

Discovery of alien water frogs (gen. *Pelophylax*) in Umbria, with first report of *P. shqipericus* for Italy

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Abstract. Allochthonous water frogs (gen. *Pelophylax*) have been repeatedly introduced in several European countries, causing dramatic consequences for the conservation of indigenous taxa. In Italy, invasive populations are known for northern regions, where they were introduced mainly for edible and scientific purposes. Here, we report the first detection of an alien population of water frogs in Central Italy, along the Resina valley (Umbria). Genetic analysis of the mitochondrial ND3 gene polymorphism assigned some specimens to two different *Pelophylax ridibundus* clades widespread in Central and Eastern Europe. By contrast, two samples matched the mitochondrial DNA (mtDNA) sequence of *Pelophylax lessonae bergeri*, an autochthonous taxon widespread in Central Italy, suggesting possible hybridization between alien and indigenous frogs. Finally, the specific haplotype of *Pelophylax shqipericus*, the Albanian Pool Frog, was also identified according to mtDNA polymorphism. This record, firstly reported for Italy, poses concerns for the conservation of this cryptic taxon, suggesting that international water frog trade may involve also particularly endangered species.

Keywords. *Pelophylax ridibundus*, *Pelophylax shqipericus*, European water frogs, alien species, conservation, mitochondrial DNA, Central Italy.

Water frogs of the genus *Pelophylax* (Fitzinger, 1843) comprise several species widespread in a multitude of freshwater habitats across Western Palearctic. Within the genus, several complexes of genetically similar taxa can naturally hybridize in the wild, posing questions for species determination and conservation (e.g., Akin et al., 2010). For instance, in Central and Eastern Europe, the Pool Frog *Pelophylax lessonae* (Camerano, 1882) can mate with the Marsh Frog *Pelophylax ridibundus* (Pallas, 1771), to produce the hybrid taxon *Pelophylax esculentus* (Linnaeus, 1758) according to a fascinating and unusual mode of reproduction (i.e., hybridogenesis). During hybridogenetic reproduction, *P. esculentus* discards the *lessonae* genome prior to meiosis in order to produce gametes containing only the *ridibundus* genome, which

is clonally transmitted in each generation (Tunner and Heppich, 1981; Pagano et al., 1997).

The absence of strong reproductive barriers coupled with the weak morphological variation of different taxa pose serious issues for conservation, because invasive lineages may rapidly pollute the gene pool of indigenous populations spreading over a large geographic range without being detected (e.g., Pagano et al., 2003; Holsbeek and Jooris, 2010; Holsbeek et al., 2010). In recent years, molecular techniques proved to be extremely useful for species determination within the *Pelophylax* genus, because distinct taxa can be identified according to genetic variation, despite the weak morphological and/or bioacoustic differentiation of several species (Lode and Pagano, 2000; Plötner and Ohst, 2001; Plötner et al., 2001, 2008, 2009; Akin et al., 2010).

Despite exotic lineages of European water frogs have been introduced repeatedly due to uncontrolled commercial trade (e.g., Belgium: Holsbeek et al., 2010; France: Schmeller et al., 2007; Germany: Mayer et al., 2013; Great Britain: Zeisset and Beebee, 2003; Spain: Arano et al., 1995), this group is currently classified in Italy as less vulnerable among amphibians (Andreone and Luiselli, 2000). From a taxonomic point of view, the situation of Italian populations is rather complicated by the presence of at least five different species and hemiclonally-reproducing hybrid lineages, living in mixed hybridogenetic populations. In Northern Italy, hybridogenetic populations of *P. lessonae* and *P. esculentus* occur, while southwards populations are assigned to two different taxa, *Pelophylax lessonae bergeri* (Günther, 1986) and the hybridogenetic *Pelophylax hispanicus* (Bonaparte, 1839). In Sicily, a different and not yet described subspecific taxon exists (Canestrelli and Nascetti, 2008), while in Sardinia, water frog populations have been introduced several times during the past two centuries from various regions of the Italian Peninsula as well as from other countries (e.g., Eastern Turkey, Bellati et al., 2012). Autochthonous populations of *P. ridibundus* occur only in the Carso Triestino (Friuli-Venezia Giulia, North-eastern Italy, Dolce, 1976; Mezzena and Dolce, 1978; Lanza, 1983), but allochthonous populations have been repeatedly introduced in many Italian regions from other European countries for edible and scientific purposes (e.g., Andreone and Sindaco, 1999; Bressi, 2006b; Razzetti et al., 2006). The most important geographic source of alien populations has been identified in the eastern Mediterranean regions, such as Turkey, Greece and the Balkans (see Ficetola and Scali, 2010 for a history of introductions).

