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Multilocus species tree analyses resolve the radiation of the widespread *Bufo bufo* species group (Anura, Bufonidae)

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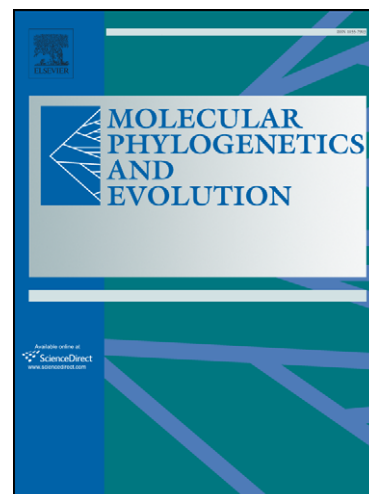
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1 **Multilocus species tree analyses resolve the radiation of the widespread *Bufo***  
2 ***bufo* species group (Anura, Bufonidae)**

3

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29

30 **Abstract**

31 New analytical methods are improving our ability to reconstruct robust species trees from  
32 multilocus datasets, despite difficulties in phylogenetic reconstruction associated with recent,  
33 rapid divergence, incomplete lineage sorting and/or introgression. In this study, we applied  
34 these methods to resolve the radiation of toads in the *Bufo bufo* (Anura, Bufonidae) species  
35 group, ranging from the Iberian Peninsula and North Africa to Siberia, based on sequences  
36 from two mitochondrial and four nuclear DNA regions (3490 base pairs). We obtained a  
37 fully-resolved topology, with the recently described *Bufo eichwaldi* from the Talysh  
38 Mountains in south Azerbaijan and Iran as the sister taxon to a clade including: 1) north  
39 African, Iberian, and most French populations, referred herein to *Bufo spinosus* based on the  
40 implied inclusion of populations from its type locality; and 2) a second clade, sister to *B.*  
41 *spinosus*, including two sister subclades: one with all samples of *Bufo verrucosissimus* from  
42 the Caucasus and another one with samples of *Bufo bufo* from northern France to Russia,  
43 including the Apennine and Balkan peninsulas and most of Anatolia. Coalescent-based  
44 estimations of time to most recent common ancestors for each species and selected subclades  
45 allowed historical reconstruction of the diversification of the species group in the context of  
46 Mediterranean paleogeography and indicated a long evolutionary history in this region.  
47 Finally, we used our data to delimit the ranges of the four species, particularly the more  
48 widespread and historically confused *B. spinosus* and *B. bufo*, and identify potential contact  
49 zones, some of which show striking parallels with other co-distributed species.

50

51 **Keywords:** Amphibia, *Bufo bufo*, *Bufo spinosus*, mitochondrial DNA, nuclear DNA, species  
52 trees

53

54

**55 1. Introduction**

56 The long-term goal of Systematics is understanding the historical relationships among  
57 organisms, and a powerful tool to achieve this goal is to estimate robust species trees  
58 (Edwards, 2009). The importance of combining information from multiple, unlinked loci in  
59 individuals from a geographically comprehensive sample to obtain robust estimates of  
60 phylogenetic relationships has been long recognized. Recently, the increased availability of  
61 molecular data and the parallel development of efficient analytical tools to extract the  
62 information have increased the accuracy of species tree estimation (Liu et al., 2008; Kubatko  
63 et al., 2009; Heled & Drummond, 2010). New analytical tools are improving our ability to  
64 reconstruct species trees from multilocus datasets, despite difficulties associated with the  
65 processes of recent, rapid divergence, incomplete lineage sorting and/or introgression in  
66 phylogenetic reconstruction (Chung & Ané, 2011). For instance, methodological advances  
67 related to estimation of the timing of population divergence, and in particular the  
68 incorporation in the analyses of the uncertainty associated to estimates of substitution rates  
69 and the temporal constraints imposed by the fossil record have greatly improved our  
70 understanding about the timescales involved in species formation in a wide variety of  
71 taxonomic groups (for example, Aldenhoven et al., 2010; Arora et al., 2010; Blackburn et al.,  
72 2010). This has also led to taxonomic revision of historically problematic groups, which  
73 include widespread and morphologically undifferentiated species (for example, Gvozdík et  
74 al., 2010; Salicini et al., in press; Sanguila et al., in press).

75       Until recently, the genus *Bufo* contained some of the largest number of amphibian  
76 species in the world, with a total of 283 described toad species. As a result of its paraphyly it  
77 was split by Frost et al. (2006) into several genera. Far from adequately solving the  
78 systematics, this highlighted the complexity of the group. As an example, the green toad  
79 (*Bufo viridis*) that is widespread throughout Eurasia and parts of northern Africa, has a  
80 complex genetic structure, including differences in ploidy levels across species and  
81 populations (Stöck et al., 2006). It was split into several species, with additional changes in  
82 green toad taxonomy expected in the near future (Stöck et al., 2006, 2008). *Bufo bufo*  
83 (Linnaeus 1758) (Anura, Bufonidae), another widespread species with an extensive Euro-  
84 Asiatic distribution, was formerly included in the *B. bufo* species group of Inger (1972),  
85 including species from Europe through Asia. Recent studies have recognized a marked  
86 distinction between western and eastern species groups, some within the latter now  
87 recognized as distinct species, like *B. japonicus* (Matsui, 1980, 1984; Igawa et al., 2006) and  
88 *B. gargarizans* (Gumilevskij, 1936; Matsui, 1986; Macey et al., 1998; Liu et al., 2000; Fu et  
89 al., 2005). Here we focus on the western species group, including *B. bufo* and related species,  
90 that ranges from the Iberian Peninsula and North Africa in the west to northern Kazakhstan  
91 and eastern Siberia in the east (IUCN, 2009, Fig. 1). However, the boundaries of the different  
92 species and their phylogenetic relationships remain poorly known, and a recent study has

93 shown that the actual species diversity in the group is underestimated (Litvinchuk et al.,  
94 2008), highlighting the need for more work.

95 Litvinchuk et al. (2008) described a new species from the Talysh Mountains in south  
96 Azerbaijan and Iran, *B. eichwaldi*, which is morphologically and genetically distinct from *B.*  
97 *bufo*, and discussed its relationships with *B. bufo* and *B. verrucosissimus*. However, their  
98 study lacked comprehensive sampling and did not include populations from western Europe,  
99 which in turn prevented a thorough re-evaluation of the status of *B. bufo* - in particular, the  
100 delineation of the ranges of the most widespread subspecies: *B. b. bufo* and *B. b. spinosus*,  
101 whose type localities are in Sweden and southern France, respectively (Frost, 2011). The three  
102 subspecies traditionally recognized in *B. bufo* (*B. b. bufo*, *B. b. gredosicola*, and *B. b.*  
103 *spinosus*) (Dubois & Bour, 2010) were originally described based on morphological  
104 differences. Additionally, Litvinchuk et al. (2008), based on allozyme data, suggested that the  
105 available name *B. b. palmarum* be used for the Italian populations of this species. In any case,  
106 the relative ranges and potential areas of overlap between the different subspecies remain  
107 unknown and their taxonomic status is disputed because of the lack of consistence of  
108 purported diagnostic characters across populations (see for instance De Lange, 1973; Lüscher  
109 et al., 2001; García-París et al., 2004). Preliminary mtDNA studies based on partial sequences  
110 of the control region did not find evidence for geographical structuring of populations of *B.*  
111 *bufo* in the Iberian Peninsula, where up to three subspecies have been reported; instead, the  
112 Iberian populations form a well-supported clade with respect to north African samples  
113 (Martínez-Solano & González, 2008). Within the Caucasian species *Bufo verrucosissimus*,  
114 three to four morphological subspecies are currently recognized (*B. v. verrucosissimus*, *B. v.*  
115 *turovi*, *B. b. circassicus*, and *B. b. tertyschnikovi*; see Orlova, Tuniyev, 1989; Kidov, 2009);  
116 but their taxonomic status remains questionable (Kuzmin, 1999; Litvinchuk et al., 2008).  
117 Also, the distribution limits of *B. verrucosissimus* and *B. bufo* in North Anatolia are unclear  
118 (Eiselt, 1965; Baran & Atatur, 1998).

119 In this paper, we present a comprehensive assessment of the diversification history of  
120 the *Bufo bufo* species group in Europe using molecular data from the mitochondrial and  
121 nuclear genomes, including all described species and subspecies with full geographic  
122 coverage. In particular, the objectives of our study are: 1) resolving phylogenetic relationships  
123 between species in the widespread *Bufo bufo* species group; 2) reconstructing well-dated and  
124 robust paleobiogeographic scenarios for the evolution of the group, and 3) use this  
125 information to delineate the ranges of the species and identify potential contact zones.

126

## 127 **2. Material and methods**

### 128 *2.1. Sampling and sequencing*

129 A total of 232 individuals of *B. bufo sensu lato* (i. e., including all currently recognized  
130 subspecies) and related species (*B. verrucosissimus* and *B. eichwaldi*, including all currently

131 recognized subspecies, see Kutrup et al., 2006; Litvinchuk et al., 2008; Sinsch et al., 2009)  
132 were included in the study (Table 1, Fig. 1). We collected samples across all the species  
133 ranges, with a focus in the three main southern peninsulas in Europe, and including  
134 populations from North Africa (Morocco and Tunisia), Caucasian isthmus and Anatolia. As  
135 outgroups we included nine samples from other species previously considered part of the *B.*  
136 *bufo* species group (in the “eastern species group” cited above): *B. bankorensis*, *B.*  
137 *gargarizans*, *B. japonicus*, and *B. torrenticola* (Table 1).

138 Genomic DNA was extracted from tissues (tail or toe clips), preserved in 95% ethanol  
139 or frozen, using commercial kits (Nucleospin). We amplified by polymerase chain reaction  
140 (PCR) and sequenced fragments of two mitochondrial genes (16S and cytochrome *b*, *cyt-b*) in  
141 all 241 samples, and four nuclear genes (proopiomelanocortin: POMC, chemokine (C-X-C)  
142 receptor 4: CXCR4, brain-derived neurotrophic factor: BDNF, and ribosomal protein L3:  
143 RPL3) in 52-55 samples representing all major mtDNA clades (see Table 1). PCR conditions  
144 followed standard procedures (see Martínez-Solano & González, 2008), with primers and  
145 annealing temperatures listed in Table 2.

146

#### 147 2.2. Molecular diversity, neutrality and recombination tests

148 Number of haplotypes and values of mean haplotype ( $h$ ) and nucleotide diversities ( $\pi$ ) (Nei,  
149 1987) for mtDNA (16S + *cyt-b*) were estimated within and between species using DnaSP v5.0  
150 (Librado & Rozas, 2009). Genetic distances within and between species were calculated with  
151 MEGA5 (Tamura et al., 2011). To test whether the data were consistent with neutral  
152 expectations, we also calculated Tajima’s D statistic (Tajima, 1989) and Fu & Li’s (1993) D  
153 and F statistics for each locus using DnaSP. Recombination in the nuclear markers was  
154 assessed using the pairwise homoplasy index (PHI - - statistic, Bruen et al., 2006)  
155 implemented in Splitstree v.4.11 (Huson & Bryant, 2006).

156

#### 157 2.3. Phylogenetic analyses

158 For each data set (16S, *cyt-b*, POMC, CXCR4, BDNF and RPL3), the best-fit model of  
159 evolution was estimated based on the Bayesian Information Criterion (BIC) as implemented  
160 in jModeltest 1.0 (Guindon & Gascuel, 2003; Posada, 2008). Phylogenetic analyses were  
161 based on Bayesian inference, maximum likelihood (ML) and maximum parsimony (MP)  
162 methods as implemented in MrBayes v3.1.2 (Huelsenbeck & Ronquist, 2001), Garli version  
163 2.0 (Zwickl, 2006) and PAUP\* v4.10 (Swofford, 2001) respectively. Several independent  
164 analyses were run: 1) a combined mtDNA (16S + *cyt-b*) dataset including only one  
165 representative of each haplotype in the dataset; 2) a combined nDNA (POMC, CXCR4,  
166 BDNF and RPL3) dataset; and 3) a combined mtDNA + nDNA dataset. Bayesian and ML  
167 analyses were run with the different genes set as independent partitions, with the models  
168 selected by jModeltest. Support for branches was based on Bayesian posterior probabilities

169 (BPPs) and bootstrap values for ML and MP analyses. For analyses with Garli, 100 bootstrap  
170 replicates were run, with five search replicates per bootstrap replicate. In PAUP, bootstrap  
171 values were estimated from 1000 replicates, with a maximum of 1000 (mtDNA, dataset 1) or  
172 20000 (datasets 2 and 3) trees saved per random addition replicate in order to keep  
173 computation time within reasonable bounds. Sequence alignments were deposited in  
174 TreeBase under accession number 11885.

175 We also estimated time to most recent common ancestor (TMRCA) of selected  
176 haplogroups in the mtDNA dataset, corresponding to each species as well as geographically  
177 based haplogroups where relevant using BEAST v1.6.1 (Drummond & Rambaut, 2007). For  
178 these analyses, we considered two partitions, one for each gene (16S and cyt-b), with  
179 substitution models selected based on jModeltest results, and a relaxed (uncorrelated-  
180 lognormal) clock model, since preliminary runs showed it fit the data better than a strict clock  
181 model (i.e., the 95% highest posterior density (HPD) interval for the parameter “coefficient of  
182 variation” did not include zero). We specified a substitution rate sampled from a normal  
183 distribution with a mean of 0.0069 and a standard deviation of 0.0017 substitutions per site  
184 per million years, based on rates estimated by Macey et al. (1998) in related species. We used  
185 fossil data as prior information to set bounds on some TMRCAs, based on the oldest fossil  
186 remains of *B. bufo* (*sensu lato*) and *B. verrucosissimus* in Europe (see Martín & Sanchiz,  
187 2011). There are Miocene records from fossil sites in Moldavia (9.7-11.1 million years ago –  
188 Mya-) that have been assigned to *B. bufo*. Since at present there are no diagnostic osteological  
189 features distinguishing between species in the *B. bufo* group (except for *B. verrucosissimus*,  
190 Chkhikvadze, 1984; Ratnikov, 2001; Pisanets et al., 2009), the precise taxonomic assignment  
191 of these remains is uncertain, so we conservatively used this information to place a minimum  
192 age for the TMRCA of all species in the group. We implemented this by setting a prior  
193 distribution for their TMRCA following a lognormal distribution with an offset of 9.7 Mya,  
194 and 95% of the values between 10.1 and 22.2 Mya. On the other hand, the oldest fossil  
195 remains confidently assigned to *B. verrucosissimus* are from the Pliocene of Russia (1.81-2.59  
196 Mya), so we used this information to set a lognormal prior with an offset of 1.81 Mya and  
197 95% of the values between 2 and 4.5 Mya. The birth-death process was specified for the tree  
198 prior, since it is well suited to a multi-species dataset with deep genetic divergence across  
199 clades and species. Four independent runs of 25 million generations, sampling every 2,500  
200 generations, were combined, after checking for convergence and adequate effective sample  
201 sizes (ESSs) of parameters of interest using the software Tracer (Rambaut & Drummond,  
202 2007), to derive the median values and 95% highest posterior density intervals (HPDIs) of the  
203 corresponding TMRCAs.

