

## First report of *Bufo bufo* (Linnaeus, 1758) from Sardinia (Italy)

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**Abstract.** The Common toad *Bufo bufo* (Linnaeus, 1758) was found for the first time in Sardinia in 2016 during herpetological surveys. The species appears to be well established in the finding area. Many adults and juveniles, tadpoles at different developmental stages, and eggs have been found during repeated monitoring. In order to infer the geographic origin of the Sardinian population, we amplified two mitochondrial markers (*16S*, *cytb*) and compared sequences with those available for the species across its natural range. We also screened samples for the presence of *Bd* pathogen to assess the risk of infection mediated by the species in the area. Results suggest that Sardinian individuals are genetically close to the Central Italian populations, although they show a unique distinct haplotype. Though the species should be considered allochthonous to the Island, further molecular and ecological data are urgently needed to assess the genetic structure and the possible impact on the local fauna, which is largely composed by endemic taxa. Particularly, possible interactions with other native amphibians like the green toad *Bufo balearicus* (Boettger, 1880), also present in the area, should be investigated, both in terms of competition for breeding sites and genetic pollution, as these species are already known to hybridize in the wild.

**Keywords.** Common toad, amphibians, early detection, Sardinia, Mediterranean Basin.

### INTRODUCTION

The Common toad *Bufo bufo* (Linnaeus, 1758) is widespread in the western Palearctic region, from North Africa to the 68°N in Finland and from the western Iberian Peninsula to the Baikal Lake in Siberia (Lizana, 2002; Böhme et al., 2007). The only exceptions are Iceland, the coldest northern parts of Scandinavia, Ireland and several Mediterranean islands, among which Malta, Crete, Corsica, Sardinia and the Balearic Islands (Böhme et al., 2007).

Until recently, the evolutionary history of this species has been controversial and so far molecular studies have revealed the existence of distinct subspecies and delimited their respective ranges (Litvinchuk et al., 2008; Garcia-Porta et al., 2012; Recuero et al., 2012; Arntzen et al., 2013a,b). In Europe, several distinct evolutionary lineages occur (at least 7, according to Garcia-Porta et al., 2012) which diversified between the late Miocene and early Pliocene (11.6-3.6 Ma). For instance, distinct phylogroups have been detected in the Italian Peninsula, one in the South, including Sicily, and the other in the northern-

central regions, as a result of the persistence of populations in multiple refugia during the Pleistocene glaciations (according to the “refugia within refugia” hypothesis, Gómez and Lunt, 2007).

*Bufo bufo* has been discovered in north-central Sardinia in late summer 2016. Several surveys, carried out in the area of the first detection, revealed a high density of both adults and larvae. Later on, adults and juveniles have been also detected far from the original detection point, suggesting the occurrence of a stable and viable population in the region. According to the composition of the Sardinian amphibian community, the species could be classified as allochthonous, likely resulting from one or multiple human-mediated introductions from the mainland. Therefore, in order to provide information concerning the geographic origin of the Common toad found in Sardinia, informative mitochondrial molecular markers were selected to compare insular haplotypes with the mainland ones.

## MATERIALS AND METHODS

### Study Area

The study area is located in north-central Sardinia at an elevation of 400-1000 m a.s.l., in a mountain woodland region characterized by a mosaic of high Mediterranean maquis, as well as coniferous and deciduous forest resulting from several anthropic interventions. Artificial small lakes and ponds occur throughout the area. The small streams flowing from the above mentioned water bodies offer a suitable habitat for amphibians. The climate is characterized by mild wet winters (rarely T min goes below 0°C), when high precipitation rate is recorded. In summer, the dry season lasts in general no longer than three months. The area therefore presents suitable ecological conditions for the species.