To date, documented introduction of alien taxa of European water frogs in Italy are the Balkan Frog *Pelophylax kurtmuelleri* (Gayda, 1940) in Liguria and Piedmont regions (Lanza, 1962), *P. ridibundus* in Friuli Venezia Giulia and Trentino Alto Adige (Lapini and Zanghellini, 1993; see also Bressi, 2006 a) and probably *Pelophylax bedriagae* (Camerano, 1882) in Emilia Romagna. Noteworthy, despite the detection of several alien populations of water frogs in Northern Italy, records from central regions (south of Emilia-Romagna) are scarce or even completely absent. In Umbria (Central Italy) no allochthonous taxa of water frogs have been reported during last decades (Ragni et al., 2006).

During summer 2012, we found large-sized frogs in a pond located near the Resina river, in the province of Perugia (43.24679°N, 12.48821°E WGS84). In late September 2012, during field work, we observed adult and juvenile water frogs of the *Pelophylax* complex. However, no correspondence of these specimens with any Italian

taxon was possible according to morphological characters. These specimens were characterized by large body size, wrinkled skin, small metatarsal tubercles, and males had black-coloured vocal sacs (Table 1, Fig. 1B). According to morphology, these frogs were classified as *P. cf. ridibundus* and clearly distinguished from the autochthonous central Italian taxa observed at the same site (*P. l. bergeri* and *P. hispanicus*), which showed bright green and yellow colours and big metatarsal tubercles (Lanza, 1983; Günther, 1990; Lanza et al., 2007, 2009). The unidentified water frogs were found together, or in immediate proximity, of a rich batrachofauna including *Lissotriton vulgaris*, *Triturus carnifex*, *Bufo bufo*, *Hyla intermedia*, *Rana italica* and *Rana dalmatina*. Morphometric measurements were recorded by photographing them on a metric board and the images were then analyzed using Quantum GIS and ArcView GIS software. This allowed to take the standard morphometric measures suggested as diagnostic by previous authors (Plötner et al., 1994): tibia length (TL); femur length (FL); inner metatarsal tubercles plus first toe length (MTFTL); snout to vent length (SVL); head width (HW); nostril to eye length (NE) and tympan-

Table 1. Morphometric measures (in mm) of the 17 specimens found in the surveyed pond within the Resina river catchment and morphologically classified as *Pelophylax cf. ridibundus*. Specific assignments of individuals analyzed with molecular techniques have been reported on the basis of mitochondrial ND3 sequence polymorphism (rid, *Pelophylax ridibundus*; ber, *Pelophylax lessonae bergeri*; shq, *Pelophylax shqipericus*).

For the explanation of different measures' acronyms, please refer to the text. juv, specimens metamorphosed in the year of sampling; ♀, female; ♂, male.