204 Finally, we used the species tree reconstruction method implemented in \*BEAST  
205 (Drummond & Rambaut, 2007; Heled & Drummond, 2010) to address phylogenetic  
206 relationships in the *B. bufo* species group. *Bufo gargarizans* was used as an outgroup. This

207 analysis considered five independent partitions: 16S + cyt-b, POMC, CXCR4, BDNF and  
208 RPL3. For each of them, models of substitution were based on results of jModeltest. Clock  
209 rate and topology were unlinked in the five partitions. The birth-death process was chosen as  
210 the coalescent prior for the species tree. Three independent analyses were run for 50 million  
211 generations each, sampling every 5,000 generations. Results were visualized in Tracer to  
212 check for convergence and adequate effective sample sizes (ESSs) of parameters.

213

### 214 3. Results

#### 215 3.1. mtDNA

216 The mtDNA alignment contained 1239 sites across the 241 specimens studied (335 of them  
217 polymorphic), defining 123 haplotypes. Those more widespread and found at higher  
218 frequencies include haplotype 2 (N=11), found in samples from the Netherlands, northern  
219 France, Germany, Belgium and Hungary (samples BB002, BB142, BB143, BB144, BB149,  
220 BB164, BB170, BB171, BB186, BB188, BB189); haplotype 6 (N=11), in samples from  
221 southern France and north-eastern Spain (Pyrenees) (samples BB006, BB012, BB117,  
222 BB118, BB147, BB166, BB191, BB193, OBYX6, OBYX9 and OBYX19); and haplotype 9  
223 (N=25), in samples from Serbia, Hungary, Slovakia, Ukraine, Russia, and Romania (samples  
224 BB14, BB33, BB34, BB35, BB44, BB52, BB53, BB74, BB75, BB76, BB105, BB123,  
225 BB126, BB127, BB128, BB159, BB160, BB161, BB162, BB163, BB180, BB181, BB185,  
226 RO1, RO3) (Table 1). Phylogenetic analyses based on mtDNA data produced a well-resolved  
227 tree (Fig. 2A), with *B. eichwaldi* as the sister group of two clades (BPP=1.0/ MP  
228 bootstrap=100/ ML bootstrap=100). Clade 1 (subsequently referred to as *Bufo spinosus*, see  
229 below) includes samples from North Africa, the Iberian Peninsula and France (1.0/100/100).  
230 *Bufo spinosus* is sister (1.0/97/100) to Clade 2 (1.0/100/100), which includes two subclades:  
231 one comprising samples from all other populations of *B. bufo*, from northern France to  
232 Siberia, including the Apennine peninsula and Sicily, the Balkan Peninsula and most of  
233 Anatolia (1.0/96/91), and another, weakly supported clade (<0.90/64/52), including all  
234 samples of *B. verrucosissimus*. At least three major subclades are recovered within *B.*  
235 *spinosus*: 1) samples from Morocco (1.0/100/100); 2) samples from Tunisia (1.0/100/99); and  
236 3) samples from the Iberian Peninsula and most of France (1.0/100/100). Samples from  
237 Morocco and Tunisia are closely related to one another (1.0/100/99). Within *B. bufo* there are  
238 several well-supported clades, including: 1) southern Italy and Sicily (1.0/90/82); 2) the  
239 remaining samples from Italy (center and north) plus samples from the Balkans in Serbia,  
240 Montenegro, Albania, Macedonia and Greece (0.95/73/58); and 3) the remaining samples  
241 (1.0/84/79), including haplotypes also found in the Balkans (Greece, Serbia, Montenegro),  
242 Anatolia, and also all samples from central, western and northern Europe (Germany,  
243 Netherlands, northern France, Belgium, Hungary, England, Poland, Russia, Ukraine, Sweden,  
244 Romania, Slovakia), which form a well-supported subclade (1.0/80/92) that also includes



245 samples from the Balkans (Serbia, Montenegro, Croatia) (Fig. 2A, Table 1). Finally, within *B.*  
 246 *verrucosissimus* there are two well-supported subclades: one mainly distributed north of the  
 247 Caucasus and comprising all samples from Russia plus one sample from Abkhazia, Georgia  
 248 (BB059, Malaya Ritza lake) (1.0/80/73); and a second one including the remaining samples  
 249 from southern Georgia and Turkey (0.99/94/91), south of the Caucasus. These groups do not  
 250 reflect current subspecific assignments (Table 1), but rather allopatric groups of populations  
 251 in the northwestern slopes of the Great Caucasus versus Lesser Caucasus and southern slope  
 252 of the Great Caucasus, respectively (Fig. 1, Table 1).

253 Mean pairwise (p-uncorrected  $\pm$  standard deviations) genetic distances between  
 254 species are presented in Table 3 and range from  $1.6 \pm 0.3\%$  (between *B. bufo* and *B.*  
 255 *verrucosissimus*) to  $7.7 \pm 0.7\%$  (between *B. spinosus* and *B. eichwaldi*). Within *B. bufo* the  
 256 mean uncorrected distance was  $0.8 \pm 0.1\%$ ; within *B. spinosus* it was  $1.5 \pm 0.1\%$ , and within  
 257 *B. eichwaldi* and *B. verrucosissimus*,  $0.1 \pm 0.07\%$  and  $0.2 \pm 0.08\%$ , respectively. The *B. bufo*  
 258 clade included 171 individuals, with 98 variable sites defining 70 haplotypes (haplotype  
 259 diversity,  $h = 0.933$ ). Nucleotide diversity ( $\pi$ ) was 0.008, and the average number of  
 260 nucleotide differences ( $k$ ) was 9.5. The *B. spinosus* clade included 43 individuals, with 102  
 261 variable sites defining 24 haplotypes, with similar values of haplotype diversity ( $h = 0.910$ )  
 262 and higher nucleotide diversity ( $\pi = 0.016$  and  $k = 19.4$ ). The corresponding values for *B.*  
 263 *eichwaldi* ( $N=5$ ) and *B. verrucosissimus* ( $N=13$ ) were, respectively,  $h = 0.800$ ,  $\pi = 0.001$  and  
 264  $k = 1.4$ ; and  $h = 0.692$ ,  $\pi = 0.002$  and  $k = 2.7$ . Neutrality test results (Tajima's D, Fu and Li's  
 265 D and F statistics) were not significant (results not shown).

266 Estimated TMRCAs (in Mya) for selected clades and subclades (median and 95%  
 267 HPDIs) are as follows: *B. eichwaldi*: 0.19 (0.0364-0.53); *B. spinosus*: 5.18 (2.48-9.00); *B.*  
 268 *bufo*: 1.70 (0.89-2.88); *B. bufo* + *B. verrucosissimus*: 2.75 (1.90-4.27); *B. spinosus* + *B. bufo*  
 269 + *B. verrucosissimus*: 9.19 (5.37-15.76); *B. spinosus* (North Africa): 2.20 (0.81-4.23); *B.*  
 270 *spinosus* (Iberia + France): 1.09 (0.50-2.05); *B. verrucosissimus* (NW Caucasus): 0.18  
 271 (0.0373-0.50); *B. verrucosissimus* (SW Caucasus): 0.11 (0.0079-0.36).

### 272 3.2 nDNA

274 The number of polymorphic sites in the nuclear markers was nine for POMC, nine for  
 275 CXCR4, five for BDNF and 65 for RPL3. Results of neutrality (Tajima's D, Fu & Li's F and  
 276 D) and recombination (PHI) tests were not significant (results not shown). Nuclear DNA  
 277 topologies can be found in the Supplementary content section. In all cases, haplotypes  
 278 grouped together according to species designations. For instance, haplotypes in *B. eichwaldi*  
 279 were well differentiated from those found in the other species and always formed a  
 280 monophyletic group. On the other hand, haplotypes in *B. spinosus* were also well  
 281 differentiated: they were not found in other species and represent considerable diversity.

282 Finally, haplotypes from *B. bufo* and *B. verrucosissimus* were closely related and in most  
 283 cases formed well-supported clades (Supplementary content). The combined nDNA topology  
 284 is consistent with these results and similar to the mtDNA tree (Fig. 2B), with *B. eichwaldi*  
 285 sister to (*B. spinosus* + (*B. bufo* + *B. verrucosissimus*)) (1.0/100/100). *Bufo eichwaldi*  
 286 (1.0/89/91) and *B. spinosus* (1.0/84/89) were recovered as monophyletic groups. A sister  
 287 group between *B. spinosus* and (*B. bufo* + *B. verrucosissimus*) was also well supported  
 288 (1.0/89/86). Samples of *B. verrucosissimus* were nested within *B. bufo* (Fig. 2B) and were  
 289 most similar to samples of *B. bufo* from Turkey (BB070) and Greece (BB117), with which  
 290 they form a subclade (0.99/-/52) that, in turn, includes another subclade with samples from  
 291 central and southern Italy and Sicily (OGAM2, OMON5, ORAG3, 5, see Table 1 and Fig. 1)  
 292 (1.0/-/76).

293

### 294 3.3. Combined mtDNA + nDNA analyses

295 The combined mtDNA + nDNA topology (Fig. 3A) is very similar to the mtDNA tree, with  
 296 *B. eichwaldi* as sister to (*B. spinosus* + (*B. verrucosissimus* + *B. bufo*)), with BPPs=1.0 and  
 297 bootstrap values=100 in all cases. However, whereas the monophyly of *B. eichwaldi*  
 298 (1.0/100/100), *B. spinosus* (1.0/100/100) and *B. verrucosissimus* (1.0/76/82) is also well  
 299 supported, the monophyly of *B. bufo* is well supported in MP (88) but not in ML (where *B.*  
 300 *verrucosissimus* is nested within *B. bufo*) or Bayesian (<0.9) analyses (Fig. 3A). On the other  
 301 hand, results of \*BEAST analyses produced a fully resolved topology, with *B. eichwaldi*  
 302 sister to (*B. spinosus* (*B. bufo* + *B. verrucosissimus*)), with all nodes having BPPs>0.95 (Fig.  
 303 3B).

304

## 305 4. Discussion

306 Our combination of comprehensive geographic sampling and choice of molecular markers  
 307 with different substitution rates and patterns of inheritance has produced a fully resolved  
 308 phylogenetic hypothesis for Eurasian and north African species in the *B. bufo* species group.  
 309 Our results indicate an old (Miocene) origin for the current species diversity in the group,  
 310 with the initial split of the ancestors of *B. eichwaldi* in the Caucasus, and of *B. bufo* (*sensu*  
 311 *lato*) in the western Mediterranean region around 9-13 Mya. The congruence between the  
 312 results of analyses based on mtDNA, nDNA, concatenated analyses of mitochondrial and  
 313 nuclear DNA, and species trees based on the multispecies coalescent (Figs. 2-3) strongly  
 314 support our phylogenetic hypothesis.

315 The inferred species tree and time estimates derived from coalescent-based analyses  
 316 of mtDNA data can be used to reconstruct the evolutionary history of the *B. bufo* species  
 317 group (*B. bufo sensu lato* plus *B. eichwaldi* and *B. verrucosissimus*). In doing so, two facts  
 318 must be kept in mind. First, TMRCA estimates refer to coalescence times in currently  
 319 observed haplotype variants, and thus necessarily predate population divergence times.

320 Although the magnitude of this difference between coalescence times and population  
321 divergence is hard to calculate (Rosenberg & Feldman, 2002), our estimates can still be  
322 informative about the relative timing of splitting events. Second, our TMRCA estimates are  
323 dependent on our choice of nodes for fossil calibration; in this respect the decision to use  
324 Miocene fossils to calibrate the minimum time for the deepest divergence between the four  
325 species seems the most appropriate with the data at hand (i. e., in the absence of diagnostic  
326 osteological characters distinguishing species). The scenarios derived from our analyses  
327 suggest a long evolutionary history of the *B. bufo* species group in western Europe, which is  
328 consistent with the fossil record, that includes abundant Pliocene sites in central Europe  
329 (Germany, Romania, Poland, Hungary, Slovakia) and the Apennine and Iberian peninsulas,  
330 and Pleistocene fossils in North Africa (Morocco) (see Martín & Sanchiz, 2011) (Fig. 4).

