDNA dataset,
192 only one of the two phased alleles per individual was included, and the optimal partitioning
193 strategies for each data type (eight for mtDNA and three for nDNA, see Table S2) were specified
194 based on PARTITIONFINDER results.

195 ML analyses of both concatenated (partitioned) and single-gene datasets were conducted
196 using GARLI v.2.0 (Zwickl, 2006). In all cases, we performed 100 bootstrap replicates (BS) and a
197 50% majority-rule consensus tree was subsequently computed with PAUP* (Swofford, 2003). BI
198 analyses were run in MRBAYES v.3.2.2 (Huelsenbeck and Ronquist, 2001; Ronquist et al., 2012).

199 Two replicate runs with four independent chains were run for 10×10^7 generations, sampling every
200 10,000 generations. A majority-rule consensus tree was computed after discarding the first 25%
201 generations. TRACER v1.6 (Rambaut and Drummond, 2007) was used to check convergence results
202 by plotting the log-likelihood values *versus* generation number. Branch support was based on
203 Bayesian Posterior Probabilities (BPPs).

204 In addition to concatenation approaches, we analyzed our multilocus dataset under the
205 multispecies coalescent as implemented in *BEAST 1.8 (Drummond and Rambaut, 2007; Heled
206 and Drummond, 2010). We defined 12 independent partitions: eight in the mitochondrial dataset, as
207 previously selected by PARTITIONFINDER (see Table S2 for details), and each of the four nuclear
208 markers independently. Substitution models were unlinked across partitions, and molecular clock
209 and tree priors were linked in the mtDNA partitions and unlinked in the nuclear genes. As species-
210 tree prior, we used the Yule speciation model. Preliminary runs indicated deviations from the strict
211 clock model in three partitions: mtDNA, *β -fibint7* and *RPL9int4*, and therefore we used in
212 subsequent analyses the uncorrelated (lognormal) relaxed clock instead of the strict clock used in
213 the remaining partitions (*PPP3CAint4* and *C-myc*).

214 The fossil record of *Alytes* includes Middle Miocene remains that have been assigned to a
215 new species predating the diversification of extant species, with a minimum age of 15.8 million
216 years (Bastir et al., 2014). In order to accommodate this prior information in the analyses, we
217 specified a lognormal prior for the root of the tree with a mean of 20 million years and a log
218 (standard deviation) of 0.1, yielding dates from 16.36 (2.5% quantile) to 24.21 (97.5% quantile)
219 million years. For substitution rates, we used a wide, largely uninformative prior with a lognormal
220 distribution (mean: 0.01 substitutions per lineage per million years; log (st. dev.): 1; 95% range:
221 1.171E^{-3} - 3.142E^{-2}).

222 Two independent analyses were run for 300 million generations in order to check for
223 convergence of results across runs. Logfiles were inspected in TRACER1.6 to assess adequate
224 mixing. All effective sample sizes (ESS) of parameters estimated were >200 , as recommended by

225 the authors. A burn-in period of 10% of the total running time was specified and a Maximum Clade
226 Credibility consensus tree was constructed with the program TREEANNOTATOR, which is distributed
227 as part of the BEAST package.

228 Since previous studies suggested the possibility of hybridization between ancestors of
229 *Baleaphryne* and *A. obstetricans* based on β -*fibint7* data (Gonçalves et al., 2007), we used the
230 posterior predictive checking approach implemented in JMLv1.02 (Joly et al., 2009; Joly, 2012) to
231 discriminate between this and the alternative hypothesis of incomplete lineage sorting in this
232 marker. JML takes as input the posterior distribution of species trees from *BEAST and simulates
233 gene trees under the coalescent with no migration. Minimum genetic distances between species in a
234 simulated β -*fibint7* dataset were used to generate a posterior predictive distribution, which was then
235 compared with empirical (observed) values.

236 Finally, in order to check for consistence of results across different species-tree inference
237 methods, we used another species-tree reconstruction approach, the pseudo-likelihood method
238 implemented in MP-EST v. 1.3 (Liu et al., 2010). To estimate support, 100 replicate gene trees for
239 each marker were subjected to MP-EST analyses to derive bootstrap proportions of inferred clades
240 in the species tree. Since we found variation in clade support in analyses of the same datasets under
241 different optimization criteria, we used as alternative inputs for these analyses: 1) 100 ML trees
242 from a bootstrap analysis in GARLI; 2) 100 post burn-in trees from a MRBAYES run; and 3) 100 post
243 burn-in trees from a *BEAST analysis.

244

245 **3. Results**

246

247 *3.1. Sequence variation and genetic distances*

248 The final mtDNA alignment totaled 9,045bp of which 7,091bp are related with protein-
249 coding genes (Table S1). A gap of 3bp (nucleotide positions 763-765, according to the reference
250 sequence of *A. obstetricans* from GenBank, Accession Number: NC_006688.1, San Mauro et al.,
251 2004) was observed at the ND1 gene (mt03 fragment, see Table S1) in the two samples of *A.*

252 *cisternasii*. This codon specifies serine in *A. obstetricans* and threonine in *Baleaphryne*. For the
253 nuclear markers, we obtained a concatenated alignment of 3,142bp, including indels in all genes.
254 No evidence of recombination or saturation was detected in any marker (results not shown).

255 Supplementary Table S3 presents an overview of genetic diversity. All markers and
256 partitions were polymorphic, with a range of 36 to 322 segregating sites in mtDNA and 74-85 in
257 nDNA. Mitochondrial and nuclear genetic distances (*p*-uncorrected) between species and subgenera
258 are in Table 3. Within *Baleaphryne*, species differed between 7.2% (*A. maurus* - *A. muletensis*) and
259 8.2% (*A. dickhilleni* - *A. muletensis*) in mtDNA and 1.0% (*A. maurus* - *A. muletensis*) to 1.2% (*A.*
260 *maurus* - *A. dickhilleni*) in nDNA (4-gene concatenated alignment). Within *A. obstetricans*, genetic
261 distances ranged from 0.9% (*A. o. obstetricans* and *A. o. pertinax*) to 3.4% (*A. o. boscai* and *A. o.*
262 *almogavarii*) in mtDNA and from 0.5% (*A. o. obstetricans* and *A. o. pertinax*) to 1.2% (*A. o. boscai*
263 and *A. o. pertinax*) in nDNA. *Alytes cisternasii* differed by >15% in mtDNA and >6% in nDNA
264 from other *Alytes* species, whereas *Baleaphryne* and *A. obstetricans* differed by over 9% in mtDNA
265 and 2% in nDNA.