ID _{sex}	mtDNA	TL	FL	MTFTL	SVL	HW	NE	TY
1♂	ber	26.3	23.8	9.9	48.5	17.7	3.7	3.8
2♀	-	26.2	26.8	9.5	49.6	17.3	3.4	4.1
3♀	rid	24.3	23.0	7.7	46.3	15.9	3.2	3.4
4♂	rid	23.1	22.9	7.7	44.7	15.2	2.8	3.4
5♂	-	24.9	23.9	8.9	47.2	16.4	3.0	3.7
6 _{juv.}	ber	13.5	14.9	5.0	30.6	11.0	1.9	2.7
7	rid	40.3	33.3	12.8	79.8	28.2	5.9	5.1
8♂	-	27.4	19.1	10.5	53.4	19.1	3.6	4.2
9♂	-	38.3	52.2	15.0	71.9	27.9	5.8	5.5
10♂	-	24.6	24.2	8.1	47.4	16.3	3.3	3.8
11♂	-	20.1	13.9	8.1	38.7	14.0	2.9	3.1
12♂	-	31.0	38.3	11.0	58.8	21.6	4.0	4.7
13♀	rid	25.7	21.0	9.3	49.9	17.3	3.5	3.9
14♂	-	35.8	43.3	13.2	68.7	23.8	4.6	5.6
15♂	-	29.4	30.9	10.1	56.6	19.9	3.8	4.5
16 _{juv.}	-	11.9	13.8	2.5	26.0	9.9	1.7	2.1
17♀	shq	34.9	36.8	12.4	77.2	25.8	4.9	5.4