331 According to our median TMRCA estimates, and taking into account the uncertainty  
332 reflected in their corresponding HPDIs, the initial split between *B. eichwaldi* and the other  
333 species would have taken place in the Miocene, between about 13 and 9 Mya [which are the  
334 estimated TMRCA of (*eichwaldi* + *spinosus* + *bufo* + *verrucosissimus*) and the latter three  
335 species, respectively], considerably older than previously estimated based on allozyme data  
336 (Litvinchuk et al., 2008) (Fig. 4A). This biogeographic pattern (Europe-Caucasus split) is  
337 recurrent across groups, with other amphibian examples including Parsley frogs (genus  
338 *Pelodytes*), which have species in the Iberian Peninsula, France and northwestern Italy (*P.*  
339 *punctatus* and *P. ibericus*) and in the Caucasus (*P. caucasicus*) (García-París et al., 2003;  
340 Veith et al., 2006); and salamanders (the Iberian *Chioglossa lusitanica* and *Mertensiella*  
341 *caucasica* in the Caucasus) (Veith et al., 1998; Zhang et al., 2008). The uplift of the Armenian  
342 Plateau and the opening of the Mid-Aegean Trench during the Miocene, which have been  
343 invoked to explain similar patterns in other groups (see Wielstra et al., 2010, and references  
344 cited therein), might also be associated with this split in the *Bufo bufo* species group. Our data  
345 show contrasting patterns of genetic diversity in the current descendants of these two ancient  
346 lineages. *Bufo eichwaldi* presents low intraspecific genetic diversity, although perhaps denser  
347 sampling across its range (particularly towards the Iranian end of the distribution) might  
348 reveal the existence of additional sublineages. On the other hand, species with restricted  
349 ranges are especially sensitive to major climate fluctuations, and Pleistocene glaciations may  
350 have had a major impact on genetic diversity in *B. eichwaldi*, as has been the case for other  
351 species that were marginally distributed in refugium areas during that period (Recuero &  
352 García-París, 2011). This isolation might have also prevented secondary contact and  
353 introgression with other species in the group. At present at least 250 km of unsuitable  
354 woodless lowland area separates populations of *B. eichwaldi* from the nearest populations of  
355 *B. verrucosissimus* (a few records of *B. bufo* species group from central Azerbaijan require  
356 confirmation). However, there is no evidence of introgression between both species based on  
357 our nDNA analyses. Litvinchuk et al. (2008) reported seven diagnostic allozyme loci out of

358 24 analyzed between the two species. The Hyrcanian broad-leaved subtropical forests of  
 359 Talysh and Elburz mountains are known as a relic of the Tertiary vegetation, which formerly  
 360 had a much broader distribution in NW Eurasia (see Tuniyev, 1999 and references cited  
 361 therein). This area harbors a number of endemic amphibian and reptile species and lineages  
 362 (e.g. *Paradactylodon persicus*, *Rana pseudodalmatina*, *Darevskia chlorogaster*, *D. defilippii*,  
 363 *Zamenis persicus*, *Gloydius (halys) caucasicus*). Our phylogenetic scenario for the *B. bufo*  
 364 species group provides further evidence that the Hyrcanian refugium played an important role  
 365 in the formation of modern herpetological diversity of the Caucasus, Anatolia and Europe.

366 After the initial split, the ancestor of western European species gave rise to a much  
 367 more genetically and morphologically diverse lineage, where three extant species can be  
 368 readily identified. Most variation in these species is concentrated in the three major  
 369 Pleistocene refugia in Western Europe: the Iberian, Apennine and Balkan peninsulas, as  
 370 typically found in other widely distributed organisms in the western Palearctic (e.g., Zeisset &  
 371 Beebee, 2008). This pattern can be explained by the isolation and persistence through climatic  
 372 cycles of old (Miocene and Pliocene) toad lineages, mainly in North Africa, the Caucasus and  
 373 the Mediterranean peninsulas. The splitting of *B. spinosus* from the ancestor of *B. bufo* + *B.*  
 374 *verrucosissimus* might be associated with the different phases of the uplift of the Pyrenees,  
 375 which isolated the Iberian peninsula from the rest of western Europe during most of the  
 376 Tertiary and ended about 5 million years ago (Plaziat, 1981; Oosterbroek & Arntzen, 1992)  
 377 (Fig. 4B). Based on our TMRCA estimates, the split between north African and Iberian  
 378 populations of *B. spinosus* probably took place at the end of the Messinian Salinity Crisis 5.3  
 379 Mya or slightly thereafter (see Hewitt, 2011, and references cited therein) (Fig. 4C). Busack  
 380 (1986) found three fixed allozyme differences between populations on both sides of the Strait  
 381 of Gibraltar, with a genetic distance ( $D_{Nei}$ ) of 0.16, which is consistent with this hypothesis,  
 382 although more detailed assessments of north African populations, which are small and  
 383 fragmented, are needed. Although no obvious pattern of genetic structure was found in  
 384 Iberian populations of *B. bufo*, as reported by Martínez-Solano and González (2008),  
 385 populations in southern Iberia and the Sistema Central Mountains in central Spain (including  
 386 Gredos, from where *B. b. gredosicola* was described) do seem to harbor considerable genetic  
 387 diversity. Future studies should use more variable markers from the nuclear genome and  
 388 denser population sampling in order to further unravel the evolutionary history of *B. spinosus*  
 389 across its range.

390 Finally, *B. bufo* and *B. verrucosissimus* appear to have split recently, in the  
 391 Pleistocene (Fig. 4D). Mitochondrial DNA distances are low with respect to typical values  
 392 found in other amphibian species pairs (but see also *T. marmoratus* / *T. pygmaeus* or  
 393 *Pelodytes punctatus* / *P. ibericus*, García-París et al., 2001, 2003; Veith et al., 2006; Wielstra  
 394 & Arntzen, 2011), but this may reflect recent divergence (as indicated by incomplete lineage  
 395 sorting in nuclear markers), perhaps with some gene flow (some of the patterns observed in

396 the nuclear DNA topologies are consistent with this interpretation, with shared alleles  
397 between *verrucosissimus* and Anatolian *B. bufo* in some markers, see also the combined  
398 nDNA tree in Fig. 2B). In spite of these patterns, previous studies have documented the  
399 distinctiveness of *B. verrucosissimus* based on morphological, ecological, and biochemical  
400 characters (Birshtein & Mazin, 1982; Orlova & Tuniyev, 1989; Kuzmin, 1999; Pisanets et  
401 al., 2009), and there is some evidence that, whereas both species can interbreed in laboratory  
402 conditions, hybrid survival may not exceed the second generation (see Pisanets, 2001, 2002;  
403 Pisanets et al., 2009).

404 During the Pleistocene, major haplogroups within *B. spinosus* in North Africa and the  
405 Iberian Peninsula originated (Fig. 4D), and the same is true for well-resolved subclades within  
406 *B. bufo* and *B. verrucosissimus*. Within the latter up to four morphological subspecies were  
407 recognized, with *B. v. verrucosissimus* occupying the major part of the species distribution in  
408 Georgia and along the southern slopes of the Great Caucasus, and *B. v. turowi*, *B. v.*  
409 *circassicus*, and *B. v. tertyschnikovi* occupying mountain areas in the northwest periphery of  
410 the species range (Orlova & Tuniyev, 1989; Kidov, 2009). Despite significant interspecific  
411 variation of certain morphological characters, their validity has been doubted recently  
412 (Litvinchuk et al., 2008; Pisanets et al., 2009). The subclades of *B. verrucosissimus* we  
413 recovered do not correspond to current subspecific designations, rather they correspond to the  
414 two major allopatric population groups from southern and northern parts of the species range  
415 and, pending more detailed morphological and ecological studies, may be regarded as  
416 independent management units in conservation planning. Our results also provide the first  
417 genetic evidence for the presence of *B. verrucosissimus* in Turkey (Karagol), which was not  
418 yet confirmed (Eiselt, 1965; Baran & Atatur, 1998; Kutrup, 2006), and also clearly indicate  
419 that most of Anatolia is inhabited by *B. bufo* (*s. str.*), in contrast with Litvinchuk et al.'s  
420 (2008) assumption that the name *B. v. verrucosissimus* should be applied to all Anatolian  
421 populations.

422 In *B. bufo* there is a sharp contrast between the high genetic diversity observed in the  
423 south (especially in the Apennine and Balkan peninsulas) and the genetic homogeneity of  
424 populations in the north, with haplotype sharing in samples from central and northern Europe  
425 being fairly common. Brede & Beebe (2006) found remarkably little genetic differentiation  
426 in *B. bufo* populations across most of Europe based on microsatellite markers. Although they  
427 did not find a deep break between populations of *B. bufo* and *B. spinosus* in their study,  
428 samples from Iberia and south France showed deviations from Hardy-Weinberg equilibrium  
429 that could reflect the occurrence of null alleles in these populations and some of them were  
430 thus not included in interpopulation comparisons. In any case, their results also suggest a very  
431 rapid colonization potential for the species, since the areas that are now occupied by  
432 populations nested in the less genetically diverse clade within *B. bufo* were covered by  
433 glaciers and tundra until the end of the Last Glacial Maximum (Hewitt, 1999; CGMW-

434 ANDRA, 1999). Based on our phylogenetic analyses, the source of this colonization could  
435 have been the Balkan Peninsula (Fig. 2) - in a pattern very similar to that observed in other  
436 amphibians, like *Lissotriton vulgaris* (see Babik et al., 2005) - or some neighboring area (see  
437 e.g. Hofman et al., 2007; Sotiropoulos et al., 2007; Canestrelli & Nascetti, 2008).

438 Our results also allow delineation of the ranges of *B. bufo* and *B. spinosus*, which has  
439 long been an issue in morphological, ecological, genetic and conservation studies (Daudin,  
440 1803; Mertens & Wermuth, 1960; Hemmer & Böhme, 1976; Lüscher et al., 2001; IUCN,  
441 2009). The type locality of *B. spinosus* is “aux environs de Brives et de Bordeaux”, in  
442 southern France (Frost, 2011), well within the range of our *spinosus* clade. According to our  
443 data, therefore, *B. spinosus* includes all populations in the Maghreb, the Iberian Peninsula and  
444 parts of France. The genetic distinctiveness in all markers examined with respect to *B. bufo*  
445 support its recognition at the species level, although a reappraisal of morphological and  
446 ecological variation within and between species in the group, based on current species limits,  
447 is still desired. Preliminary location of contact zones with *B. bufo* based on spatial patterns of  
448 mtDNA haplotype sharing points to: 1) the Maritime Alps between SE France and NW Italy  
449 (sample codes: OBYX, see Table 1 and Fig. 1), which is a well-known suture zone (*sensu*  
450 Swenson & Howard, 2005) for other species (see for example, Canestrelli et al., 2006), and 2)  
451 northern France, where there is a striking parallelism with the contact zone between the newts  
452 *Triturus cristatus* and *T. marmoratus* (Arntzen & Wallis, 1991; Arntzen et al., 2009). These  
453 areas are worth of further fine-scale studies on the formation and maintenance of reproductive  
454 barriers between species. On the other hand, *B. bufo* would be distributed in northern France  
455 and the rest of Western Europe to Siberia, including the Apennine and Balkan peninsulas and  
456 most of Anatolia. The eastern limits and the potential existence of contact zones with *B.*  
457 *verrucosissimus* are still unclear, although our study will also help identify relevant areas for  
458 further study.

459 Our study is the first to analyze a geographically comprehensive sampling including  
460 all described species and subspecies in the *B. bufo* species group. The combined use of  
461 molecular markers from the nuclear and mitochondrial genomes and the adoption of a variety  
462 of analytical approaches including species tree estimation produced a fully resolved topology  
463 which will set the basis for the clarification of the taxonomy, systematics, and evolutionary  
464 history of the group and will serve as a foundation for further studies on the process of species  
465 formation.

466

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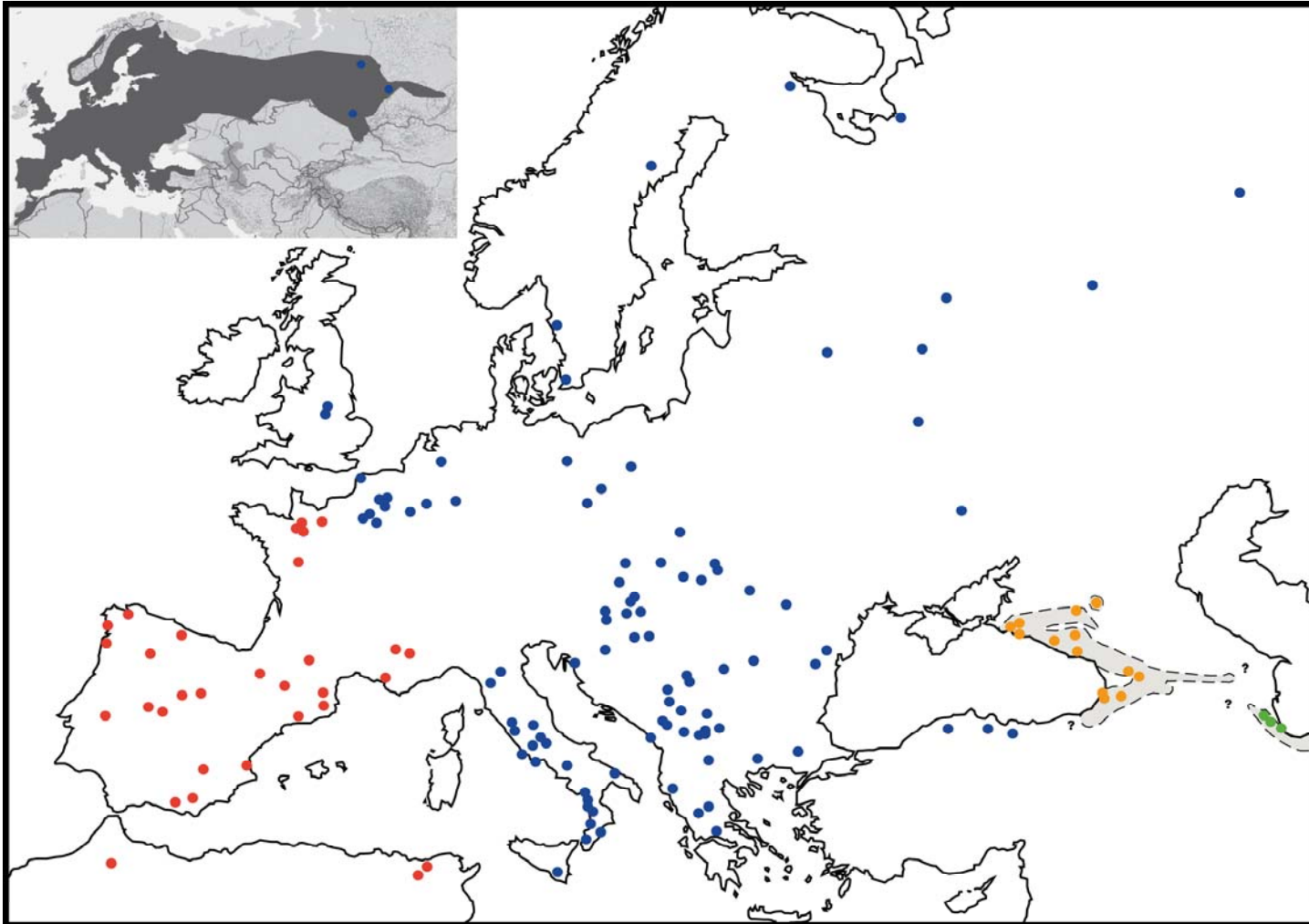
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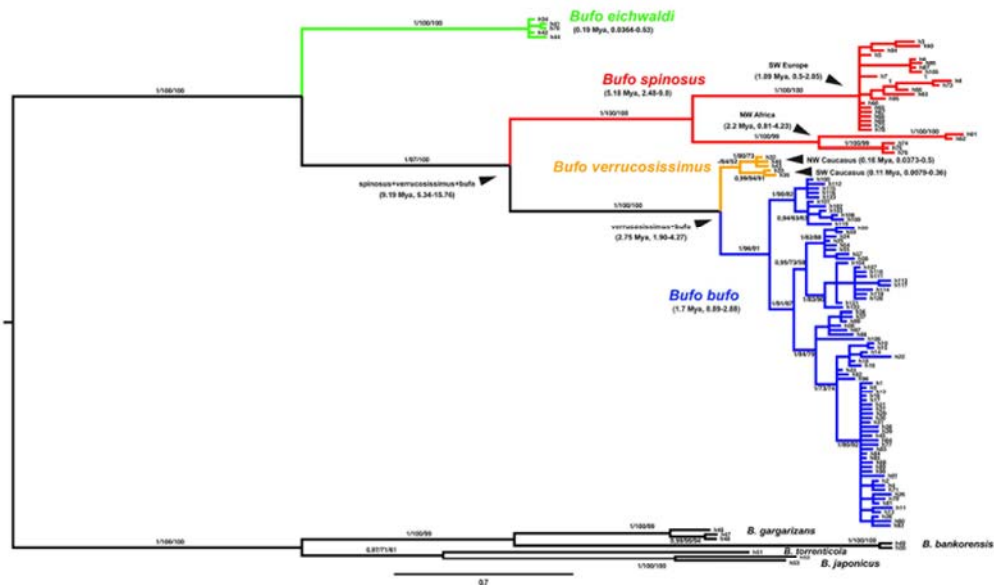
726 Figure 1. Sampling localities (see also Table 1). Blue dots represent samples of *Bufo bufo*, and red dots, *B. spinosus*. The distribution of *B. bufo* and *B.*  
727 *spinosus* is shown in the inset, which shows also the location of the three easternmost samples in our dataset. The distributions of *B. verrucosissimus* (orange  
728 dots) and *B. eichwaldi* (green dots) are shaded.