266

267 3.2. Phylogenetic analyses

268 The ML and BI trees based on the concatenated mtDNA dataset are highly concordant,
269 including a fully resolved tree (Figure 2a). Three main clades were recovered with high support
270 (BPP $\geq$ 0.9, BS $\geq$ 75), corresponding to the three subgenera: *Ammoryctis* (*A. cisternasii*), *Alytes* (*A.*
271 *obstetricans*), and *Baleaphryne* (*A. muletensis*, *A. dickhilleni* and *A. maurus*). *Alytes cisternasii* is
272 the sister taxon of *A. obstetricans* + *Baleaphryne*, as in previous studies. Within *Baleaphryne*, *A.*
273 *muletensis* is recovered as the sister group of *A. maurus* + *A. dickhilleni* (BS=76; BPP=1.0). Within
274 *A. obstetricans*, *A. o. pertinax* and *A. o. obstetricans* are recovered as monophyletic and grouped as
275 sister taxa. On the other hand, subspecies *boscai* and *almogavarii* are not recovered as
276 monophyletic groups (Fig. 2a). *Alytes o. boscai* is divided into a well-supported, highly divergent
277 southern group (samples 02S and 03S) and a northern group (sample 01N); however, in *A. o.*

278 *almogavarii* the sample HUE from the Pyrenees in Huesca does not group with the other two
279 samples (alm01 and alm02), which form a well-supported clade.

280 Regarding each nuclear marker, individual gene trees had similar topologies based on ML
281 or BI. Support for some of the clades recovered in analyses of mtDNA, based on BS and BPPs, was
282 lower (Supplementary Figures S1 and S2). For instance, *A. obstetricans* was recovered as a
283 monophyletic group in *PPP3CAint4* and *C-myc* gene trees, but not in *β-fibint7* or *RPL9int4*.
284 Monophyly of *Baleaphryne* was supported by all markers in both BI and ML analyses. However,
285 alternative resolutions of the trichotomy in *Baleaphryne* were recovered by different genes. For
286 instance, *C-myc* recovers *A. muletensis* as the sister group of *A.maurus* + *A.dickhilleni* with
287 moderate (BS: 64) to high (BPP: 0.94) support, as in mtDNA, whereas *RPL9int4* groups *A.*
288 *muletensis* and *A. dickhilleni* instead, although with low support (BPP<0.9, BS<75, Figures S1 and
289 S2). On the other hand, some *β-fibint7* alleles of *A. o. almogavarii* cluster with *Baleaphryne* in a
290 well-supported clade (BPP: 1.0; BS: 79). According to JML results, this is consistent with the
291 coalescent with no migration model, thus supporting the hypothesis of incomplete lineage sorting as
292 the source of discordance. *PPP3CAint4*, on the other hand, does not resolve the relationships within
293 *Baleaphryne*. The differences across nuclear gene trees are reflected in the lack of resolution of the
294 concatenated nDNA tree, where the *Baleaphryne* trichotomy remains unresolved (Figure 2b).

295 Regarding subspecies of *A. obstetricans*, there is some discordance between the different
296 nuclear gene trees. Some clades are recovered with strong support in both ML and BI inference,
297 although there is no correspondence with described subspecies: no subspecies is recovered as a
298 monophyletic group across all markers (Figures S1 and S2). When nDNA fragments are
299 concatenated into a single alignment, BPPs and BS values for some clades increase. Samples of *A.*
300 *o. pertinax* group with part of *A. o. obstetricans* in a well-supported clade (BPP: 1.0; BS: 85), the
301 monophyly of *A. o. boscai* is well supported by BI (BPP: 0.92) but not in ML analyses, and *A. o.*
302 *almogavarii* (excluding alleles from Huesca) is recovered as a monophyletic group (BPP: 1.0; BS:
303 94).

304 The concatenated mtDNA + nDNA tree is well-resolved (Fig. 3b) and similar to the
305 mtDNA tree. Both *Baleaphryne* and *A. obstetricans* are recovered as monophyletic, and
306 relationships within *Baleaphryne* are well-resolved, with *A. maurus* being the sister taxon to *A.*
307 *dickhilleni*. Within *A. obstetricans*, *A. o. pertinax* is more closely related to *A. o. obstetricans*,
308 whereas the other two subspecies (*A. o. boscai* and *A. o. almogavarii*) are not recovered as
309 monophyletic, as in the mtDNA tree.

310 Results of *BEAST analyses are shown in Figure 3a. The monophyly of subgenera *Alytes*
311 and *Baleaphryne* is supported by BPPs of 1.0, and there is also support for a sister-group
312 relationship between *A. dickhilleni* and *A. maurus* (BPP=0.91). Time estimates (time to most recent
313 common ancestor, or TMRCA) for major splits include median values of 3.39 million years for
314 *Baleaphryne* (95% highest posterior density interval, HPD: 2.04-5.11 million years) and 5.57 for
315 *Baleaphryne*+*A. obstetricans* (95% HPD: 3.43-8.16 million years). MP-EST recovered the same
316 species tree, including a sister-group relationship between *A. maurus* and *A. dickhilleni*, although
317 the level of support for this clade varied depending on the sources of gene trees used in
318 bootstrapping analyses. When ML trees were used, this clade was recovered only 41% of the time,
319 but this value increased to 52% with MRBAYES trees and up to 84% with trees from the *BEAST
320 run used as input.