### Sampling and molecular analysis

Surveys were carried out from late summer 2016 until June 2017. Toads were searched by active search (VES = Visual Encounter System; Crump and Scott, 1994) at night under rainy weather conditions to facilitate the detection of adults. Several adults were observed throughout the study area as well as, at least 150, post-metamorphs on the shore of a small water body. Swabs and tissues were collected in order to perform genetic characterization by molecular analysis. Specifically, biological samples were obtained from one tadpole, three post-metamorphs, and two adults. Tissue samples were collected only from death animals. The first buccal swab was collected on an adult on January 12<sup>th</sup>, 2017. All swabs and tissue samples (N = 6) were preserved in ethanol 96% until DNA extraction. In the laboratory, genomic DNA was extracted using the commercial kit GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-

Aldrich, Saint Louis, USA) and following the manufacturer's instructions.

To infer the putative geographic origin of the sampled individuals, we amplified two different diagnostic mitochondrial markers, corresponding to a portion (504 bp) of the ribosomal 16S rDNA gene (16S) and a portion (722 bp) of the protein-encoding cytochrome *b* (*cytb*) region, using primer pairs already available in the literature (Palumbi et al., 1991; Recuero et al., 2012). The selected markers are known to be diagnostic for discriminating Italian populations and lineages (Recuero et al., 2012; Arntzen et al., 2017). PCR products were purified with GenElute Gel Extraction Kit (Sigma-Aldrich, Saint Louis, USA) following the manufacturer's instructions, and sequenced using the forward primer of each pair (Eurofins Genomics/Applied Genomics, Ebersberg, Germany).

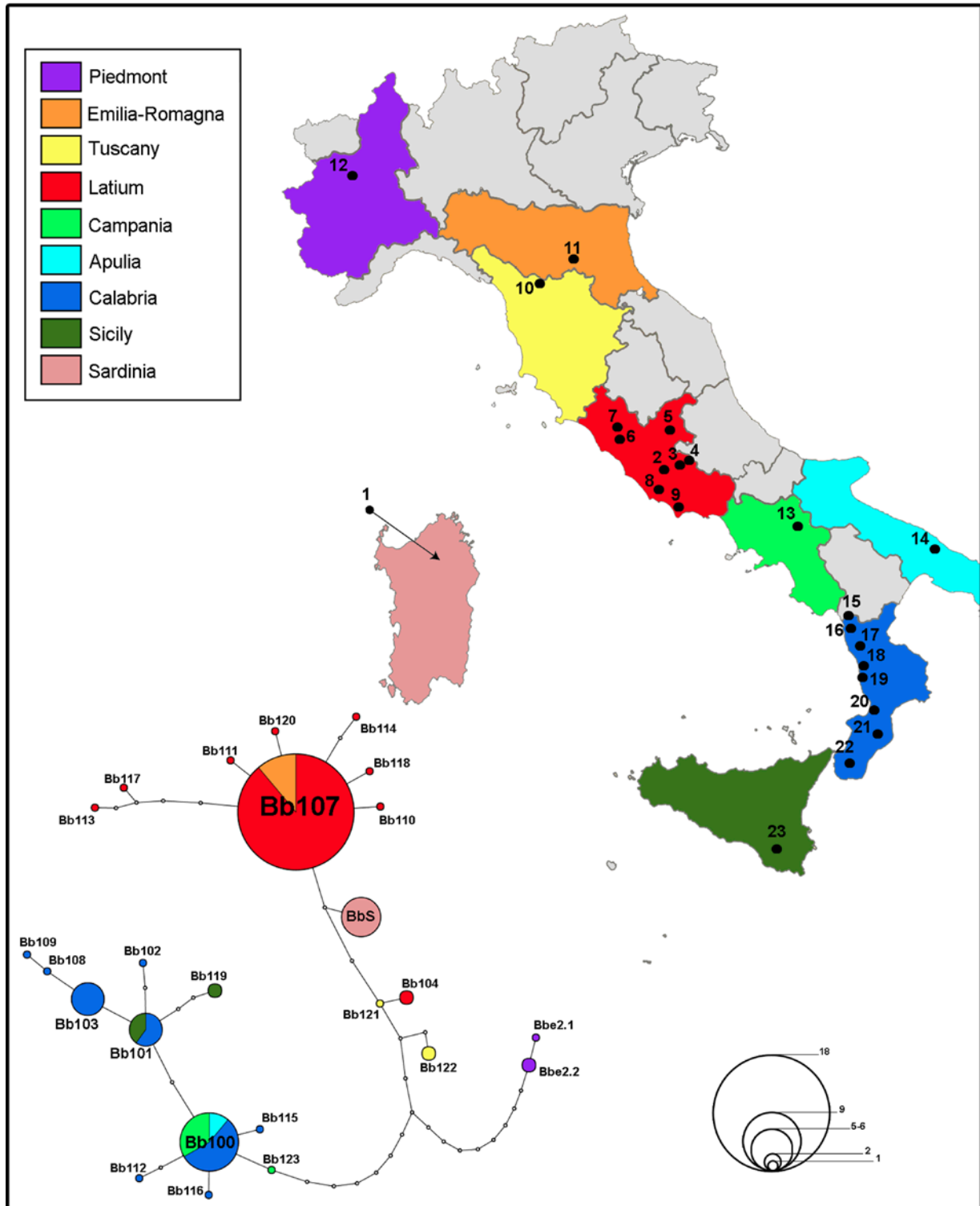
Chromatograms were imported in Geneious 10.0.9 (Biomatters Ltd.) and checked manually for insertions or deletions (indels) and ambiguous positions in protein-encoding mitochondrial gene fragments. The latter were also translated into amino acidic sequences to exclude the presence of non-functional copies of target markers (i.e., pseudogenes), which can be detected by premature stop codons or non-sense codons occurring in the coding frame. Sequences were aligned for each gene independently with the online version of MAFFT (Katoh and Toh, 2008) applying parameters by default (Auto strategy, Gap opening penalty: 1.53, Offset value: 0.0).