729

730 Figure 2. Bayesian consensus phylograms based on mtDNA data (A) and the concatenated nuclear  
 731 dataset (B). Support values in relevant branches are Bayesian posterior probabilities (only those >0.9 are  
 732 shown), maximum likelihood (ML) and maximum parsimony (MP) bootstrap values, respectively.  
 733 Estimated times to most recent common ancestor (TMRCA) for selected clades are shown in parentheses  
 734 (median, 95% highest posterior density interval).

### A) mtDNA data



### B) Concatenated nDNA data

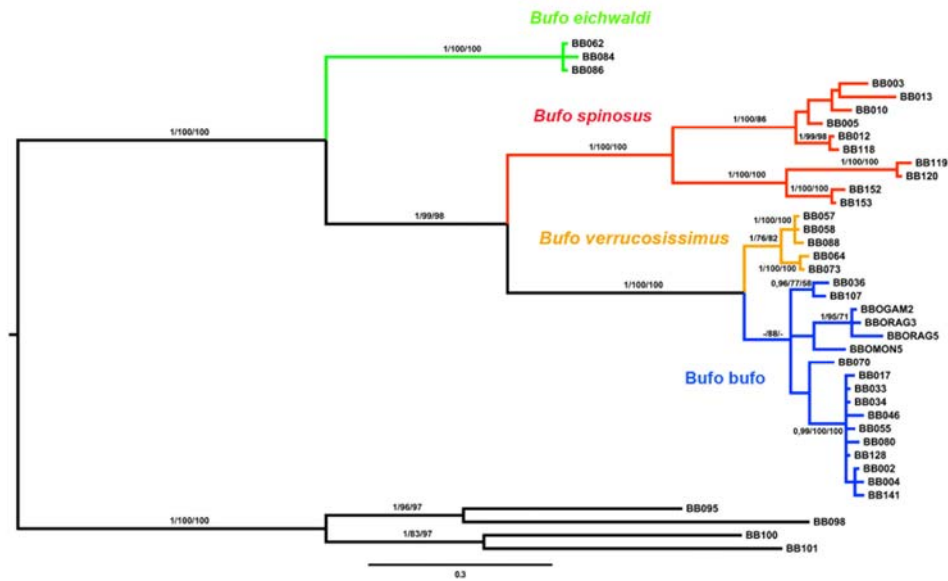


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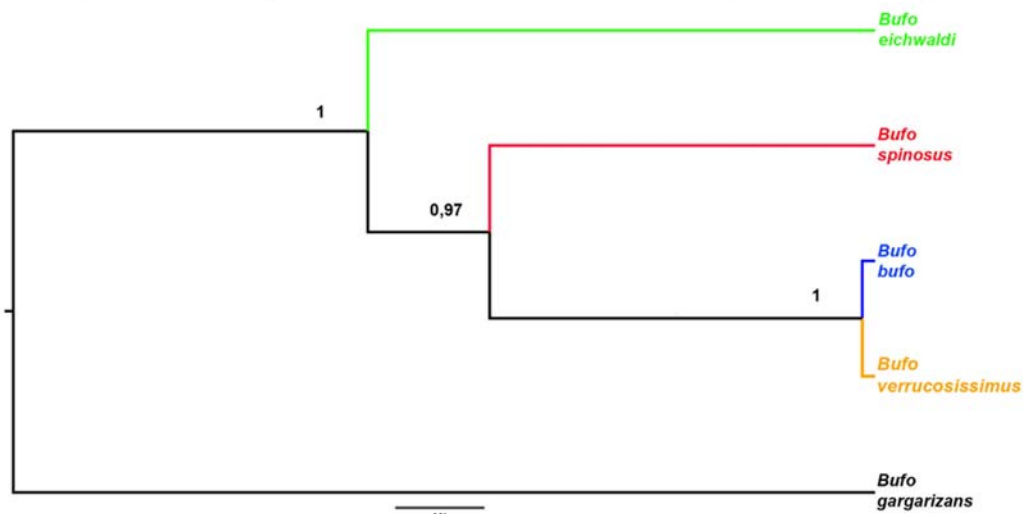


736 Figure 3. Bayesian consensus phylogram based on concatenated analysis of mitochondrial and nuclear  
 737 DNA (A), and species tree based on the multispecies coalescent implemented in \*BEAST (B). Support  
 738 values in relevant branches are Bayesian posterior probabilities (only those >0.9 are shown), and, where  
 739 applicable (Fig. 3A), maximum likelihood (ML) and maximum parsimony (MP) bootstrap values,  
 740 respectively.

### A) Concatenated mtDNA + nDNA

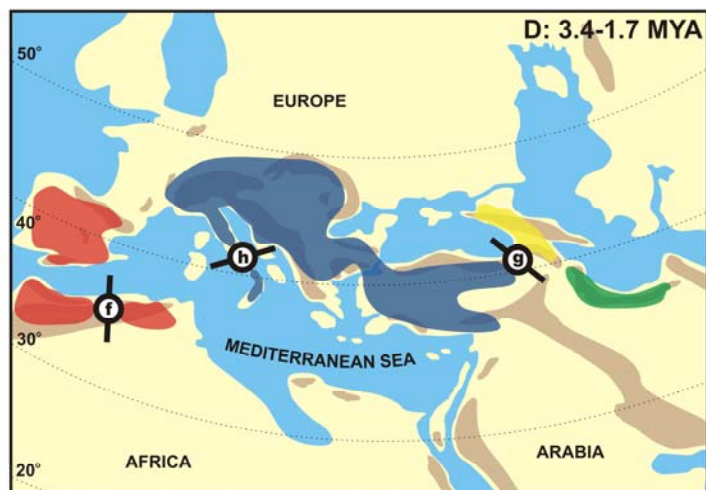
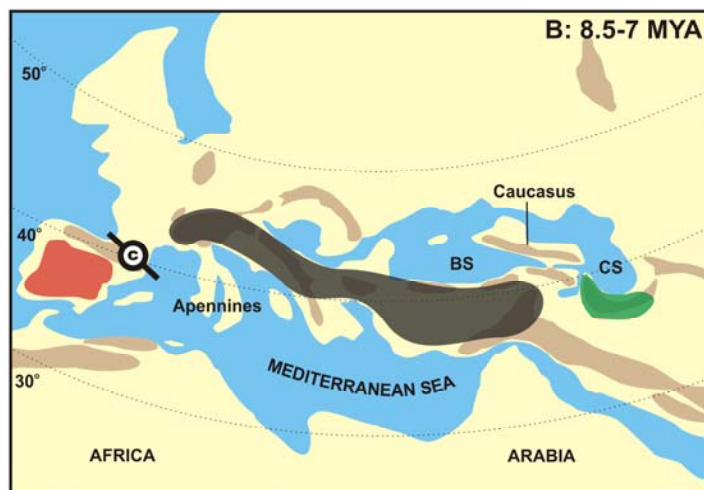
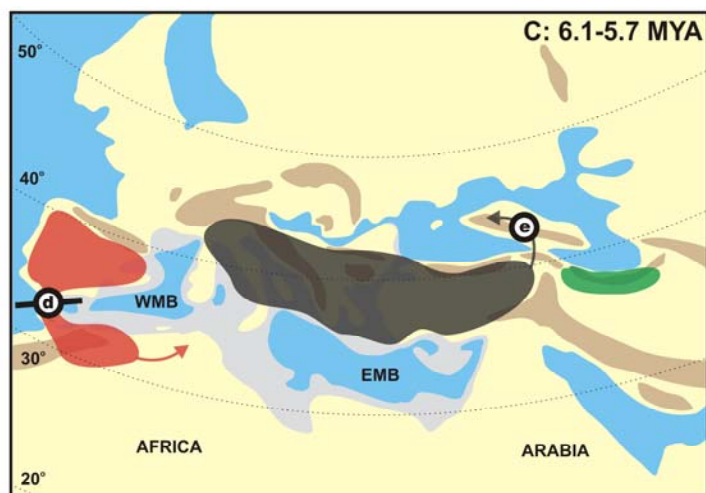
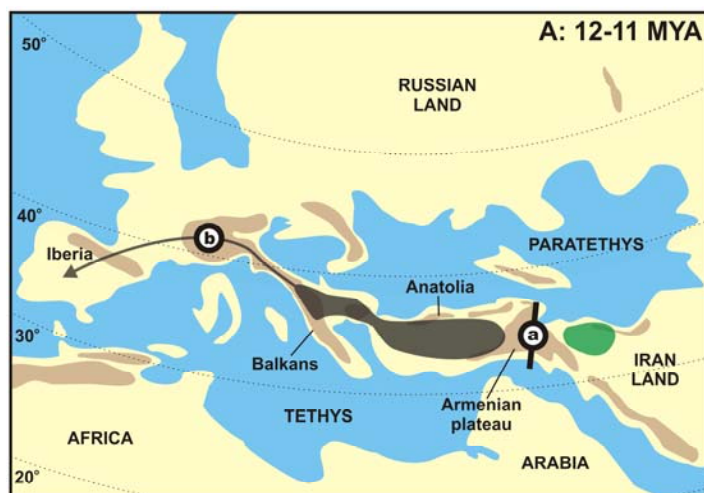


### B) Multispecies coalescent (\*BEAST)



741

742 Figure 4. Paleogeographic reconstructions and major cladogenetic events in *Bufo bufo* (modified from Popov et al. (2004) and Wielstra et al. (2010)).  
743 Phylogenetic splits (see Fig. 2A) are marked with letters (a-g); range colors as in Figs. 1-3; inferred ancestor ranges in black. A) Late-Middle Miocene: the  
744 movement of African and Arabian plates towards the European archipelago leads to extensive orogenesis (shaded light brown) in the Near and Middle East  
745 and the uplift of the Armenian Plateau. B) Mid-Late Miocene: extensive orogenesis and formation of the Neo-Pyrenees. C) Late Miocene: the Messinian  
746 Salinity Crisis led to dramatic sea level drop in the Mediterranean Basin (exposed parts of sea bottom shaded in light gray), including a land bridge between  
747 Iberia and N Africa; Caucasia gradually becomes connected with Anatolian land. D) Middle-Late Pliocene: the marine connection between the Atlantic  
748 ocean, Mediterranean and Ponto-Caspian basins is re-established, Caucasia gradually becomes an isthmus between the Black and Caspian seas; intensification  
749 of orogenesis, periodical drops in global sea level and climatic oscillations during the Pleistocene. Abbreviations: WMB - West Mediterranean Basin; EMB -  
750 East Mediterranean Basin; BS - Black Sea; CS - Caspian Sea.



751  
752

753 Table 1. Sampling localities: species, sample codes, latitude, longitude, and GenBank Accession numbers for the sequences generated in this study.