321

322 4. Discussion

323 Recently Biton et al. (2013) presented a comprehensive molecular phylogeny for Alytidae
324 based on six genes (2,500 bp), which recovered a well-supported *A. maurus* + *A. dickhilleni* clade.
325 Our mtDNA data confirm this result in an enlarged dataset including a good representation of
326 intraspecific variation in *A. obstetricans*, and it appears to be robust to different methods of
327 analysis. On the other hand, our four nuclear markers, despite presenting significant variation,
328 provided less resolution, although all species, including the three species in *Baleaphryne*, were
329 recovered as monophyletic groups in most nuclear-gene trees (Figures S1 and S2), with the only

330 exception of *β-fibint7*, as previously reported by Gonçalves et al. (2007). Lack of reciprocal
331 monophyly in this marker can be explained, based on JML results, which suggest absence of
332 hybridization, by a deep coalescence event.

333 Increased data sampling, especially in mtDNA, has been shown to increase phylogenetic
334 accuracy, even in situations of rapid, simultaneous divergence that make inference of population
335 history extremely challenging (see Pabijan et al., 2013; Williams et al., 2013). In our case, it
336 allowed full resolution of the *Baleaphryne* radiation with very high support. This contrasts with the
337 lower support derived from nuclear data, which may stem from several factors, apart from the
338 higher number of base pairs sequenced for mtDNA. It has been long known that the higher mutation
339 rate and lower effective population size of mitochondrial DNA permit more precise phylogenetic
340 resolution compared to nuclear markers (Avise, 2000; Zhang et al., 2008; Belfiore et al., 2008).
341 Conflicting gene trees are more common among nuclear markers due to incomplete lineage sorting,
342 as in *Alytes*. Even with informative markers, as in our study, many additional independent nuclear
343 markers may be required to resolve short internodes, such as those in the *Baleaphryne* radiation.

344 Despite the lack of resolution in nuclear DNA markers (both individually and concatenated),
345 implementation of the multispecies coalescent in *BEAST produced a species tree with good
346 support for the *maurus + dickhilleni* clade. In general, methods that estimate a species tree from
347 independently estimated gene trees (*BEAST), including those that account for the stochastic
348 nature of genetic drift in the lineage-sorting process, may be more likely to reconstruct the true
349 species phylogeny, even where there is strong discordance among gene trees (Edwards et al., 2007;
350 Williams et al., 2013). Leaché and Rannala (2011) suggested that Bayesian implementations of the
351 multispecies coalescent could produce very accurate estimates of the species tree. Whether support
352 for a *maurus-dickhilleni* clade in our species tree results from dominance of the mitochondrial
353 marker in *BEAST analyses (which simultaneously estimates gene trees and the species tree) is
354 unclear, although simulation studies suggest that this should not be the case (Heled and Drummond,
355 2010). In fact, results of MP-EST, which does not infer gene trees and thus is expected to weight

356 them equally when inferring the species tree, also reveal a sister-group relationship between *A.*
357 *maurus* and *A. dickhilleni*, although with weaker support. Support for this clade increased
358 significantly when using as input gene trees from a *BEAST analysis, highlighting the potential for
359 biases from the faster-evolving marker in the *BEAST approach during co-estimation of gene and
360 species trees. In any event, one of the nuclear markers (*C-myc*) also provides robust support for that
361 clade (Figs. S1c and S2c), and the other markers do not provide strong evidence for conflicting
362 relationships. Therefore, in the absence of evidence for introgression or any other potentially
363 confounding factor in either mtDNA or *C-myc*, we consider our results well supported. This is in
364 agreement with previous analyses of rapid radiations using mitogenomes, in which mtDNA
365 recovered the same pattern of phylogenetic relationships than that estimated by multilocus
366 coalescence analyses (see Steinfartz et al., 2007; Zhang et al., 2008).

367 The new phylogenetic hypothesis is consistent with palaeogeological evidence regarding the
368 fragmentation of the Betic-Rifean Massif (see for instance, Martín et al., 2009), with the species
369 endemic to the Betic (*A. dickhilleni*) and Rifean (*A. maurus*) mountains sharing a more recent
370 common ancestor with respect to the other species, endemic to the Balearic islands (*A. muletensis*).
371 The opening of the Strait of Gibraltar might be associated with the split of *A. maurus* and *A.*
372 *dickhilleni*. Our time estimates have wide confidence intervals that encompass the extended
373 timeframe of this geological event, although median values tend to be somewhat recent and
374 probably underestimate the actual divergence time. In any case, the overseas dispersal event
375 postulated by Martínez-Solano et al. (2004) to explain the differentiation of *A. muletensis* is no
376 longer required.

377 Phylogenetic relationships between subspecies of *A. obstetricans* were not resolved in our
378 study. Subspecies *pertinax* and *obstetricans* are recovered as monophyletic groups and form a clade
379 in most gene trees. On the contrary, subspecies *boscai* and *almogavarii* are not recovered as
380 monophyletic in any of the gene trees. Both taxa include very divergent lineages whose affinities
381 are uncertain (see also Maia-Carvalho et al., 2014). Delineating major clades within *A. obstetricans*

382 and resolving their evolutionary history requires extensive sampling and is thus beyond the scope of
383 this paper; however, the nuclear markers used here are informative at the intraspecific level.
384 Phylogenetic structure of lineages within *A. obstetricans* is nonetheless expected to be complicated
385 by demographic fluctuations, secondary contact and admixture across lineages during the
386 Quaternary.

387

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404

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619 **Figure legends**

620 **Fig. 1.** Distribution map of *Alytes* species and subspecies with indication of the geographical origin
621 of all samples analyzed in the present study. Light gray shading represents areas where the
622 assignment to the different *Alytes obstetricans* subspecies is doubtful (see for instance Maia-
623 Carvalho et al., 2014). Sample codes are described in Table 1.

624
625 **Fig. 2.** Phylogenetic relationships of all recognized *Alytes* species and subspecies based on (a)
626 mtDNA and (b) nDNA concatenated sequenced data under BI and ML analyses. Phylograms are
627 Bayesian consensus trees; the outgroup (*A. cisternasii*) was omitted for clarity. Posterior probability
628 and bootstrap values of well-supported nodes ($BPP \geq 0.9/BS \geq 75$) are shown at nodes. Sample codes
629 as in Table 1. RefSeq is the reference mitogenome of *A. o. pertinax* (GenBank Accession:
630 NC_006688.1, San Mauro et al., 2004), which was included for reference.