Obtained sequences were compared with all the available homologous sequences from GenBank (<https://www.ncbi.nlm.nih.gov/>) using the Basic Local Alignment Search Tool (BLAST, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and setting the nucleotide blast (nblast) algorithm with default parameters.

Concatenated alignment was built for including 61 homologous sequences from 22 Italian populations (Recuero et al., 2012; Arntzen et al., 2017), then individual sequences were merged into single gene haplotypes using the on-line web tool DNACollapser 1.0 available at FaBox site (<http://users-birc.au.dk/biopv/php/fabox/>) (please refer to Table 1 for all the accession numbers and information concerning populations and haplotypes). Relationships among mitochondrial DNA (mtDNA) haplotypes were visualized by reconstructing a statistical parsimony haplotype network, selecting the 95% connection limit as a reliable parsimony threshold as implemented in TCS 1.21 (Clement et al., 2000). This procedure allowed examining the extent of haplotype sharing between the Sardinian population and those from the mainland, under the statistical parsimony method (Templeton et al., 1992). Finally, tissues were screened for the presence of the diagnostic molecular marker (300 bp long) in order to detect the presence of the chytrid fungus *Batrachochytrium dendrobatidis* in our samples according to Annis et al. (2004).

## RESULTS

All the biological samples collected in the field were successfully amplified, nor gap nor premature stop codon were detected in the protein-encoding *cytb* sequences.



**Fig. 1.** Distribution of the Sardinian and other Italian populations of *Bufo bufo* analysed in this study. Different regions for which mtDNA data were available have been drawn using different colours. Population numbers refer to Table 1. Bottom-left: haplotype network reconstruction of concatenated mtDNAs (*16S+cytb*) showing phylogeographic relationships among populations and haplotypes. Each circle corresponds to a distinct haplotype, and size is proportional to the number of samples sharing the same mtDNA sequence. Colours refer to distinct Italian regions. Each bar refers to a single point mutation connecting two haplotypes, each dot corresponds to missing haplotypes (not sampled or extinct). Reference haplotype names have been modified from Recuero et al. (2012) and Arntzen et al. (2017).