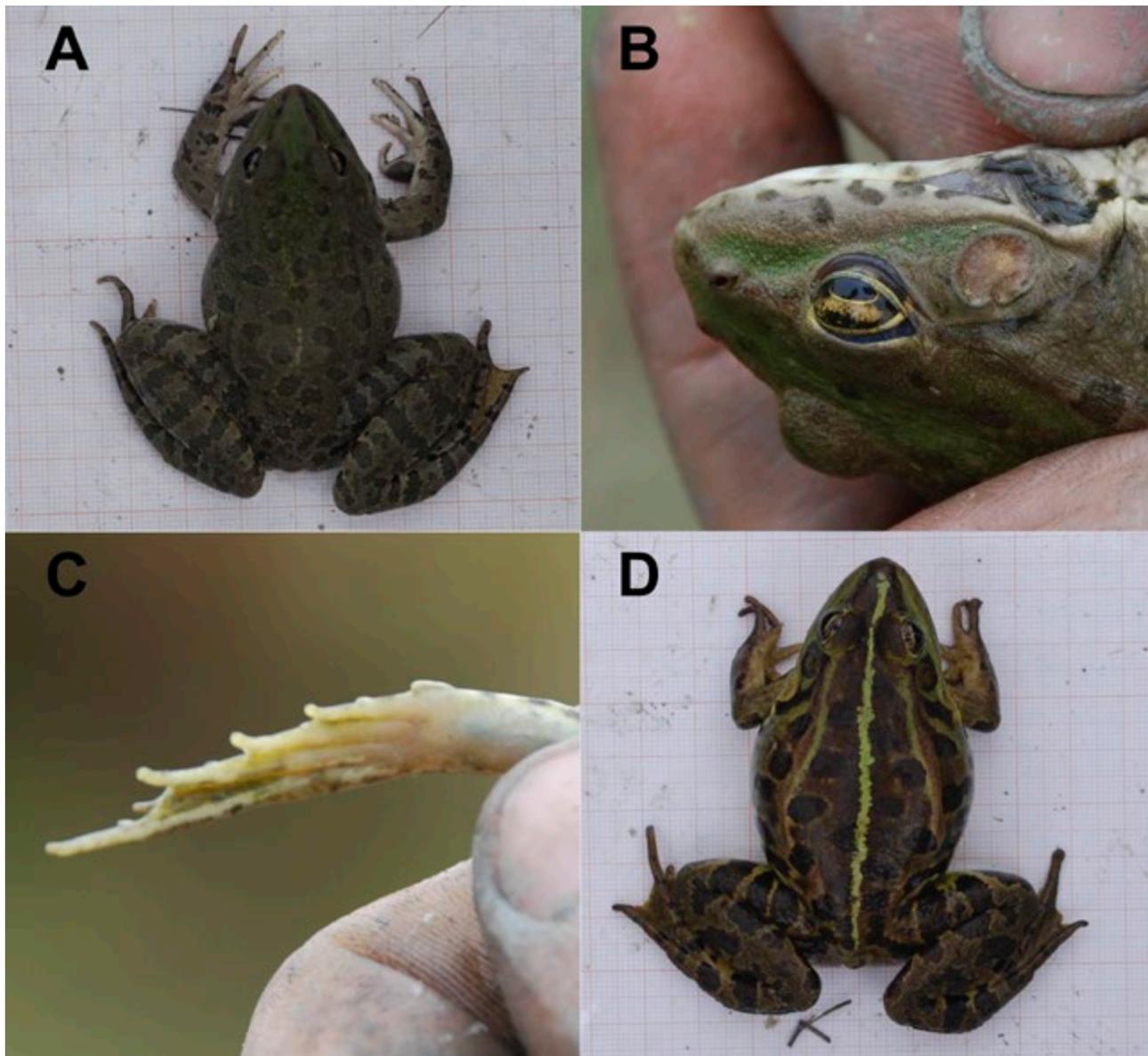


Fig. 1. Pictures of (A) *Pelophylax ridibundus* specimen number 9, (B) *P. ridibundus* specimen number 9, detail of the vocal sac black-coloured, (C) *P. ridibundus* specimen number 13, showing a short and flat metatarsal tubercle and (D) *P. shqipericus* specimen number 17.

num diameter (TY) (Table 1). Overall, we measured 17 of these questionable specimens.

A small piece of tissue was also collected from seven specimens by toe-clipping and stored in 96% ethanol for genetic analysis. In the laboratory, genomic DNA was purified with a standard salt-extraction procedure using the commercial kit “ArchivePure DNA Cell /Tissue” kit (5 PRIME, Hamburg, Germany) and following the manufacturer’s instructions. A portion (340 base pair long) of the mitochondrial ND3 gene encoding for the subunit 3 of NADH dehydrogenase was amplified with already published primer pair (Plötner et al., 2008)

modified from Meyer (1993). This marker appears to be diagnostic for *Pelophylax* species (Akin et al., 2010), allowing also the recognition of distinct geographic clades of *P. ridibundus* (Plötner et al., 2008; Akin et al., 2010). PCR products were purified with “GelElute Extraction” kit (5 PRIME, Hamburg, Germany) and sequenced on an ABI 3730XL using the forward primer (Macrogen Europe, Amsterdam, the Netherlands). Sequences were compared with all the available homologous sequences available from GenBank (www.ncbi.nlm.nih.gov/genbank/) 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.

Genetic analysis assigned four individuals (number 3, 4, 7 and 13) to European clades of *P. ridibundus* widespread in Central and Eastern Europe, for which several geographic mitochondrial haplogroups have been described (Plötner et al., 2008). In particular, sample 3, 7 and 13 (Accession number: HG763871, HG763872 and HG763873 respectively) yielded a 100% match with the previously identified haplotype R17 (distributed in France, Bulgaria, Serbia, Slovakia, Romania, Latvia, Poland and Russia), which is the most widespread haplotype in central European *P. ridibundus*, and with R18 and R19 (occurring in Bulgaria and Greece, respectively), while sample 4 (Accession number: HG763874) was identical with haplotypes R13 (distributed in Germany, Lithuania, Macedonia, Romania, Ukraine and Latvia) and to R14 from Greece, which is widely distributed in Eastern Europe (Plötner et al., 2008).