631
632 **Fig. 3.** (a). Species tree of *Alytes* based on *BEAST results of the analysis of mtDNA and four
633 nuclear genes (*β -fibint7*; *C-myc*; *PPP3CAint4* and *RPL9int4*). Values at nodes indicate Bayesian
634 posterior probabilities. (b) Bayesian phylogram based on analysis of concatenated mtDNA and
635 nDNA data. *= $BPP: \geq 0.95$ and $BS: \geq 85$.

636
637 **Fig. S1.** Bayesian nuclear gene trees of 16 samples representing all species and subspecies of *Alytes*
638 (a: *β -fibint7*; b: *PPP3CAint4*; c: *C-myc*; and d: *RPL9int4*). The outgroup *A. cisternasii* was omitted
639 for clarity. Numbers at nodes represent Bayesian posterior probabilities (only values ≥ 0.9 are
640 shown).

641
642 **Fig. S2.** Maximum likelihood gene trees of 16 samples representing all species and subspecies of
643 *Alytes* based on GARLI analyses (a: *β -fibint7*; b: *PPP3CAint4*; c: *C-myc*; and d: *RPL9int4*). The
644 outgroup *A. cisternasii* was omitted for clarity. RAXML results were very similar in topology and

645 branch support (not shown). The phylogram represents the best tree obtained by GARLI for each
646 nuclear marker. Numbers at nodes represent bootstrap values (only values ≥ 75 are shown).
647
648

Table 1

Table 1. Locality information of *Alytes* samples used in the study. The “captivity” samples (*A. muletensis*) are from a captive breeding programme at Jersey Zoo. Taxon code as in figures 2, 3B, S1 and S2. Map code as in Figure 1.

Taxa	Taxon code	Voucher	Population	Country	Map code	Latitude	Longitude
<i>A. o. pertinax</i>	A.o.pert	MAD4	Belmonte de Tajo, Madrid	Spain	9	40.13	-3.33
		CUE1	Buenache de la Sierra, Cuenca	Spain	8	40.13	-2.00
		VLC02	Algar de Palancia, Valencia	Spain	7	39.78	-0.36
<i>A. o. obstetricans</i>	A.o.obst	FRAN14	St Pierre-de-la-Fage	France	2	43.79	3.42
		FRAN01	Jublains	France	1	48.25	-0.50
		SAN01	Puerto de la Magdalena, Cantabria	Spain	3	43.35	-3.33
<i>A. o. boscai</i>	A.o.boscN	OUR03	Penalba, Ourense	Spain	13	43.42	-7.73
	A.o.boscS	MTM02	Cinfães, Serra de Montemuro	Portugal	14	41.07	-8.02
		MTM05	Cinfães, Serra de Montemuro	Portugal	14	41.07	-8.02
		SMA25	Rib. S. Bento, Serra S. Mamede	Portugal	16	39.31	-7.41
<i>A. o. almogavarii</i>	A.o.alm	HUE1	Ibón de Piedrafita, Huesca	Spain	4	42.70	-0.33
		BER1	Rasos de Peguera, Berga, Barcelona	Spain	5	42.13	1.76
		BER3	Rasos de Peguera, Berga, Barcelona	Spain	5	42.13	1.76

<i>Alytes maurus</i>	A.mau	MAR03	Bab Bou Idir, Taza	Morocco	20	34.06	-4.11
		MAR04	Bab Bou Idir, Taza	Morocco	20	34.06	-4.11
		MAR05	Bab Bou Idir, Taza	Morocco	20	34.06	-4.11
		MAR06	Bab Bou Idir, Taza	Morocco	20	34.06	-4.11
<i>Alytes dickhilleni</i>	A.dic	HG103	Puerto de las Crucetillas, Sierra de Alcaraz, Albacete	Spain	18	38.54	-2.38
		HG104	Puerto de las Crucetillas, Sierra de Alcaraz, Albacete	Spain	18	38.54	-2.38
		Aly120D	Fuente Alta, Illora, Granada	Spain	19	37.31	-3.86
		IMS4189	Riopar Viejo, Albacete	Spain	21	38.50	-2.44
<i>Alytes muletensis</i>	A.mul	CAP01	captivity	Spain	6	-	-
		AM02	captivity	Spain	6	-	-
		MAI06	Sierra de Tramuntana, Mallorca	Spain	6	39.58	2.50
<i>Alytes cisternasii</i>	A.cis	CER01	Cercal	Portugal	17	37.75	-8.65
		CER17	Cercal	Portugal	17	37.75	-8.65
		AVI02	Río Adaja, Ávila	Spain	11	40.65	-4.70
		AVI01	Río Adaja, Ávila	Spain	11	40.65	-4.70
		HG37	Montejo de la Sierra, Madrid	Spain	10	41.06	-3.53
		IDN03	Idanha-a-Nova	Portugal	15	40.00	-7.23

NAV22	Las Navas de la Concepción, Constantina	Spain	12	37.86	-5.61
IMS2004	Alcaracejos, Córdoba	Spain	22	38.38	-4.96

Table 2

Table 2. Primers used for the PCR amplification of ten mtDNA and four nuclear gene fragments in *Alytes*.