**Table 1.** List of localities and mtDNA haplotypes (*16S*+*cytb*) used as reference for the Sardinian Common toad population, with specification of sample sizes (xN), accession numbers and reference ([1] Recuero et al., 2012; [2] Arntzen et al., 2017). In bold: reference sequences showing 100% (*16S*) and 99% (*cytb*) sequence similarity with the Sardinian haplotypes.

Map	Locality	ID <sub>haplo</sub> xN	Accession numbers		Reference
			16S	Cytb	
1	north-central Sardinia	BbS(x6)	MG199041	MG199042	this study
2	Doganella, Latium	Bb107(x4)	<b>JN647054-57</b>	<b>JN647367-70</b>	[1]
3	Subiaco, Latium	Bb120(x1)	<b>JN647094</b>	JN647407	[1]
		Bb107(x1)	<b>JN647095</b>	<b>JN647408</b>	
4	Jenne, Latium	Bb113(x1)	JN647071	<b>JN647384</b>	[1]
		Bb107(x1)	<b>JN647072</b>	<b>JN647385</b>	
5	Rocca Sinibalda, fiume Turano, Latium	Bb114(x1)	JN647073	<b>JN647386</b>	[1]
		Bb107(x3)	<b>JN647099-101</b>	<b>JN647412-14</b>	
6	Canale Monterano, Latium	Bb107(x3)	<b>JN647082-83/85</b>	<b>JN647395-96/98</b>	[1]
		Bb118(x1)	JN647084	<b>JN647397</b>	
7	Barbarano, Latium	Bb104(x2)	JN647179-80	<b>JN647365-66</b>	[1]
8	Bosco del Foglino, Latium	Bb110(x1)	JN647064	<b>JN647377</b>	[1]
		Bb107(x1)	<b>JN647065</b>	<b>JN647378</b>	
9	Molella, Fonti di Locullo, Latium	Bb111(x1)	<b>JN647066</b>	JN647379	[1]
		Bb107(x3)	<b>JN647078-79/81</b>	<b>JN647391-92/94</b>	
10	Campo Tizzoro, Tuscany	Bb117(x1)	JN647080	<b>JN647393</b>	[1]
		Bb121(x1)	JN647096	<b>JN647409</b>	
11	Monghidoro, Emilia-Romagna	Bb122(x2)	JN647097-98	JN647410-11	[1]
		Bb107(x2)	<b>JN647069-70</b>	<b>JN647382-83</b>	
12	Favero, Piedmont	Bbe2.1(x1)	MF461242	MF461270	[2]
		Bbe2.2(x2)	MF461243-44	MF461271-72	
13	Pian di Verteglia, Campania	Bb100(x3)	JN647102/04-05	JN647415/17-18	[1]
		Bb123(x1)	JN647103	JN647416	
14	Alberobello, Apulia	Bb100(x1)	JN647175	JN647361	[1]
		Bb115(x1)	JN647074	JN647387	
15	Tortora, C.da Massadita, Calabria	Bb114(x1)	JN647075	JN647388	[1]
		Bb100(x2)	JN647076-77	JN647389-90	
16	Orsomarso, Calabria	Bb100(x3)	JN647086-87-88	JN647399-400-01	[1]
17	Fagnano Castello, Lago Paglia, Calabria	Bb108(x1)	JN647058	JN647371	[1]
		Bb109(x1)	JN647059	JN647372	
18	Fiumefreddo Bruzio, Calabria	Bb103(x4)	JN647060-63	JN647373-76	[1]
19	Bel Monte Calabro Marina, Calabria	Bb103(x1)	JN647178	JN647364	[1]
20	Lago dell'Antigola, Calabria	Bb101(x1)	JN647176	JN647362	[1]
		Bb102(x1)	JN647177	JN647363	
21	Stilo, Calabria	Bb101(x1)	JN647093	JN647406	[1]
22	Gambarie, Calabria	Bb101(x1)	JN647068	JN647381	[1]
		Bb112(x1)	JN647067	JN647380	
23	Fiume Irminio, Sicily	Bb101(x2)	JN647089-92	JN647402-05	[1]
		Bb119(x2)	JN647090-91	JN647403-04	

We detected one single *16S* haplotype (accession number: MG199041) and one *cytb* haplotype (accession number: MG199042) in all Sardinian samples.