The genetic analysis of individuals 1 (Accession number: HG763869) and 6 (Accession number: HG763870, a young individual metamorphosed in the same year) assigned these specimens to *P. l. bergeri* with 100% probability, matching one published sequence from Italy (Akin et al., 2010). Interestingly, these individuals, possessing *bergeri*-specific mtDNA, but morphologically looking very similar to frogs genetically identified as *P. ridibundus* (tonality of brown colour, no yellow pigment on the hind legs, little metatarsal tubercles), may indicate hybridization between alien *P. ridibundus* and autochthonous *P. l. bergeri*. Recent molecular analyses of serum albumin intron-1 polymorphism according to the technique by Hauswaldt et al. (2012) for the detection of hybrids within the *P. esculentus* complex seem to support this conclusion (Bellati A., unpubl. data).

Finally, individual 17 (Accession number: HG763875, an adult female) was identical with the ND3 sequence of *Pelophylax shqipericus* (Hotz, Uzzell, Günther, Tunner & Heppich, 1987), the Albanian Pool Frog, from lake Skutari, Montenegro (Akin et al., 2010). This species inhabits a restricted geographic area between Greece and Southern Montenegro, its populations are currently endangered due to rapid wetlands loss and harvesting for commercial purpose, particularly food consumption, and it is therefore under consideration for CITES listing (Gratwicke et al., 2010).

The discovery of a frog carrying *P. shqipericus* mtDNA was unexpected, but at the same time it posed major concerns about consequences of uncontrolled water frog trade. Indeed, according to our results, translocations of individuals outside their native range could negatively affect not only indigenous populations, but also the per-

sistence of cryptic and endangered taxa with very localized spatial distribution. Further nuclear and eventually morphological analyses are needed to clarify this aspect. Noteworthy, unpublished molecular investigations of serum albumin intron-1 polymorphism seem to corroborate mitochondrial evidence (Bellati A., unpubl. data), since this individual showed a unique nuclear profile which also differs from all the species analyzed so far (Hauswaldt et al., 2012), including *P. ridibundus*, *P. kurtmuelleri*, *P. bedriagae*, *P. lessonae* and *P. l. bergeri*.

As suggested by several studies, the main risk for the genetic integrity of autochthonous populations results from the release of allochthonous *Pelophylax* species and their hybridization with native water frogs (Pagano et al., 2003; Schmeller et al., 2007; Holsbeek et al., 2008, 2010; Holsbeek and Jooris, 2010; Luquet et al., 2011). Accordingly, Italian hybridogenetic populations could be threatened by the introduction of allochthonous European *P. ridibundus* lineages, which may provoke a rapid genetic loss of *P. lessonae* and *P. l. bergeri* due to hemiclinal exclusion of the *lessonae* genome in the hybrids (Plötner et al., 2008). Finally, we are aware that the syntopic presence of native Italian taxa, *P. ridibundus* and *P. shqipericus* in the same small studied pond (approximately 20 square meters) could eventually lead to the production of hybrids whose genealogy would be difficult to assess. Natural hybrids between *P. ridibundus* and Balkan species such as *P. shqipericus* have been occasionally reported (Hotz and Uzzell, 1982). These individuals are obviously hybrids in constitution, do not exclude any parental genome, encounter meiotic disturbances and decrease in fitness. Similarly, they are unable to reproduce hybridogenetically (Ragghianti et al., 1999). Inter-specific crosses between *P. shqipericus* and other European taxa (like *P. lessonae*) may also generate high hatching rate (Plötner et al., 2010). Therefore, further investigations with co-dominant nuclear markers (such as hypervariable microsatellites) are strongly needed to assess the real hybridization rate in central Italian ponds of the Resina river, in order to undertake efficient measures to control their possible spread.

In conclusion, our work allowed for the first time the detection of non-native water frog taxa in Central Italy, whose invasive potential will be assessed in future by more extensive surveys and greater sampling effort. Moreover, we confirmed the importance of molecular analyses for water frog identification, which appeared not possible just according to morphology and biometric features.

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