Fragment amplified	Estimated size (bp)	Primer Name	Sequence (5' - 3')	Reference
mtDNA				
RNA1	1000	Aly406F	GTCGCACGCTTCAGTTG	Present study
		Aly1405R	TAGGCTTGTCACCTCTACTC	
RNA2	1017	Aly1184F	GTAAGGGAAAGATGAAATAGC	Present study
		Aly2200R	CTTGCTTGTTAGATGAG	
ND1	1220	Aly2623F	GACAAAAATTGCAACTAAGC	Present study
		Aly3842R	ACTTTACTAGGAAGTGGCATA	
ND2	1343	Aly3825F	GCCACTTCCTAGTAAAGTCA	Present study
		Aly5167R	GGAGAAGTAGAATGAAGCTC	
COX1	1811	Aly5129F	GCTAAACGCTCAATCCAG	Present study
		Aly6940R	AATGACAGAGTGGTGATGTG	
COX2-APT6	1915	Aly6867F	GCCACTAACGAGAAAAGAG	Present study
		Aly8781R	TCGTTAGAAGTATGGTGATTAG	
COX3-ND4L	1611	Aly8617F	GTCTAATGGCACACCAAG	Present study

		Aly10227R	GTCATAGGGCTGGAATAAG	
<i>ND4-rRNALEU</i>	880	ND4	CACCTATGACTACCAAAAGCTCATGTAGAAGC	Arévalo et al. (1994)
		Leu	CATTACTTTTACTTGGATTTCACCA	
<i>ND5</i>	1185	Aly11732F	CAAAGCCTCTTGGTGCAACT	Present study
		Aly12916R	GGTTCCGGTTAGGGCTAGG	
<i>ND6</i>	718	Aly13407F	AGCTCTAGTACCACAAACAAA	Present study
nDNA				
<i>β-fibint7</i>	900	FIBX7	GGAGANAACAGNACNATGACAATNCAC	Sequeira et al. (2006)
		FIBX8	ATCTNCCATTAGGNTTGGCTGCATGGC	
	700	BFXF	CAGYACTTTYGAYAGAGACAAYGATGG	
		BFXR	TTGTACCACCAKCCACCACCRCTCTC	
<i>PPP3CAint4</i>	700	PPP3CA4F1	CTGTAYTTGTGGGCCTTGAAAATTC	Pinho et al. (2010)
		PPP3CA5R1	AAGGCATCCATGCAGGCATCATATA	
<i>C-myc</i>	1200	Cmyc1U	GAGGACATCTGGAARAARTT	Crawford (2003)
		Cmyc3cat	GTTGYTGCTGATCTGTTTGAG	Brunes et al. (2010)
<i>RPL9int4</i>	500	RPL94F	CGTGTGACAAATGGTGGGGTAA	Pinho et al. (2010)
		RPL95R	ATGGGAAAGTGAGCRTACACAGA	

Table 3

Table 3. Average number of pairwise sequence differences (*p*-uncorrected distance) across all *Alytes* taxa. Mitochondrial estimates are above diagonal and nuclear estimates (four markers concatenated) are below diagonal. Taxon “*A.o.bosc*” includes samples from the Southern and Northern lineages in *A. o. boscai* (see Maia-Carvalho et al., 2014).

	<i>A.obstetricans</i>	<i>A.o.pertinax</i>	<i>A.o.obstetricans</i>	<i>A.o.boscai</i>	<i>A.o.almogavarii</i>	<i>Baleaphryne</i>	<i>A.maurus</i>	<i>A.dickhilleni</i>	<i>A.muletensis</i>	<i>A.cisternasii</i>
<i>A.obst</i>	0	*	*	*	*	0.091	0.091	0.096	0.088	0.153
<i>A.o.pert</i>	*	0	0.009	0.024	0.029	0.092	0.092	0.096	0.088	0.154
<i>A.o.obst</i>	*	0.005	0	0.027	0.031	0.093	0.094	0.096	0.090	0.153
<i>A.o.bosc</i>	*	0.012	0.010	0	0.034	0.091	0.091	0.095	0.088	0.152
<i>A.o.alm</i>	*	0.010	0.010	0.011	0	0.090	0.088	0.096	0.085	0.153
<i>Baleap</i>	0.021	0.022	0.021	0.022	0.021	0	*	*	*	0.158
<i>A.mau</i>	0.021	0.022	0.021	0.021	0.021	*	0	0.081	0.072	0.162
<i>A.dic</i>	0.022	0.023	0.022	0.023	0.022	*	0.012	0	0.082	0.156
<i>A.mul</i>	0.021	0.021	0.021	0.021	0.020	*	0.010	0.011	0	0.155
<i>A.cist</i>	0.067	0.066	0.066	0.068	0.067	0.066	0.067	0.070	0.068	0

Table S1

Table S1. GenBank accession numbers and sequence information. Taxon code as in Table 1. Voucher numbers (in parentheses, see Table 1) indicate the main samples representing each lineage. +: indicates that the sequence was obtained from the main voucher; other codes indicate additional samples used to complement the dataset. FA: fragment amplified; FS: Fragment sequenced. Capital letters in parentheses indicate sequencing direction (F: Forward, R: Reverse). NP: nucleotide position based on the mtDNA genome (reviewed reference sequence, San Mauro et al., 2004) of *A. o. pertinax* (GI: NC_006688.1). Lowercase letters in parentheses refer to sequences obtained from GenBank: (a) Martínez-Solano et al. (2004); (b) Gonçalves et al. (2007); (c) Gonçalves et al. (2009); (d) Pinho et al. (2010); ***: no data.