754

Species	Sample code	Country	Locality	Latitude	Longitude	mtDNA haplotype	16S	cytb	POMC	CXCR4	BDNF	RPL3
<i>Bufo bufo</i>	BB194	Albania	near Tepelena	40°17'25.20"N	20°01'30.30"E	25	JN647155	JN647341				
<i>Bufo bufo</i>	BB188	Belgium	Liege prov: Solwaster	50°31'23.00"N	5°58'32.00"E	2	JN647153	JN647339				
<i>Bufo bufo</i>	BB189	Belgium	Liege prov: Marche-en-Famenne	50°15'49.00"N	5°22'7.00"E	2	JN647154	JN647340				
<i>Bufo bufo</i>	BB157	Croatia	Svika	44°52'20.73"N	15°10'56.39"E	17	JN647136	JN647319				
<i>Bufo bufo</i>	BB158	Croatia	Zutica	45°37'51.68"N	16°26'43.62"E	77	JN647137	JN647320				
<i>Bufo bufo</i>	BB141	France	Erloy, Foret de Regnaval	49°54'60.00"N	3°50'30.00"E	71	JN647129	JN647313	JN646935	JN646884	JN646979	JN647505
<i>Bufo bufo</i>	BB142	France	Bruyères et Montbérault	49°32'6.00"N	3°40'40.00"E	2	JN647130	JN647314				
<i>Bufo bufo</i>	BB143	France	Le Boujon	49°56'55.00"N	3°50'15.00"E	2	JN647131	JN647315				
<i>Bufo bufo</i>	BB144	France	Le Chevalet	50°1'0.00"N	3°52'30.00"E	2	JN647132	JN647316				
<i>Bufo bufo</i>	BB145	France	Nouvion	50°0'20.00"N	3°46'45.00"E	23	JN647133	JN647317				
<i>Bufo bufo</i>	BB146	France	Vorges	49°31'0.84"N	3°38'49.08"E	23	JN647134	JN647318				
<i>Bufo bufo</i>	BB149	France	Chigny	49°17'57.75"N	3°46'30.00"E	2	JN647135	JN647329				
<i>Bufo bufo</i>	BB164	France	Adresselles, dept. Pas-de-Calais, France	50°49'16.10"N	1°36'8.30"E	2	JN647143	JN647326				
<i>Bufo bufo</i>	OBYX12	France	Saint Bonnet en Champsaur	44°40'58.42"N	6°4'34.13"E	106	JN647224	JN647419				
<i>Bufo bufo</i>	BB001	Germany	Eberswalde, Bad Freienwald, ~50 km NE Berlin	52°49'32.52"N	13°48'58.32"E	1	JN647011	JN647247				
<i>Bufo bufo</i>	BB170	Germany	Bonn, W Rhine	50°41'52.00"N	7°7'42.00"E	2	JN647147	JN647333				
<i>Bufo bufo</i>	BB171	Germany	Bonn, W Rhine	50°41'52.00"N	7°7'42.00"E	2	JN647148	JN647334				
<i>Bufo bufo</i>	BB026	Greece	3 km W Nea Zoi	39°43'28.40"N	21°45'14.44"E	20	JN647026	JN647262				
<i>Bufo bufo</i>	BB107	Greece	Thessalia, Desi	39°33'37.51"N	21°22'18.70"E	54	JN647117	JN647301	JN646932	JN646881	JN646977	JN647503
<i>Bufo bufo</i>	BB108	Greece	Thessalia, Desi	39°33'37.51"N	21°22'18.70"E	55	JN647118	JN647302				
<i>Bufo bufo</i>	BB109	Greece	Thraki, Sofiko	41°26'21.95"N	26°36'30.96"E	56	JN647119	JN647303				
<i>Bufo bufo</i>	BB111	Greece	Makedonia, Lepida timberyard	41°23'22.52"N	24°38'15.43"E	57	JN647120	JN647304				
<i>Bufo bufo</i>	BB112	Greece	Makedonia, Lepida timberyard	41°23'22.52"N	24°38'15.43"E	58	JN647121	JN647305				
<i>Bufo bufo</i>	BB114	Greece	Stereia Ellada, Gkiona mt., at stream by Agios Nikolaos church, on the road	38°33'51.77"N	22°20'56.69"E	59	JN647122	JN647306	JN646933	JN646882	JN646978	

			from Ptosilio to Amfissa										
<i>Bufo bufo</i>	BB032	Hungary	Öcs	47°0'34.10"N	17°37'49.21"E	23	JN647032	JN647268					
<i>Bufo bufo</i>	BB033	Hungary	Öcs	47°0'34.10"N	17°37'49.21"E	9	JN647033	JN647269					
<i>Bufo bufo</i>	BB034	Hungary	Pécs, Jakab-Hill, Mecsek Mts.	46°5'35.34"N	18°8'28.98"E	9	JN647034	JN647270					
<i>Bufo bufo</i>	BB179	Hungary	Vinye, Bakony Mts.	47°21'11.28"N	17°46'44.64"E	88	JN647158	JN647344	JN646930	JN646886	JN646988	JN647492	
<i>Bufo bufo</i>	BB180	Hungary	Erd bénye, Zemplén Mts.	48°18'50.70"N	21°20'4.96"E	9	JN647159	JN647345	JN646931	JN646887	JN646981	JN647493	
<i>Bufo bufo</i>	BB181	Hungary	Bakonybél, Bakony Mts.	47°16'7.38"N	17°41'41.28"E	9	JN647160	JN647346					
<i>Bufo bufo</i>	BB182	Hungary	Szendr	48°23'44.88"N	20°44'27.84"E	89	JN647161	JN647347					
<i>Bufo bufo</i>	BB183	Hungary	Csákánydoroszló	46°58'1.50"N	16°30'29.40"E	88	JN647162	JN647348					
<i>Bufo bufo</i>	BB184	Hungary	rség, Bârkâs-lake	46°53'59.40"N	16°27'53.29"E	90	JN647163	JN647349					
<i>Bufo bufo</i>	BB185	Hungary	Tihany	46°54'33.12"N	17°50'57.26"E	9	JN647164	JN647350					
<i>Bufo bufo</i>	BB186	Hungary	Bâtaapâti	46°12'54.12"N	18°36'41.25"E	2	JN647165	JN647351					
<i>Bufo bufo</i>	BB187	Hungary	Bâtaapâti	46°12'54.12"N	18°36'41.25"E	91	JN647166	JN647352					
<i>Bufo bufo</i>	OALB4	Italy	Alberobello	40°47'10.59"N	17°14'14.84"E	100	JN647175	JN647361					
<i>Bufo bufo</i>	OANG10	Italy	Lago dell' Antigola	38°44'22.41"N	16°14'9.51"E	101	JN647176	JN647362					
<i>Bufo bufo</i>	OANG8	Italy	Lago dell' Antigola	38°44'22.41"N	16°14'9.51"E	102	JN647177	JN647363					
<i>Bufo bufo</i>	OBEL2	Italy	Bel Monte Calabro Marina	39°10'2.52"N	16°4'44.87"E	103	JN647178	JN647364					
<i>Bufo bufo</i>	OBRB15	Italy	Barbarano	42°16'11.63"N	12°3'38.28"E	104	JN647179	JN647365					
<i>Bufo bufo</i>	OBRB2	Italy	Barbarano	42°16'11.63"N	12°3'38.28"E	104	JN647180	JN647366					
<i>Bufo bufo</i>	ODOG10	Italy	Doganella	41°45'00.35"N	12°45'39.60"E	107	JN647054	JN647367					
<i>Bufo bufo</i>	ODOG5	Italy	Doganella	41°45'00.35"N	12°45'39.60"E	107	JN647055	JN647368					
<i>Bufo bufo</i>	ODOG8	Italy	Doganella	41°45'00.35"N	12°45'39.60"E	107	JN647056	JN647369					
<i>Bufo bufo</i>	ODOG9	Italy	Doganella	41°45'00.35"N	12°45'39.60"E	107	JN647057	JN647370					
<i>Bufo bufo</i>	OFAG2	Italy	Fagnano Castello-Lago Paglia	39°33'57.59"N	16°3'15.42"E	108	JN647058	JN647371					
<i>Bufo bufo</i>	OFAG5	Italy	Fagnano Castello-Lago Paglia	39°33'57.59"N	16°3'15.42"E	109	JN647059	JN647372					
<i>Bufo bufo</i>	OFIUB1	Italy	Fiumefreddo Bruzio	39°15'28.62"N	16°5'44.61"E	103	JN647060	JN647373					
<i>Bufo bufo</i>	OFIUB2	Italy	Fiumefreddo Bruzio	39°15'28.62"N	16°5'44.61"E	103	JN647061	JN647374	JN646917	JN646869		JN647483	
<i>Bufo bufo</i>	OFIUB3	Italy	Fiumefreddo Bruzio	39°15'28.62"N	16°5'44.61"E	103	JN647062	JN647375	JN646918				
<i>Bufo bufo</i>	OFIUB4	Italy	Fiumefreddo Bruzio	39°15'28.62"N	16°5'44.61"E	103	JN647063	JN647376					
<i>Bufo bufo</i>	OFOG4	Italy	Bosco del Foglino	41°28'14.95"N	12°43'6.02"E	110	JN647064	JN647377					
<i>Bufo bufo</i>	OFOG5	Italy	Bosco del Foglino	41°28'14.95"N	12°43'6.02"E	107	JN647065	JN647378					
<i>Bufo bufo</i>	OFOG9	Italy	Bosco del Foglino	41°28'14.95"N	12°43'6.02"E	111	JN647066	JN647379					
<i>Bufo bufo</i>	OGAM2	Italy	Gambarie	38°10'51.59"N	15°50'45.32"E	101	JN647068	JN647381	JN646919	JN646870	JN646967	JN647485	
<i>Bufo bufo</i>	OGAM22	Italy	Gambarie	38°10'51.59"N	15°50'45.32"E	112	JN647067	JN647380	JN646920	JN646871	JN646968		
<i>Bufo bufo</i>	OGHI2	Italy	Monghidoro	44°14'53.89"N	11°20'46.43"E	107	JN647069	JN647382		JN646872	JN646969	JN647484	

<i>Bufo bufo</i>	OGHI4	Italy	Monghidoro	44°14'53.89"N	11°20'46.43"E	107	JN647070	JN647383				
<i>Bufo bufo</i>	OJEN1	Italy	Jenne	41°53'23.47"N	13°10'14.33"E	113	JN647071	JN647384	JN646921	JN646873		JN647486
<i>Bufo bufo</i>	OJEN2	Italy	Jenne	41°53'23.47"N	13°10'14.33"E	107	JN647072	JN647385				
<i>Bufo bufo</i>	OJEN3	Italy	Jenne	41°53'23.47"N	13°10'14.33"E	114	JN647073	JN647386				
<i>Bufo bufo</i>	OMAS2	Italy	Tortora -C.da Massadita-	39°56'30.93"N	15°48'21.54"E	115	JN647074	JN647387				
<i>Bufo bufo</i>	OMAS3	Italy	Tortora -C.da Massadita-	39°56'30.93"N	15°48'21.54"E	116	JN647075	JN647388				
<i>Bufo bufo</i>	OMAS4	Italy	Tortora -C.da Massadita-	39°56'30.93"N	15°48'21.54"E	100	JN647076	JN647389				
<i>Bufo bufo</i>	OMAS5	Italy	Tortora -C.da Massadita-	39°56'30.93"N	15°48'21.54"E	100	JN647077	JN647390				
<i>Bufo bufo</i>	OMOL1	Italy	Molella- Fonti di Locullo	41°16'5.09"N	13°2'44.20"E	107	JN647078	JN647391				
<i>Bufo bufo</i>	OMOL2	Italy	Molella- Fonti di Locullo	41°16'5.09"N	13°2'44.20"E	107	JN647079	JN647392				
<i>Bufo bufo</i>	OMOL4	Italy	Molella- Fonti di Locullo	41°16'5.09"N	13°2'44.20"E	117	JN647080	JN647393				
<i>Bufo bufo</i>	OMOL6	Italy	Molella- Fonti di Locullo	41°16'5.09"N	13°2'44.20"E	107	JN647081	JN647394				
<i>Bufo bufo</i>	OMON1	Italy	Canale Monterano	42°8'25.68"N	12°5'49.46"E	107	JN647083	JN647396	JN646922	JN646874	JN646970	
<i>Bufo bufo</i>	OMON16	Italy	Canale Monterano	42°8'25.68"N	12°5'49.46"E	107	JN647082	JN647395				
<i>Bufo bufo</i>	OMON4	Italy	Canale Monterano	42°8'25.68"N	12°5'49.46"E	118	JN647084	JN647397	JN646923	JN646875	JN646971	
<i>Bufo bufo</i>	OMON5	Italy	Canale Monterano	42°8'25.68"N	12°5'49.46"E	107	JN647085	JN647398	JN646924	JN646876	JN646972	JN647487
<i>Bufo bufo</i>	OORS10	Italy	Orsomaso	39°48'1.37"N	15°54'28.58"E	100	JN647086	JN647399				
<i>Bufo bufo</i>	OORS2	Italy	Orsomaso	39°48'1.37"N	15°54'28.58"E	100	JN647087	JN647400				
<i>Bufo bufo</i>	OORS6	Italy	Orsomaso	39°48'1.37"N	15°54'28.58"E	100	JN647088	JN647401				
<i>Bufo bufo</i>	ORAG3	Italy	Fiume Irminio	36°55'44.01"N	14°40'25.03"E	101	JN647089	JN647402	JN646925	JN646877	JN646973	JN647488
<i>Bufo bufo</i>	ORAG5	Italy	Fiume Irminio	36°55'44.01"N	14°40'25.03"E	119	JN647090	JN647403	JN646926	JN646878	JN646974	JN647489
<i>Bufo bufo</i>	ORAG8	Italy	Fiume Irminio	36°55'44.01"N	14°40'25.03"E	119	JN647091	JN647404	JN646927	JN646879	JN646975	
<i>Bufo bufo</i>	ORAG9	Italy	Fiume Irminio	36°55'44.01"N	14°40'25.03"E	101	JN647092	JN647405		JN646880	JN646976	JN647490
<i>Bufo bufo</i>	OSTI1	Italy	Stilo	38°28'41.27"N	16°28'9.20"E	101	JN647093	JN647406				
<i>Bufo bufo</i>	OSUB2	Italy	Subiaco	41°50'28.12"N	13°2'20.36"E	120	JN647094	JN647407				
<i>Bufo bufo</i>	OSUB5	Italy	Subiaco	41°50'28.12"N	13°2'20.36"E	107	JN647095	JN647408				
<i>Bufo bufo</i>	OTIZ2	Italy	Campo Tizzoro	44°2'19.22"N	10°51'42.19"E	121	JN647096	JN647409				
<i>Bufo bufo</i>	OTIZ3	Italy	Campo Tizzoro	44°2'19.22"N	10°51'42.19"E	122	JN647097	JN647410				
<i>Bufo bufo</i>	OTIZ5	Italy	Campo Tizzoro	44°2'19.22"N	10°51'42.19"E	122	JN647098	JN647411				
<i>Bufo bufo</i>	OTUR3	Italy	Rocca Sinibalda-fiume Turano	42°16'29.47"N	12°55'32.25"E	107	JN647099	JN647412				
<i>Bufo bufo</i>	OTUR4	Italy	Rocca Sinibalda-fiume Turano	42°16'29.47"N	12°55'32.25"E	107	JN647100	JN647413				
<i>Bufo bufo</i>	OTUR5	Italy	Rocca Sinibalda-fiume	42°16'29.47"N	12°55'32.25"E	107	JN647101	JN647414				