The *16S* haplotype yielded a 100% match with homologous sequences from Latium and Emilia-Romagna (Recuero et al., 2012). By contrast, no exact match was found in GenBank for the Sardinian *cytb* haplotype, which returned 99% match probability with homologous

sequences from the same localities as *16S*, and from Tuscany (Recuero et al., 2012; see Table 1 for all the accession numbers).

In the final concatenated alignment of 67 mitochondrial sequences (1226 bp), including Sardinian samples and homologous sequences from the Italian Peninsula and Sicily (Recuero et al., 2012), 25 unique haplotypes were detected, corresponding to 23 populations (Table 1). The

reconstruction of the haplotype network allowed a better resolution of phylogeographic relationships between the Sardinian population and the other Italian ones, as the Sardinian haplotype clearly clustered together with samples from Central Italy (Fig. 1).

The most closely related haplotypes were Bb107, already found in 8 localities in Latium by previous authors, and Bb121 recovered in the only population screened from Tuscany (Recuero et al., 2012) (Table 1).

Finally, none of the samples was positive for the presence of diagnostic chytrid marker (not shown).

## DISCUSSION

The Common toad, never recorded for Sardinia (Sindaco et al., 2006), was found on the Island in 2016. Therefore, a genetic analysis was performed in order to assess the origin of the Sardinian population. According to our molecular results, the low genetic variation detected at both markers (one single haplotype shared by all the samples) attests a general low genetic variation of the population, as expected when founder effect occurs after the arrival of few colonizers.

The occurrence of *B. bufo* in Sardinia could be due to direct release of captive individuals. However, considering the distribution and the high density of the individuals detected, it is also possible to assume alternative introduction routes. Toads could have been introduced on the Island along with plants imported from Central Italy in the '70s and '80s of the last Century, but the most reliable cause could be the introduction of eggs and tadpoles along with fish stocks (particularly, the rainbow trout *Oncorhynchus mykiss*) introduced for game fishing about 30-40 years ago. Indeed, this activity is still widely carried out in the study area.

According to the molecular results, Central Italy should be considered as the most likely source area of the Sardinian Common toad population. Indeed, haplotypes previously recovered in this area, belonging to the native populations of the species, were close to the one found in Sardinia according to both markers. Nevertheless, although the 16S haplotype perfectly matches mainland sequences from several localities (mainly in Central Italy), the *cytb* haplotype of the Sardinian samples is at present unique of this population, suggesting that the source population has not yet been sampled.

Repeated field surveys allowed to observe different life stages showing the reproductive success of the Common toad in Sardinia and its relatively wide distribution in the area, at present corresponding approximately to 17.000 ha (further surveys will be carried out in order to better define the distribution of the Sardinian *B. bufo* popula-

tion). All the ecological requirements of the species seem to be fulfilled in the study area (e.g., occurrence of streams and different kinds of water bodies, favorable climate, and trophic availability). The Common toad in fact successfully reproduces as larval stages were detected in late winter and juveniles occurred in late spring following the reproductive ecology of the species. According to our results, none of the samples turned out to be positive to chytridiomycosis. However, we acknowledge that considering the limited number of samples screened, and in the absence of histological data, we could not rule out the occurrence of the pathogen in the whole population. Nevertheless, as most of the samples were collected from adults and post-metamorphs, we are sufficiently confident that the population is not currently undergoing a massive epidemic.