Taxa	Taxon code	mtDNA fragments										nDNA - fragments								
		FA	<i>RNA1</i>	<i>RNA2</i>	<i>ND1</i>	<i>ND2</i>		<i>COX1</i>		<i>COX2-APT6</i>		<i>COX3-ND4L</i>		<i>ND4</i>	<i>ND5</i>	<i>ND6</i>	<i>β-fibin7</i>	<i>PPP3CAint4</i>	<i>RPL9int4</i>	<i>C-myc</i>
		FS	mt01(F+R)	mt02(F+R)	mt03(F+R)	mt04(F)	mt05(R)	mt06(F)	mt07(R)	mt08(F)	mt09(R)	mt10(F)	mt11(R)	mt12(F+R)	mt13(F+R)	mt14(F+R)				
		NP	482-1387	1952-2156	2688-3780	3878-4477	4855-5115	5181-5913	6516-6898	6920-7607	8202-8703	8731-9313	9624-10170	10880-11693	11788-12861	13468-14119				
<i>A. o. pertinax</i>	A.o.pert01		+	+	+	+	+	+	+	+	+	+	+	(b)	+	+	(b)	+	+	VLC22
	(MAD04)		KJ858769	KJ858787	KJ858803	KJ858821	KJ858839	KJ858857	KJ858875	KJ858893	KJ858911	KJ858929	KJ858947	EF441298	KJ858970	KJ858988	EF441322/EF441323	KJ859033	KJ859047	KJ859009/KJ859010
	A.o.pert02		+	+	+	+	+	+	+	+	+	+	+	(b)	+	+	(b)	(d)	(d)	+
	(CUE01)		KJ858770	KJ858788	KJ858804	KJ858822	KJ858840	KJ858858	KJ858876	KJ858894	KJ858912	KJ858930	KJ858948	EF441299	KJ858971	KJ858989	EF441324	GU181146	GU181179	KJ859011/KJ859012
<i>A. o. obstetricans</i>	A.o.obst01		+	+	+	+	+	+	+	+	+	+	+	+	+	+	SAN01	+	+	SAN01
	(FRAN14)		KJ858771	KJ858789	KJ858805	KJ858823	KJ858841	KJ858859	KJ858877	KJ858895	KJ858913	KJ858931	KJ858949	KJ858965	KJ858972	KJ858990	KJ859005	KJ859034	KJ859048	KJ859013
	A.o.obst02		+	+	+	+	+	+	+	+	+	+	+	(b)	+	+	(b)	(d)	(d)	+
	(FRAN01)		KJ858772	KJ858790	KJ858806	KJ858824	KJ858842	KJ858860	KJ858878	KJ858896	KJ858914	KJ858932	KJ858950	EF441292	KJ858973	KJ858991	EF441316	GU181145	GU181178	KJ859014/KJ859015
<i>A. o. boscai</i>	A.o.bosc01N		+	+	+	+	+	+	+	+	+	+	+	(b)	+	+	(b)	(d)	(d)	+
	(OUR03)		KJ858773	KJ858791	KJ858807	KJ858825	KJ858843	KJ858861	KJ858879	KJ858897	KJ858915	KJ858933	KJ858951	EF441295	KJ858974	KJ858992	EF441319	GU181147/KJ859035	GU181180	KJ859016
	A.o.bosc02S		+	+	+	+	+	+	+	+	+	+	+	(b)	+	+	(b)	+	+	+
	(SMA25)		KJ858774	KJ858792	KJ858808	KJ858826	KJ858844	KJ858862	KJ858880	KJ858898	KJ858916	KJ858934	KJ858952	EF441303	KJ858975	KJ858993	EF441330	KJ859036/KJ859037	KJ859049/KJ859050	KJ859017

	A.o.bosc03S	+	+	+	+	+	+	+	+	+	+	+	+	MTM02	+	MTM02	+	+	+
	(MTM05)	KJ858775	KJ858793	KJ858809	KJ858827	KJ858845	KJ858863	KJ858881	KJ858899	KJ858917	KJ858935	KJ858953	KJ858966	KJ858976	KJ858994	KJ859006/KJ859007	KJ859038	KJ859051	KJ859018
<i>A. o. almogavarii</i>	A.o.alm01	+	+	+	+	+	+	+	+	+	+	+	(b)	+	+	(b)	(d)	(d)	+
	(BER01)	KJ858776	KJ858794	KJ858810	KJ858828	KJ858846	KJ858864	KJ858882	KJ858900	KJ858918	KJ858936	KJ858954	EF441305	KJ858977	KJ858995	EF441332/EF441333	GU181144	KJ859052/GU181177	KJ859019/KJ859020
	A.o.alm02	+	+	+	+	+	+	+	+	+	+	+	(b)	+	+	(b)	+	+	+
	(BER03)	KJ858777	KJ858795	KJ858811	KJ858829	KJ858847	KJ858865	KJ858883	KJ858901	KJ858919	KJ858937	KJ858955	EF441306	KJ858978	KJ858996	EF441334/EF441335	KJ859039/KJ859040	KJ859053	KJ859021
	A.o.almHUE	+	+	+	+	+	+	+	+	+	+	+	(b)	+	+	(b)	+	+	+
	(HUE01)	KJ858778	KJ858796	KJ858812	KJ858830	KJ858848	KJ858866	KJ858884	KJ858902	KJ858920	KJ858938	KJ858956	EF441304	KJ858979	KJ858997	EF441331	KJ859041/KJ859042	KJ859054/KJ859055	KJ859022
<i>Alytes maurus</i>	A.mau01	+	+	+	+	+	+	+	+	+	+	+	(b)	+	+	(b)	+	+	MAR06
	(MAR04)	KJ858779	KJ858797	KJ858813	KJ858831	KJ858849	KJ858867	KJ858885	KJ858903	KJ858921	KJ858939	KJ858957	EF441312	KJ858980	KJ858998	EF441341	KJ859043	KJ859056	KJ859023
	A.mau02	+	+	+	+	+	+	+	+	+	+	+	MAR03 (b)	+	+	MAR03 (b)	MAR03 (d)	+	+
	(MAR05)	KJ858780	KJ858798	KJ858814	KJ858832	KJ858850	KJ858868	KJ858886	KJ858904	KJ858922	KJ858940	KJ858958	EF441311	KJ858981	KJ858999	EF441340	GU181141	KJ859057	KJ859024
	<i>Alytes dickhilleni</i>	A.dic01	+	+	+	+	+	+	+	+	+	+	+	+	+	HG103 (b)	+	+	+
	(HG104)	KJ858781	KJ858799	KJ858815	KJ858833	KJ858851	KJ858869	KJ858887	KJ858905	KJ858923	KJ858941	KJ858959	KJ858967	KJ858982	KJ859000	EF441338	KJ859044	KJ859058	KJ859025/KJ859026
	A.dic_02	+	+	+	+	+	+	+	+	+	+	+	+	+	+	IMS4189	+	+	+
	(Aly120D)	KJ858782	KJ858800	KJ858816	KJ858834	KJ858852	KJ858870	KJ858888	KJ858906	KJ858924	KJ858942	KJ858960	KJ858968	KJ858983	KJ859001	KJ859008	KJ859045	KJ859059	KJ859027
<i>Alytes muletensis</i>	A.mul01	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	(CAP01)	KJ858783	KJ858801	KJ858817	KJ858835	KJ858853	KJ858871	KJ858889	KJ858907	KJ858925	KJ858943	KJ858961	KJ858969	KJ858984	KJ859002	***	KJ859046	KJ859060/KJ859061	KJ859028
	A.mul02	+	MAI06	+	+	+	+	MAI06	+	MAI06	+	+	MAI06 (b)	+	+	MAI06 (b)	MAI06 (d)	MAI06 (d)	MAI06
	(AM02)	KJ858784	KJ858802	KJ858818	KJ858836	KJ858854	KJ858872	KJ858890	KJ858908	KJ858926	KJ858944	KJ858962	EF441310	KJ858985	KJ859003	EF441339	GU181142	GU181175	KJ859029
	<i>Alytes cisternasii</i>	A.cis01	+	IMS2004 (a)	+	+	+	+	+	+	+	+	HG112 (b)	+	+	HG112 (b)	HG112 (d)	HG112 (d)	HG112
	(MAD01)	KJ858785	AY442027	KJ858819	KJ858837	KJ858855	KJ858873	KJ858891	KJ858909	KJ858927	KJ858945	KJ858963	EF441313	KJ858986	KJ859004	EF441342	GU181140	GU181173	KJ859030
	A.cis02	+		+	+	+	+	+	+	+	+	+	CER01 (b)	+		CER01 (b)	CER01 (c)	CER01	NAV22
	(IDN03)	KJ858786	***	KJ858820	KJ858838	KJ858856	KJ858874	KJ858892	KJ858910	KJ858928	KJ858946	KJ858964	EF441314	KJ858987	***	EF441342	GU086791	KJ859062	KJ859031/KJ859032