			Turano											
<i>Bufo bufo</i>	OVER1	Italy	Piani di Verteglia	41°1'10.64"N	15°0'23.11"E	100	JN647102	JN647415						
<i>Bufo bufo</i>	OVER2	Italy	Piani di Verteglia	41°1'10.64"N	15°0'23.11"E	123	JN647103	JN647416						
<i>Bufo bufo</i>	OVER3	Italy	Piani di Verteglia	41°1'10.64"N	15°0'23.11"E	100	JN647104	JN647417						
<i>Bufo bufo</i>	OVER4	Italy	Piani di Verteglia	41°1'10.64"N	15°0'23.11"E	100	JN647105	JN647418						
<i>Bufo bufo</i>	BB196	Macedonia	Prilep - Dunje	41°20'13.00"N	21°42'1.50"E	25	JN647157	JN647343						
<i>Bufo bufo</i>	BB019	Montenegro	Bjelasica mt-Biogradsko jezero	42°54'5.60"N	19°35'49.20"E	14	JN647019	JN647255						
<i>Bufo bufo</i>	BB029	Montenegro	Bjelasica mt-Dolovi	42°53'55.20"N	19°38'1.80"E	21	JN647029	JN647265						
<i>Bufo bufo</i>	BB030	Montenegro	Bjelasica mt-Svatovsko Groblje	42°53'46.80"N	19°38'38.16"E	22	JN647030	JN647266						
<i>Bufo bufo</i>	BB195	Montenegro	Bjelosi	42°22'10.40"N	18°53'26.60"E	25	JN647156	JN647342						
<i>Bufo bufo</i>	BB002	Netherlands	Elspeet	52°18'15.35"N	5°49'31.22"E	2	JN647012	JN647248	JN646916	JN646866	JN646964	JN647500		
<i>Bufo bufo</i>	BB174	Poland	Zgorzelec	51°8'12.70"N	15°1'3.80"E	82	JN647149	JN647335						
<i>Bufo bufo</i>	BB176	Poland	Stara Wies	51°47'20.76"N	15°43'41.52"E	83	JN647150	JN647336						
<i>Bufo bufo</i>	BB177	Poland	Szubin	53°0'5.35"N	17°44'13.26"E	84	JN647151	JN647337						
<i>Bufo bufo</i>	BB178	Poland	Wroclen - Pruszwice	50°12'0.46"N	20°47'37.76"E	85	JN647152	JN647338						
<i>Bufo bufo</i>	BB105	Romania	M cin Mountains National Park	45°13'59.90"N	28°18'31.30"E	9	JN647116	JN647300						
<i>Bufo bufo</i>	BB122	Romania	C l ra i-Chiciu	44°51'48.60"N	27°52'33.24"E	13	JN647123	JN647307						
<i>Bufo bufo</i>	BB123	Romania	Co nea	47°52'0.85"N	24°30'55.44"E	9	JN647124	JN647308						
<i>Bufo bufo</i>	BB125	Romania	Insula Mic a Br ilei	44°51'48.60"N	27°52'33.24"E	64	JN647125	JN647309						
<i>Bufo bufo</i>	BB126	Romania	Lacul Ro u	46°46'57.72"N	25°47'1.68"E	9	JN647126	JN647310						
<i>Bufo bufo</i>	BB127	Romania	Motru Sec Clo ani	45°7'36.48"N	22°48'29.52"E	9	JN647127	JN647311						
<i>Bufo bufo</i>	BB128	Romania	Vidraru Reservoir, F g ra Mts.	45°26'32.65"N	24°36'21.96"E	9	JN647128	JN647312	JN646934	JN646883	JN646963	JN647504		
<i>Bufo bufo</i>	BBUINSULA_B	Romania	Insula Mic a Br ilei	44°51'48.60"N	27°52'33.24"E	18	JN647171	JN647357						
<i>Bufo bufo</i>	RO1	Romania	Luncavi a	45°14'20.51"N	28°17'49.19"E	9	JN647168	JN647354						
<i>Bufo bufo</i>	RO2	Romania	Luncavi a	45°14'20.51"N	28°17'49.19"E	96	JN647169	JN647355						
<i>Bufo bufo</i>	RO3	Romania	Luncavi a	45°14'20.51"N	28°17'49.19"E	9	JN647170	JN647356						
<i>Bufo bufo</i>	BB053	Russia	Russia, Moscow province, Zvenigorod	55°42'39.20"N	36°46'16.40"E	9	JN647050	JN647286						
<i>Bufo bufo</i>	BB054	Russia	Russia, Krasnoyarsk territory, Mirnyi	62°16'55.50"N	89°2'2.20"E	31	JN647051	JN647287						
<i>Bufo bufo</i>	BB055	Russia	Russia, Sverdlovsk prov., Ekaterinburg	56°50'25.40"N	60°33'20.30"E	31	JN647052	JN647288	JN646939	JN646890	JN646984	JN647496		
<i>Bufo bufo</i>	BB056	Russia	Russia, Novosibirsk prov., Novosibirsk	55°3'41.30"N	82°52'31.40"E	31	JN647053	JN647289						
<i>Bufo bufo</i>	BB074	Russia	Russia, Tver province, Toropets	56°31'48.00"N	31°32'6.50"E	9	JN647108	JN647292						
<i>Bufo bufo</i>	BB075	Russia	Russia, Yaroslavl province, Yaroslavl	57°35'54.50"N	39°53'17.40"E	9	JN647109	JN647293						

<i>Bufo bufo</i>	BB076	Russia	Russia, Orel province, Orlovskiy Zaseki NP	53°20'57.50"N	35°33'30.50"E	9	JN647110	JN647294		JN646892		JN647498
<i>Bufo bufo</i>	BB077	Russia	Russia, Krasnoyarsk territory, Kryuchkovo	56°5'42.50"N	92°8'10.50"E	38	JN647111	JN647295			JN646986	
<i>Bufo bufo</i>	BB079	Russia	Russia, Karelia, Kindo peninsula	66°32'8.40"N	33°9'41.00"E	39	JN647112	JN647296				
<i>Bufo bufo</i>	BB080	Russia	Russia, Arkhangelsk province, Arkhangelsk environs	64°32'39.50"N	40°41'22.50"E	39	JN647113	JN647297	JN646941	JN646893	JN646987	JN647499
<i>Bufo bufo</i>	BB081	Russia	Russia, Tatarstan, Kazan environs	55°52'9.30"N	49°8'40.60"E	39	JN647114	JN647298				
<i>Bufo bufo</i>	BB014	Serbia	Avala mt - Tresnja	44°36'26.60"N	20°34'14.00"E	9	JN647014	JN647250				
<i>Bufo bufo</i>	BB015	Serbia	Avala mt - Tresnja	44°36'26.60"N	20°34'14.00"E	10	JN647015	JN647251				
<i>Bufo bufo</i>	BB016	Serbia	Avala mt - Zuce	44°40'55.90"N	20°33'55.90"E	11	JN647016	JN647252				
<i>Bufo bufo</i>	BB017	Serbia	Avala mt - Zuce	44°40'55.90"N	20°33'55.90"E	12	JN647017	JN647253	JN646936	JN646885	JN646980	JN647491
<i>Bufo bufo</i>	BB018	Serbia	Avala mt - Zuce	44°40'55.90"N	20°33'55.90"E	13	JN647018	JN647254				
<i>Bufo bufo</i>	BB020	Serbia	Pozega-Rupeljevo 1	43°46'44.58"N	19°59'49.74"E	15	JN647020	JN647256				
<i>Bufo bufo</i>	BB021	Serbia	Pozega-Rupeljevo 1	43°46'44.58"N	19°59'49.74"E	15	JN647021	JN647257				
<i>Bufo bufo</i>	BB022	Serbia	Pozega-Rupeljevo 1	43°46'44.58"N	19°59'49.74"E	16	JN647022	JN647258				
<i>Bufo bufo</i>	BB023	Serbia	Pozega-Rupeljevo 2	43°46'48.30"N	19°59'21.90"E	17	JN647023	JN647259				
<i>Bufo bufo</i>	BB024	Serbia	Pozega-Rupeljevo 2	43°46'48.30"N	19°59'21.90"E	18	JN647024	JN647260				
<i>Bufo bufo</i>	BB025	Serbia	Valjevo-Petnica	44°14'50.00"N	19°55'51.00"E	19	JN647025	JN647261				
<i>Bufo bufo</i>	BB027	Serbia	Leskovac-Pasina Cesma	43°2'36.30"N	21°52'10.20"E	14	JN647027	JN647263				
<i>Bufo bufo</i>	BB028	Serbia	Golija mt-Devici	43°25'18.84"N	20°22'47.16"E	15	JN647028	JN647264				
<i>Bufo bufo</i>	BB031	Serbia	Avala mt - Tresnja	44°36'26.60"N	20°34'14.00"E	21	JN647031	JN647267				
<i>Bufo bufo</i>	BB038	Serbia	Vranje - Bustranje (1)	42°26'3.00"N	21°54'13.00"E	17	JN647037	JN647273				
<i>Bufo bufo</i>	BB039	Serbia	Vranje - Bustranje (2)	42°25'60.00"N	21°54'17.00"E	17	JN647038	JN647274				
<i>Bufo bufo</i>	BB040	Serbia	Vranje-Rusce	42°24'50.00"N	21°56'48.00"E	13	JN647039	JN647275				
<i>Bufo bufo</i>	BB041	Serbia	Vranje-Gornje Zupsko	42°27'3.00"N	21°53'21.00"E	25	JN647040	JN647276				
<i>Bufo bufo</i>	BB042	Serbia	Bosilegrad	42°29'58.40"N	22°28'23.30"E	25	JN647041	JN647277				
<i>Bufo bufo</i>	BB043	Serbia	Pozega-Rupeljevo 1	43°46'44.58"N	19°59'49.74"E	15	JN647042	JN647278				
<i>Bufo bufo</i>	BB036	Serbia-Kosovo	Novo Brdo-Prekovce	42°36'58.00"N	21°26'6.40"E	24	JN647036	JN647272	JN646937	JN646888	JN646982	JN647494
<i>Bufo bufo</i>	BB035	Slovakia	Bratislava - Zelezna studnicka	48°10'60.00"N	17°4'60.00"E	9	JN647035	JN647271				
<i>Bufo bufo</i>	BB044	Slovakia	Donovaly	48°52'0.00"N	19°13'60.00"E	9	JN647043	JN647279				
<i>Bufo bufo</i>	BBUSLO5	Slovakia	Sastin-Straze	48°37'60.00"N	17°7'60.00"E	92	JN647167	JN647353				
<i>Bufo bufo</i>	BB046	Sweden	Umea - Frederika	64°5'59.99"N	18°22'59.88"E	26	JN647044	JN647280	JN646938	JN646889	JN646983	JN647495
<i>Bufo bufo</i>	BB048	Sweden	Skane-Stensoffa (Lund)	55°40'59.88"N	13°26'60.00"E	27	JN647045	JN647281				
<i>Bufo bufo</i>	BB049	Sweden	Umea - Frederika	64°5'59.99"N	18°22'59.88"E	28	JN647046	JN647282				