Sardinia hosts an extremely valuable herpetofauna, six amphibian species are endemic, the Sardinian mountain newt (*Euproctus platycephalus*) and five species of cave salamanders (*Speleomantes flavus*, *S. imperialis*, *S. sarrabusensis* and *S. supramontis* and *Atylodes genei*). Others could be considered sub-endemic while occurring also in Corsica and on some North-Tyrrhenian and Ligurian islands, like the Tyrrhenian painted frog (*Discoglossus sardus*) and the Tyrrhenian tree frog (*Hyla sarda*). Among the Bufonids, the Balearic green toad (*Bufo balearicus*) is thought to be autochthonous to the Island (Poggesi et al., 1995; Lanza et al., 2007). The presence of *H. sarda* and *B. balearicus* has been directly assessed during the surveys, and it is likely to assume that other amphibians like *D. sardus* should also be present in the area. Therefore, considering the peculiarity of some Sardinian faunal elements, which originate from the long lasting isolation of the Island, a recent introduction of the Common toad may raise questions about the possible interaction with other species, particularly *Bufo balearicus*. However, reduced competition was observed between the two toad species because of their different ecology (Sinisch et al., 1999).

*Bufo balearicus* (identified in the literature as *Bufo viridis* or *Bufo* gr. *B. viridis*) appears rather continuously throughout the paleontological Sardinian record from the late Pliocene (Capo Mannu D1 Local Fauna, Mandriola, western Sardinia, Delfino et al., 2011) to the Pleistocene (Monte Tuttavista, Nuoro, Abbazzi et al., 2004; Grotta di Dragunara, Capo Caccia, western Sardinia, Kotsakis, 1980) and Holocene (Grotta Corbeddu, Oliena, central Sardinia, Sondaar et al., 1984; Porto Leccio, Trinità d'Agultu, northern Sardinia, Delfino, 2002; Grotta di Su Guanu, Gonagosa, Oliena, central Sardinia, Sanchiz, 1979).

By contrast, fossils ascribed to *Bufo bufo* have been reported to date only once for the Neolithic site of Su Guanu (Sanchiz, 1979), co-occurring with remains undoubtedly referable to *B. balearicus*. The presence of *B.*

*bufo* at Su Guanu is based on fragmentary evidence represented only by two humeri (a male and a female), two radioulnae and two femura that were partly described and figured by a competent anuran expert. The referral of the material to *B. bufo*, the femura in particular, could be therefore correct and was considered as such in the list of the fossil Salientia published nearly two decades later by the same author (Sanchiz, 1998).

According to these findings, despite the most supported scenario is suggesting human-mediated translocation of *B. bufo* to Sardinia happened in very recent times, the fact that the species already occurred in the past could not be ruled out, and the relative proximity (about 50 km) of the Neolithic site of Su Guanu and the recently discovered population here reported is curious at least. Moreover, amphibian dispersal across the sea was reported several times despite the limitations related to the osmotic stress by salt water (Vences et al., 2003; Measey et al., 2007). It is also known that brackish waters do not prevent Common toads from swimming in open water in the North Baltic Sea, even allowing gene flow between islands, indicating a certain degree of salt tolerance in this species (Seppa and Laurila, 1999).

To date, even if no evidence of negative impact of *Bufo bufo* on the Sardinian population of *B. balearicus* was detected, further ecological and distributional studies are needed to better detail possible interactions between the Common toad and the Green toad that also coexist on the mainland. However, it is likely that the Common toad will not be able to spread far beyond the limits imposed by its ecological requirements, which do not allow this species to persist in the xeric habitats that characterize most of the Sardinian territory (Costantini et al., 2005).

At present, it should be acknowledged that, although possible drawbacks could not be ruled out (e.g., F1 hybrids have been sporadically detected in the wild by Bressi et al., 2000; Duda, 2007), the two toads actually coexist on the mainland without interfering. Moreover, the arrival of a Palearctic element such as *Bufo bufo* in Sardinia should not be evaluated so negatively as, e.g., the presence of *Xenopus laevis*, which instead is alien to the Palearctic, in Sicily (Faraone et al., 2008).

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