Table S2

Table S2. Optimal partition schemes (eight for mtDNA and three for nDNA) and corresponding nucleotide substitution models selected by PARTITIONFINDER for MRBAYES and GARLI analyses, based on the Bayesian Information Criterion. The ordinal numbers 1st, 2nd and 3rd correspond to codon positions in the respective gene. FS: Fragment sequenced. FC: functional category.

Marker	FS	FC	Input file	Partition	Best fit model for partition (PFinder)	Best fit model (jModeltest)
mtDNA						
	mt01	<i>RNA</i>	mt01	mtDNA1	HKY + I + G	
	mt02	<i>RNA</i>	mt02	mtDNA2	TrNef + I	
	mt03	<i>RNA</i>	mt03a	mtDNA3	JC	
		<i>ND1</i>	mt03b 1st	mtDNA1		
			mt03b 2nd	mtDNA4	F81	
			mt03b 3rd	mtDNA5	TrN +I + G	
		<i>RNA</i>	mt03c	mtDNA1		
	mt04	<i>RNA</i>	mt04a	mtDNA6	TrN +I	
		<i>ND2</i>	mt04b 1st	mtDNA1		
			mt04b 2nd	mtDNA6		
			mt04b 3rd	mtDNA5		

mt05	<i>ND2</i>	mt05a 1st	mtDNA5
		mt05a 2nd	mtDNA1
		mt05a 3rd	mtDNA6
	<i>RNA</i>	mt05b	mtDNA1
mt06	<i>RNA</i>	mt06a	mtDNA1
	<i>COX1</i>	mt06b 1st	mtDNA2
		mt06b 2nd	mtDNA4
		mt06b 3rd	mtDNA5
mt07	<i>COX1</i>	mt07a 1st	mtDNA4
		mt07a 2nd	mtDNA5
		mt07a 3rd	mtDNA2
	<i>RNA</i>	mt07b	mtDNA1
mt08	<i>RNA</i>	mt08a	mtDNA1
	<i>COX2</i>	mt08b 1st	mtDNA2
		mt08b 2nd	mtDNA4
		mt08b 3rd	mtDNA5
mt09	<i>APT6</i>	mt09a 1st	mtDNA5

		mt09a 2nd	mtDNA1
		mt09a 3rd	mtDNA4
	<i>COX3</i>	mt09b 1st	mtDNA3
		mt09b 2nd	mtDNA4
		mt09b 3rd	mtDNA1
mt10	<i>COX3</i>	mt10 1st	mtDNA6
		mt10 2nd	mtDNA5
		mt10 3rd	mtDNA2
mt11	<i>ND3</i>	mt11a 1st	mtDNA1
		mt11a 2nd	mtDNA6
		mt11a 3rd	mtDNA5
	<i>RNA</i>	mt11b	mtDNA1
	<i>ND4L</i>	mt11c 1st	mtDNA1
		mt11c 2nd	mtDNA4
		mt11c 3rd	mtDNA5
mt12	<i>ND4</i>	mt12a 1st	mtDNA5
		mt12a 2nd	mtDNA1

		mt12a 3rd	mtDNA6		
	<i>RNA</i>	mt12b	mtDNA1		
mt13	<i>ND5</i>	mt13 1st	mtDNA5		
		mt13 2nd	mtDNA1		
		mt13 3rd	mtDNA6		
mt14	<i>ND5</i>	mt14a 1st	mtDNA5		
		mt14a 2nd	mtDNA1		
		mt14a 3rd	mtDNA4		
	<i>ND6</i>	mt14b 1st	mtDNA7	TIM + G	
		mt14b 2nd	mtDNA6		
		mt14b 3rd	mtDNA8	TrN + G	
	<i>RNA</i>	mt14c	mtDNA1		
nDNA					
	<i>β-fibint7</i>		nDNA1	HKY+G	F81
	<i>PPP3CAint4</i>		nDNA1		HKY
	<i>RPL9int4</i>		nDNA1		F81+G

C-myc

Exon2 1st nDNA2

JC

F81+G

Exon2 2nd nDNA2

Exon2 3rd nDNA2

Intron2 nDNA3

F81

Exon3 1st nDNA2

Exon3 2nd nDNA2

Exon3 3rd nDNA2

Table S3

1 **Table S3.** Summary results of genetic diversity in mtDNA and nuclear gene sequences. N: Number of samples, S: Number of polymorphic sites; Eta:
2 Total number of mutations; h: Total number of haplotypes; π : nucleotide diversity; SD: (standard deviation), and θ : Theta. Text in parentheses refers to
3 the fragment sequenced - FS (see Table S1).