<i>Bufo bufo</i>	BB050	Sweden	Skane-Stensoffa (Lund)	55°40'59.88"N	13°26'60.00"E	29	JN647047	JN647283				
<i>Bufo bufo</i>	BB051	Sweden	Umea - Frederika	64°5'59.99"N	18°22'59.88"E	30	JN647048	JN647284				
<i>Bufo bufo</i>	BB167	Sweden	Vastra Götaland, Göteborg, Slätta damm	57°43'60.00"N	11°55'0.00"E	79	JN647144	JN647330				
<i>Bufo bufo</i>	BB168	Sweden	Vastra Götaland, Göteborg, Slätta damm	57°43'60.00"N	11°55'0.00"E	80	JN647145	JN647331				
<i>Bufo bufo</i>	BB169	Sweden	Vastra Götaland, Göteborg, Slätta damm	57°43'60.00"N	11°55'0.00"E	81	JN647146	JN647332				
<i>Bufo bufo</i>	BB070	Turkey	Belalankoyu - Havza	41°10'50.50"N	35°46'26.60"E	36	JN647106	JN647290	JN646940	JN646891	JN646985	JN647497
<i>Bufo bufo</i>	BB071	Turkey	Erbaa	40°49'41.40"N	36°36'5.80"E	37	JN647107	JN647291				
<i>Bufo bufo</i>	BBUTURK2A	Turkey	Kiriklar-Eflani (loc. 2)	41°23'27.90"N	32°49'3.80"E	97	JN647172	JN647358				
<i>Bufo bufo</i>	BBUTURK3A	Turkey	Belalankoyu - Havza	41°10'50.50"N	35°46'26.60"E	98	JN647173	JN647359				
<i>Bufo bufo</i>	BBUTURK4A	Turkey	Erbaa	40°49'41.40"N	36°36'5.80"E	99	JN647174	JN647360				
<i>Bufo bufo</i>	BB004	UK	Wymeswold "C"	52°48'20.00"N	1°6'24.00"W	4	JN647013	JN647249	JN646928	JN646867	JN646965	JN647501
<i>Bufo bufo</i>	BB008	UK	Holly Hayes	52°40'17.00"N	1°6'52.00"W	-	X	-	JN646929	JN646868	JN646966	JN647502
<i>Bufo bufo</i>	BB052	Ukraine	Ukraine, Zakarpatska prov., Perechin district, polonina Runa	48°47'21.00"N	22°47'51.50"E	9	JN647049	JN647285				
<i>Bufo bufo</i>	BB092	Ukraine	Ukraine, Zakarpatska prov., Perechin district, Lumshory environs	48°48'36.50"N	22°45'10.30"E	45	JN647115	JN647299				
<i>Bufo bufo</i>	BB159	Ukraine	Iskiv pond	49°37'33.97"N	36°17'7.89"E	9	JN647138	JN647321				
<i>Bufo bufo</i>	BB160	Ukraine	Iskiv pond	49°37'33.97"N	36°17'7.89"E	9	JN647139	JN647322				
<i>Bufo bufo</i>	BB161	Ukraine	Koryakin pond	49°37'10.94"N	36°18'50.04"E	9	JN647140	JN647323				
<i>Bufo bufo</i>	BB162	Ukraine	Koryakin pond	49°37'10.94"N	36°18'50.04"E	9	JN647141	JN647324				
<i>Bufo bufo</i>	BB163	Ukraine	Koryakin pond	49°37'10.94"N	36°18'50.04"E	9	JN647142	JN647325				
<i>Bufo eichwaldi</i>	BB062	Azerbaijan	Azerbaijan, Lerik district, Agoshapeshta env., Talysh mts.	38°39'34.20"N	48°38'9.30"E	34	JN647240	JN647476	JN646953	JN646908	JN647002	
<i>Bufo eichwaldi</i>	BB084	Azerbaijan	Azerbaijan, Astara district, Kizhaba environs, Talysh mts.	38°33'55.50"N	48°47'51.50"E	41	JN647239	JN647475	JN646955	JN646906	JN647003	JN647518
<i>Bufo eichwaldi</i>	BB086	Azerbaijan	Azerbaijan, Lerik district, Tankevan, Shovu and Zarikumandjo environs, Talysh mts.	38°41'44.50"N	48°35'22.30"E	42	JN647241	JN647474	JN646954	JN646909	JN647005	
<i>Bufo eichwaldi</i>	BB091	Azerbaijan	Azerbaijan, Lerik district, Agoshapeshta env., Talysh mts.	38°39'34.20"N	48°38'9.30"E	44	JN647238	JN647477				
<i>Bufo eichwaldi</i>	BB140	Azerbaijan	Azerbaijan, Astara	38°33'55.50"N	48°47'51.50"E	70	JN647242	JN647478		JN646907	JN647004	

			district, Kizhaba environs, Talysh mts.									
<i>Bufo spinosus</i>	BB005	France	Beauzelle	43°39'56.70"N	1°23'39.50"E	5	JN647219	JN647420	JN646943	JN646895	JN646990	JN647507
<i>Bufo spinosus</i>	BB006	France	Mount Canigou	42°33'56.20"N	2°25'11.00"E	6	JN647220	JN647421				
<i>Bufo spinosus</i>	BB116	France	Juigny	46°58'24.95"N	0°19'50.56"W	60	JN647212	JN647426				
<i>Bufo spinosus</i>	BB117	France	Embrun	44°33'18.72"N	6°30'26.50"E	6	JN647213	JN647427		JN646897	JN646992	JN647509
<i>Bufo spinosus</i>	BB118	France	La Manouesse	43°29'17.88"N	5°26'3.08"E	6	JN647214	JN647428	JN646945	JN646898	JN647001	JN647510
<i>Bufo spinosus</i>	BB132	France	French Pyrenees (near Arguenos)	42°58'14.48"N	0°43'26.29"E	67	JN647207	JN647435				
<i>Bufo spinosus</i>	BB147	France	Fougerolles de Plessis	48°28'31.70"N	0°58'5.10"W	6	JN647197	JN647327				
<i>Bufo spinosus</i>	BB148	France	St. Mars sur la Futane	48°25'56.10"N	1°1'4.60"W	72	JN647198	JN647328				
<i>Bufo spinosus</i>	BB165	France	St Pierre des Nids near Pre-en-Pail, dept. Mayenne, France	48°23'54.57"N	0°6'0.82"W	78	JN647195	JN647445				
<i>Bufo spinosus</i>	BB166	France	In between Vautorte and St Denis de Gastines, dept. Mayenne, France	48°18'42.00"N	0°50'40.08"W	6	JN647196	JN647446				
<i>Bufo spinosus</i>	BB190	France	Moulin ouest	43°53'20.23"N	3°23'50.39"E	86	JN647191	JN647447				
<i>Bufo spinosus</i>	BB191	France	Bergerie Hôpital	43°51'38.06"N	3°22'44.75"E	6	JN647192	JN647448				
<i>Bufo spinosus</i>	BB192	France	Sorbs (Ville Vieille)	43°53'56.04"N	3°24'19.81"E	87	JN647193	JN647449				
<i>Bufo spinosus</i>	BB193	France	Sotch de Caylus	43°53'21.11"N	3°23'13.24"E	6	JN647194	JN647450				
<i>Bufo spinosus</i>	OBYX19	France	Saint Bonnet en Champsaur	44°40'58.42"N	6°4'34.13"E	6	JN647181	JN647457				
<i>Bufo spinosus</i>	OBYX6	France	Saint Bonnet en Champsaur	44°40'58.42"N	6°4'34.13"E	6	JN647182	JN647458				
<i>Bufo spinosus</i>	OBYX8	France	Saint Bonnet en Champsaur	44°40'58.42"N	6°4'34.13"E	105	JN647183	JN647459				
<i>Bufo spinosus</i>	OBYX9	France	Saint Bonnet en Champsaur	44°40'58.42"N	6°4'34.13"E	6	JN647184	JN647460				
<i>Bufo spinosus</i>	BB119	Morocco	Ifrane	33°31'51.74"N	5°6'19.58"W	61	JN647215	JN647429	JN646946	JN646899	JN646993	JN647511
<i>Bufo spinosus</i>	BB120	Morocco	Ifrane	33°31'51.74"N	5°6'19.58"W	62	JN647216	JN647430	JN646948	JN646900	JN646995	JN647512
<i>Bufo spinosus</i>	BB121	Portugal	Portalegre	39°19'52.68"N	7°19'23.81"W	63	JN647217	JN647431	JN646949	JN646901	JN646996	
<i>Bufo spinosus</i>	BB150	Portugal	Portalegre	39°19'52.68"N	7°19'23.81"W	73	JN647199	JN647440			JN646998	JN647515
<i>Bufo spinosus</i>	BB003	Spain	35 km NE Capileira	36°58'26.20"N	3°19'21.30"W	3	JN647218	JN647425	JN646942	JN646894	JN646989	JN647506
<i>Bufo spinosus</i>	BB010	Spain	A Pobra do Caramiñal	42°36'26.01"N	8°57'39.18"W	7	JN647221	JN647422	JN646944	JN646905	JN646991	JN647508
<i>Bufo spinosus</i>	BB012	Spain	Sadernes	42°16'43.30"N	2°35'24.60"E	6	JN647222	JN647423	JN646947	JN646896	JN646994	JN647513
<i>Bufo spinosus</i>	BB013	Spain	Laguna Grande de Gredos	40°15'11.00"N	5°16'33.00"W	8	JN647223	JN647424	JN646950	JN646902	JN646997	JN647514
<i>Bufo spinosus</i>	BB129	Spain	Plá dels Corral (Simat de la Vallidigna)	39°2'36.77"N	0°18'38.16"W	65	JN647204	JN647432				
<i>Bufo spinosus</i>	BB130	Spain	A Coruña	43°22'17.37"N	8°23'45.49"W	60	JN647205	JN647433				

<i>Bufo spinosus</i>	BB131	Spain	Laguna de los Peces (Parque de Sanabria). San Martín de los Gallegos	42°10'26.91"N	6°43'47.73"W	66	JN647206	JN647434					
<i>Bufo spinosus</i>	BB133	Spain	Majaelrayo a Cantalojas	41°9'39.70"N	3°18'53.90"W	68	JN647208	JN647436					
<i>Bufo spinosus</i>	BB134	Spain	Hecho	42°44'11.10"N	0°45'37.40"W	69	JN647209	JN647437					
<i>Bufo spinosus</i>	BB135	Spain	Fanlo a Escalona	42°35'15.40"N	0°0'47.30"W	60	JN647210	JN647438					
<i>Bufo spinosus</i>	BB137	Spain	Laújar	37°5'6.60"N	2°54'17.00"W	3	JN647211	JN647439					
<i>Bufo spinosus</i>	MNCN_10071	Spain	Pelahustán. Carretera de Cenicientos	40°10'34.56"N	4°35'48.97"W	93	JN647186	JN647452					
<i>Bufo spinosus</i>	MNCN_15574	Spain	San Esteve ses Rovires	41°29'40.38"N	1°52'22.08"E	94	JN647187	JN647453					
<i>Bufo spinosus</i>	MNCN_15581	Spain	Lago Enol. Covadonga	43°16'20.68"N	4°59'28.21"W	60	JN647188	JN647454					
<i>Bufo spinosus</i>	MNCN_15600	Spain	Mougás, Pontevedra	42°3'4.46"N	8°52'21.41"W	95	JN647189	JN647455					
<i>Bufo spinosus</i>	MNCN_15622	Spain	Río Madera. Orcera	38°19'2.58"N	2°39'53.68"W	3	JN647190	JN647456					
<i>Bufo spinosus</i>	MNCN_8003	Spain	Valdemanco	40°52'21.09"N	3°39'46.64"W	93	JN647185	JN647451					
<i>Bufo spinosus</i>	BB152	Tunisia	Beni M'Tir (loc. 2)	36°44'13.30"N	8°43'3.70"E	74	JN647200	JN647441	JN646951	JN646903	JN646999	JN647516	
<i>Bufo spinosus</i>	BB153	Tunisia	Beni M'Tir (loc. 4)	36°44'27.10"N	8°42'54.30"E	75	JN647201	JN647442	JN646952	JN646904	JN646000	JN647517	
<i>Bufo spinosus</i>	BB154	Tunisia	Feija N. P. (loc. 1)	36°29'11.60"N	8°18'28.50"E	75	JN647202	JN647443					
<i>Bufo spinosus</i>	BB155	Tunisia	Feija N. P. (loc. 3)	36°29'51.10"N	8°18'19.20"E	76	JN647203	JN647444					
<i>Bufo verrucosissimus circassicus</i>	BB083	Russia	Russia, Krasnodar territory, Gelenjik district, Aderbiyevka	44°36'4.50"N	38°7'15.50"E	40	JN647233	JN647469	JN646956				
<i>Bufo verrucosissimus circassicus</i>	BB089	Russia	Russia, Krasnodar territory, Gelenjik, environs of Vozrozhdeniye, Zhene gorge	44°32'48.00"N	38°14'35.00"E	32	JN647236	JN647472					
<i>Bufo verrucosissimus circassicus</i>	BB090	Russia	Russia, Krasnodar territory, Gelenjik, Dzhankot environs	44°28'1.30"N	38°9'0.90"E	32	JN647237	JN647473					
<i>Bufo verrucosissimus tertyschnikovi</i>	BB058	Russia	Russia, Stavropol territory, Shpakovskiy district, Strizhament mt., environs	44°38'44.20"N	41°32'36.30"E	32	JN647226	JN647462	JN646959	JN646912	JN647008	JN647520	
<i>Bufo verrucosissimus tertyschnikovi</i>	BB085	Russia	Russia, Stavropol territory, Shpakovskiy district, Novoyekaterinovskaya environs	44°45'58.50"N	42°2'4.50"E	32	JN647234	JN647470					
<i>Bufo</i>	BB057	Russia	Russia, Krasnodar	43°55'59.40"N	40°39'45.30"E	32	JN647225	JN647461	JN646958	JN646911	JN647010	JN647519	

<i>verrucosissimus turowi</i>			territory, Caucasian Nature Reserve, environs of Chernorechye									
<i>Bufo verrucosissimus turowi</i>	BB061	Russia	Russia, Krasnodar territory, Caucasian Nature Reserve, environs of Chernorechye	43°55'59.40"N	40°39'45.30"E	32	JN647229	JN647465				
<i>Bufo verrucosissimus verrucosissimus</i>	BB059	Georgia	Georgia, Abkhazia, Bzypi gorge, Malaya Ritza lake	43°28'34.30"N	40°28'21.40"E	32	JN647227	JN647463	JN646914			JN647521
<i>Bufo verrucosissimus verrucosissimus</i>	BB060	Georgia	Georgia, Adjaria, Charnali gorge, env. of Charnali	41°31'52.30"N	41°37'27.40"E	33	JN647228	JN647464	JN646960			JN647522
<i>Bufo verrucosissimus verrucosissimus</i>	BB063	Georgia	Georgia, Borjomi valley, Bakuriani, Jagoras-Veli	41°43'30.20"N	43°31'8.20"E	33	JN647230	JN647466				
<i>Bufo verrucosissimus verrucosissimus</i>	BB064	Georgia	Georgia, Borjomi valley, Akhaldaba river	41°52'56.50"N	43°21'30.50"E	35	JN647231	JN647467	JN646957	JN646910	JN647006	JN647523
<i>Bufo verrucosissimus verrucosissimus</i>	BB088	Russia	Russia, Krasnodar territory, Sochi district, Agura environs	43°46'20.50"N	39°37'22.70"E	43	JN647235	JN647471	JN646961	JN646915	JN647009	JN647524
<i>Bufo verrucosissimus verrucosissimus</i>	BB073	Turkey	Turkey, Karagol	41°18'25.60"N	42°28'56.00"E	33	JN647232	JN647468	JN646962	JN646913	JN647007	JN647525
<i>Outgroups</i>												
<i>Bufo bankorensis</i>	BB098	China	China, Taiwan, Taipei environs, Fu Yang Shan N.P.	25°01'14.50"N	121°33'36.50"E	49	X	X	X	X	X	X
<i>Bufo bankorensis</i>	BB099	China	China, Taiwan, Taipei environs, Fu Yang Shan N.P.	25°01'14.50"N	121°33'36.50"E	50	X	X	X		X	X
<i>Bufo gargarizans gargarizans</i>	BB096	China	China, Jilin province, SongJiangHe river, 50 km W from BaiTouShan (PekTuSan) volcano	42°9'3.50"N	127°30'44.50"E	47	JN647245	JN647481	X			X
<i>Bufo gargarizans gargarizans</i>	BB095	Russia	Russia, Jewish autonomous region, Bastak Nature Reserve, sq. 140	47°59'55.60"N	132°4'36.50"E	46	JN647244	JN647480	X	X	X	X

<i>Bufo gargarizans gargarizans</i>	BB097	Russia	Russia, Primorskiy (Maritime) territory, Partizansk district, Tigrovoy environs	43°9'17.30"N	132°53'0.30"E	48	JN647246	JN647482				
<i>Bufo gargarizans sachalinensis</i>	BB094	Russia	Russia, Sakhalin province, Sakhalin island, Tymovskiy district, Tym river, environs of Tymovskoye settlement	50°52'32.70"N	142°37'30.70"E	46	JN647243	JN647479				
<i>Bufo japonicus formosus</i>	BB101	Japan	Japan, Tokyo prefecture, Tokyo city, Sendagi	35°43'15.40"N	139°45'37.50"E	52	X	X	X	X	X	X
<i>Bufo japonicus formosus</i>	BB102	Japan	Japan, Aomori prefecture, Hirosaki city environs, Hirakawa	40°34'41.50"N	140°34'43.50"E	53	X	X	X	X	X	
<i>Bufo torrenticola</i>	BB100	Japan	Japan, Nara prefecture, Tenkawa environs	34°14'16.50"N	135°51'4.50"E	51	X	X	X	X	X	X

755 Table 2. Primer sequences used in this study.

756

Primer name	Sequence	Reference
16S-ar	CGC CTG TTT ATC AAA AAC AT	Palumbi et al. 1991
16S-br	CCG GTC TGA ACT CAG ATC ACG T	Palumbi et al. 1991
Cyt Bufo F	ATC TAC CTT CAC ATC GGA CGA G	This study
Cyt Bufo R	AGT TTR TTT TCT GTG AGT CC	This study
POMC_DRV_F1	ATA TGT CAT GAS CCA YTT YCG CTG GAA	Vieites et al. 2007
POMC_DRV_R1	GGC RTT YTT GAA WAG AGT CAT TAG WGG	Vieites et al. 2007
BUFO_CXCR4F	TTC TGG TCT GTG GAY GCY GCC ATT	This study
BUFO_CXCR4_R	TTG AGG CAG CAG TGG AAG AAG GCC	This study
BDNF_F1	ACC ATC CTT TTC CTK ACT ATG	This study
BDNF_R1	CTA TCT TCC CCT TTT AAT GGT	This study
RPL3buF1	AGG TGG CAG TGT CTG GAG TAT T	This study
RPL3buR1	GTC ACC TTA CTA CAG CTT GTT CTC A	This study

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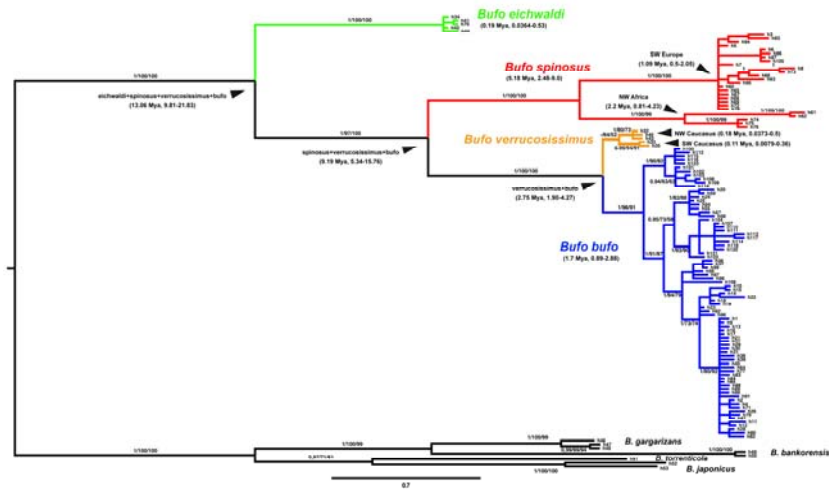
759

760 Table 3. Estimates of evolutionary divergence over sequence pairs between species as estimated by MEGA5. Mean values of p-uncorrected genetic distances  
761 and the corresponding standard errors are shown.  
762

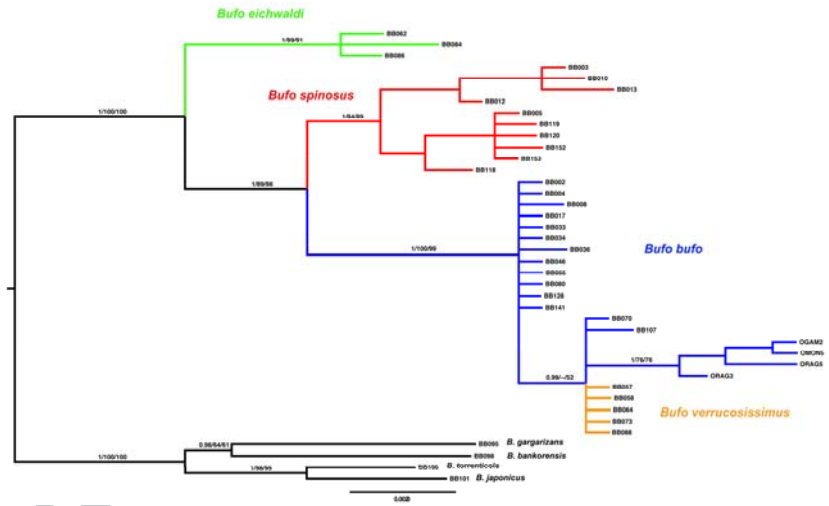
Species 1	Species 2	mean p-uncorrected	std. error
<i>B. bufo</i>	<i>B. spinosus</i>	7.0%	0.6%
<i>B. bufo</i>	<i>B. verrucosissimus</i>	1.6%	0.3%
<i>B. spinosus</i>	<i>B. verrucosissimus</i>	6.3%	0.6%
<i>B. bufo</i>	<i>B. eichwaldi</i>	7.3%	0.7%
<i>B. spinosus</i>	<i>B. eichwaldi</i>	7.7%	0.7%
<i>B. verrucosissimus</i>	<i>B. eichwaldi</i>	6.9%	0.7%

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## A) mt-DNA data

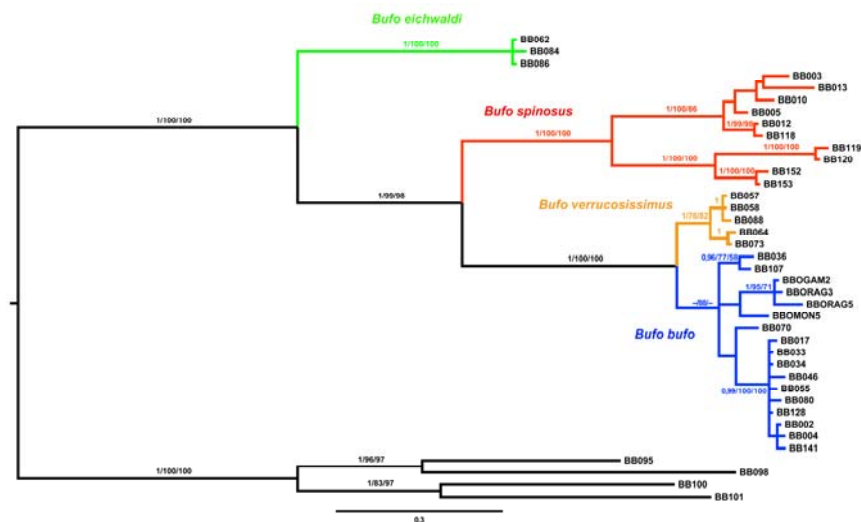


## B) Concatenated nuclear DNA data

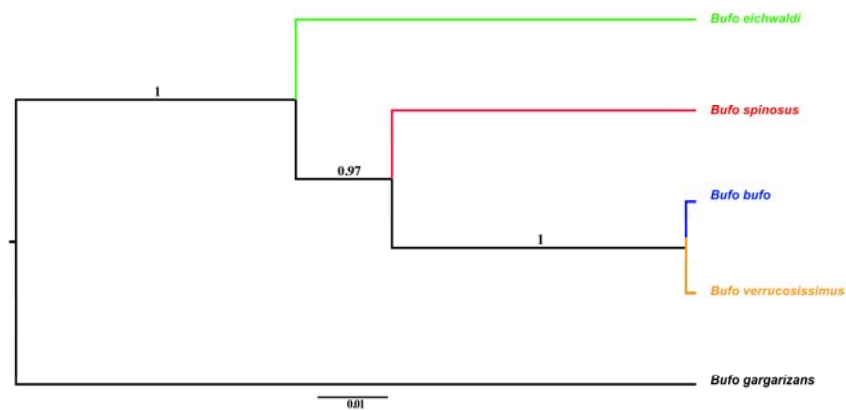


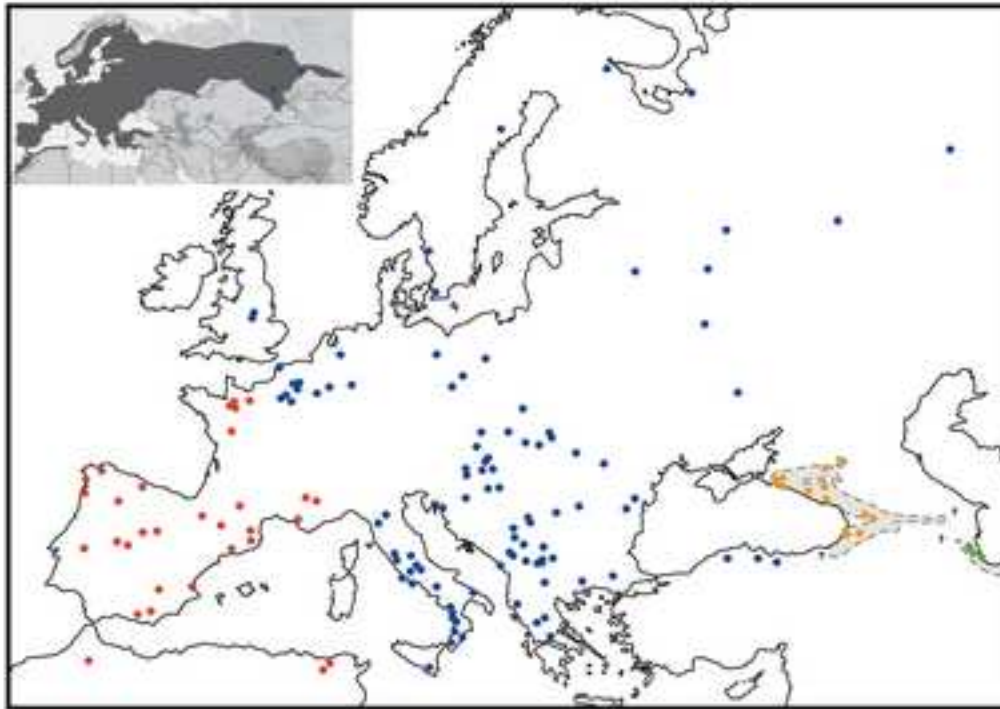


## A) Concatenated mt-DNA + n DNA



## B) Multispecies coalescent (\*BEAST)





*Bufo eichwaldi*



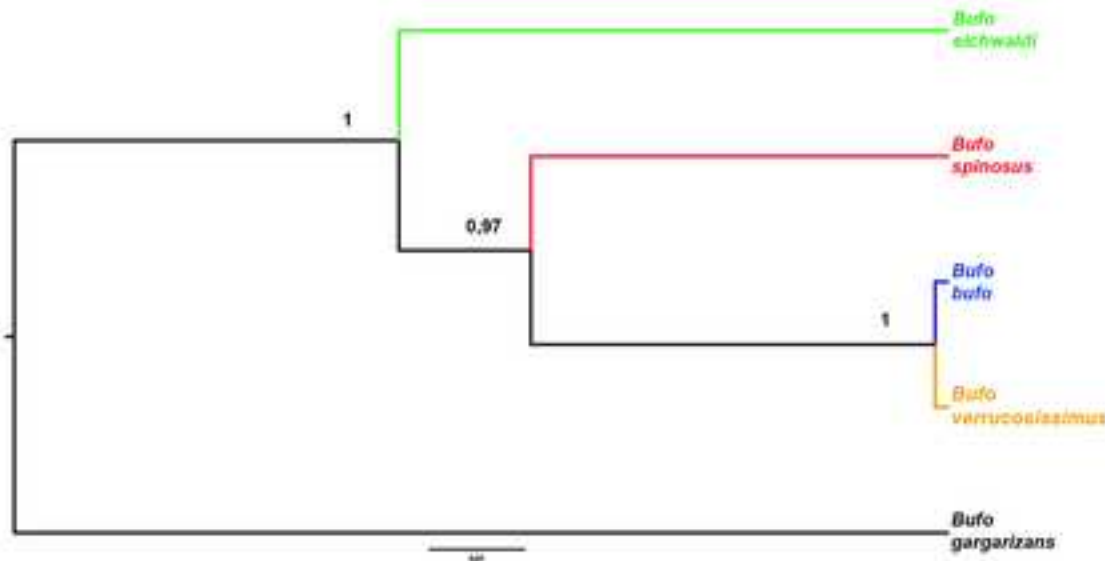
*Bufo spinosus*



*Bufo bufo*



*Bufo verrucosissimus*



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771 **Highlights**

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773 Species tree analyses resolve phylogenetic relationships in *Bufo bufo* species group

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775 Overall agreement between mtDNA and nDNA markers

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777 The evolutionary history of *B. bufo* dates back to the Miocene

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779 *Bufo spinosus* is distributed in N Africa, Iberian Peninsula and France

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ACCEPTED MANUSCRIPT