4

Marker	Functional Categories	Clade	Length	Polymorphism						
				N	S	Eta	h	π	SD(π)	θ
mtDNA		<i>Alytes</i>	960-963	18	254	285	16	0.0849	0.0133	0.0769
	(mt03)	<i>Alytes obstetricans</i>	963	9	57	57	8	0.0234	0.0032	0.0218
		<i>Baleaphryne</i>	963	6	122	127	5	0.0686	0.0109	0.0555
		<i>Alytes</i>	665	18	36	42	16	0.0270	0.0133	0.0843
	(mt04+ mt05)	<i>Alytes obstetricans</i>	665	9	50	50	8	0.0282	0.0045	0.0277
		<i>Baleaphryne</i>	665	6	91	93	5	0.0714	0.0113	0.0599
		<i>Alytes</i>	949	18	228	259	18	0.0805	0.0114	0.0699
	mt06+07	<i>Alytes obstetricans</i>	949	9	53	53	9	0.0210	0.0031	0.0206
		<i>Baleaphryne</i>	949	6	94	95	6	0.0516	0.0077	0.0539
		<i>Alytes</i>	590	18	142	164	17	0.0772	0.0120	0.0700
	(mt08)	<i>Alytes obstetricans</i>	590	9	39	39	9	0.0261	0.0034	0.0243
		<i>Baleaphryne</i>	590	6	65	66	5	0.0596	0.0090	0.0483

<i>ATP6</i>	<i>Alytes</i>	420	18	114	130	16	0.0865	0.0135	0.0789
(mt09)	<i>Alytes obstetricans</i>	420	9	22	22	8	0.0194	0.0030	0.0193
	<i>Baleaphryne</i>	420	6	51	53	5	0.0641	0.0096	0.0532
<i>COX3</i>	<i>Alytes</i>	665	18	140	164	16	0.0665	0.0096	0.0612
mt09+10	<i>Alytes obstetricans</i>	665	9	38	38	8	0.0221	0.0031	0.0210
	<i>Baleaphryne</i>	665	6	61	64	5	0.0491	0.0073	0.0402
<i>ND3</i>	<i>Alytes</i>	195	18	43	52	14	0.1246	0.0179	0.1069
(mt11)	<i>Alytes obstetricans</i>	195	9	18	18	8	0.0362	0.0057	0.0340
	<i>Baleaphryne</i>	195	6	27	27	4	0.1214	0.0188	0.1011
<i>ND4L</i>	<i>Alytes</i>	282	18	76	87	15	0.0859	0.0128	0.0784
(mt11)	<i>Alytes obstetricans</i>	282	9	17	17	8	0.0219	0.0044	0.0222
	<i>Baleaphryne</i>	282	6	40	42	4	0.0768	0.0115	0.0621
<i>ND4</i>	<i>Alytes</i>	677	18	206	241	17	0.1036	0.0149	0.0958
(mt12)	<i>Alytes obstetricans</i>	677	9	70	74	8	0.0395	0.0054	0.0381
	<i>Baleaphryne</i>	677	6	90	93	6	0.0754	0.0111	0.0630
<i>ND5</i>	<i>Alytes</i>	1174	18	322	374	18	0.1022	0.0152	0.0872
(mt13)	<i>Alytes obstetricans</i>	1174	9	64	64	9	0.0235	0.0038	0.0219
	<i>Baleaphryne</i>	1174	6	141	145	6	0.0692	0.0104	0.0575
<i>ND6</i>	<i>Alytes</i>	510	17	157	187	16	0.0978	0.0142	0.0911
(mt14)	<i>Alytes obstetricans</i>	510	9	39	39	8	0.0301	0.0045	0.0281

nDNA	Non coding	<i>Baleaphryne</i>	510	6	83	87	6	0.0873	0.0129	0.0713
		<i>Alytes</i>	1954	17	245	267	17	0.0310	0.00622	0.03736
		<i>Alytes obstetricans</i>	1954	9	61	63	9	0.0112	0.00159	0.01152
	Coding	<i>Baleaphryne</i>	1954	6	66	68	6	0.0178	0.00266	0.01486
		<i>Alytes</i>	7091	17	1898	2185	17	0.0816	0.0125	0.08069
		<i>Alytes obstetricans</i>	7091	9	478	482	9	0.0259	0.00364	0.02481
	<i>β-fibint7</i>	<i>Baleaphryne</i>	7091	6	878	905	6	0.0669	0.00995	0.05523
		<i>Alytes</i>	634	36	76	77	17	0.02977	0.00261	0.02995
		<i>Alytes obstetricans</i>	634	18	37	37	12	0.01744	0.00139	0.01707
		<i>Baleaphryne</i>	634	12	12	12	3	0.00932	0.00088	0.00637
		<i>PPP3CAint4</i>	712	36	74	77	15	0.0252	0.0051	0.0262
		<i>Alytes obstetricans</i>	712	18	14	15	8	0.0059	0.0006	0.0059
		<i>Baleaphryne</i>	712	12	14	14	5	0.0088	0.0008	0.0067
		<i>C-myc</i>	1317	36	85	86	20	0.0150	0.00247	0.01601
		<i>Alytes obstetricans</i>	1317	18	21	21	12	0.0045	0.00038	0.00473
		<i>Baleaphryne</i>	1317	12	15	15	6	0.0047	0.0004	0.0039
	<i>RPL9int4</i>	<i>Alytes</i>	479	36	76	81	18	0.04803	0.0116	0.0513
		<i>Alytes obstetricans</i>	479	18	19	19	10	0.0141	0.0017	0.0123
		<i>Baleaphryne</i>	479	12	15	15	6	0.0134	0.0015	0.0111

Figure1
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Figure2
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Figure 3
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(a)

(b)

(a) *β-fibin7*

(b) *PPP3CAint4*

(c) *C-myc*

(d) *RPL9int4*