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Research Article

Detection of alien *Pelophylax* species in Sardinia (western Mediterranean, Italy)

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Abstract

Sardinian herpetofauna represent a valuable biodiversity resource in the Mediterranean Basin. Among amphibians, most of the taxa are indeed endemic to the island. In contrast, water frogs of the *Pelophylax* genus are considered allochthonous to Sardinia, and are traditionally assigned to the Italian pool frog (*P. bergeri*) and its hybridogenetic hybrid (*P.* kl. *hispanicus*). Members of this genus were recently found in certain sites in northern and southern Sardinia, and ascribed to the "*ridibundus*" group (the "Marsh frog" species complex). As the absence of clear phenotypic diagnostic features complicates the identification of distinct *Pelophylax* species, particularly European *P. ridibundus*, *P. kurtmuelleri* and several still undescribed "*bedriagae*" taxa, an informative diagnostic mitochondrial marker (*ND3*) was isolated in this work in order to detect the occurrence of distinct lineages in alien Sardinian populations. As water frogs are known to readily hybridize in the wild, two further diagnostic nuclear markers (*SAI-1* and 4 microsatellite loci) were subsequently screened in order to exclude the occurrence of hybrid genotypes. According to our results, three distinct taxa were detected on the island, and assigned to *P. kurtmuelleri* (the Balkan frog), and two cryptic taxa of the "*bedriagae*" group. Nuclear markers only successfully amplified alleles diagnostic for the "*ridibundus*"-type (R) genome. Accordingly, we can exclude hybridization with the Italian pool frog already introduced on the island, although further studies are needed to better address the occurrence of new hybrid lineages stemming from the cross between the taxa detected in this work. As eggs and tadpoles have been detected in the field, alien populations can thus be considered successfully established in Sardinia. Our study revealed for the first time the occurrence of the alien Balkan frog in the western Mediterranean. It is worthy of note that this taxon shows a high invasion potential in mainland Europe. The establishment of alien *Pelophylax* populations in Sardinia may be favored by vacant niches. Nonetheless, the dry hot season could limit their expansion along rivers.

Key words: *Pelophylax ridibundus*, *Pelophylax bedriagae*, *Pelophylax kurtmuelleri*, multiple-introduction, conservation, island

Introduction

Amphibians have declined dramatically in many areas of the world and are now more threatened than mammals and birds (Beebee and Griffiths 2005). According to the 2008 IUCN Red List Status, nearly one-third of the amphibian species are globally threatened or extinct. The release of alien species represents a major threat to amphibian conservation, as it involves competition, predation, hybridization and genetic swamping, as well as the spread of pathogens at the expense of native taxa (Kiesecker and Semlitsch 2003; Kraus 2009, 2015; Bucciarelli et al. 2014).

Mediterranean islands represent a biodiversity hotspot for amphibians, most of which are endemisms differentiated from mainland populations starting from the end of the Messinian Salinity Crisis (5.33 Ma, Hewitt 2011). A particularly high concentration of endemic taxa is present in Sardinia and Corsica (western Mediterranean, Corti et al. 1999). Together, these islands today host 11 native species (Delaugerre and Cheylan 1992; Bassu et al. 2010). Six species are strictly endemic to Sardinia, such as the Sardinian mountain newt (*Euproctus platycephalus*) and five species of cave salamanders (*Speleomantes flavus*, *S. genei*, *S. imperialis*, *S. sarrabusensis* and *S. supramontis*). Other species also exist in Corsica and on certain North-Tyrrhenian islands, e.g., the Tyrrhenian painted frog (*Discoglossus sardus*) and the Tyrrhenian tree frog (*Hyla sarda*). The Balearic green toad (*Bufotes balearicus*) inhabits Sardinia, Corsica, the Balearic Islands and part of mainland Italy.

In contrast, water frogs of the *Pelophylax* genus, with several species distributed throughout Europe, Asia, and North Africa, are considered allochthonous in Sardinia (Andreone et al. 2009). Indeed, previous authors reported the introduction of the Italian pool frog, *P. bergeri*, and its hybridogenetic hybrid *P.* kl. *hispanicus* in northern Sardinia at the end of 19th Century, as the result of human-mediated translocation from central Italy (Lanza 1983).

Recently, the detection of these taxa have occurred sporadically on the island (e.g., only two individuals reported from the “Grotta di San Giovanni” at Domusnovas in southwestern Sardinia by Li Vigni et al. 2011). Moreover, no updated distributional data are available, apart from two sites listed in the “Atlas of Italian amphibians and reptiles” (Sindaco et al. 2006), i.e., one in the south, 40 kilometers from Cagliari, and one in the north, near Porto Torres.

Recently, new alien *Pelophylax* populations were detected in Sardinia and ascribed to the “Marsh frog” group (*P. ridibundus sensu lato*) according to preliminary bioacustic and morphological analyses. It is noteworthy that phylogenetic and phylogeographic studies have recently pointed out that this taxon is instead a complex of several species whose determination is challenging in the field, but feasible via molecular analysis (e.g., Lymberakis et al. 2007; Akın et al. 2010a; Plötner et al. 2010; Hoffmann et al. 2015). For instance, the Balkan frog *P. kurtmuelleri* proved to be phylogenetically not distinguishable from the European *P. ridibundus* (Lymberakis et al. 2007), although it possesses typical mitochondrial (mtDNA) haplotypes (Plötner et al. 2008). By contrast, the Levantine frog *P. bedriagae*, formerly considered a subspecies of *P. ridibundus*, has been recognized as a complex of several evolutionary distinct taxa still deserving formal description (Akın et al. 2010a; Plötner et al. 2010).

In mainland Europe, *P. ridibundus* has been repeatedly introduced—mainly from the eastern countries—to Italy (Domeneghetti et al. 2013; Laghi et al. 2013), France (Pagano et al. 2001, 2003), Switzerland (Grossenbacher 1988; Dubey et al. 2014) and Belgium (Holsbeek et al. 2008, 2010). The main reason for exportation is the international food trade (i.e., frog leg consumption, Schmeller et al. 2007). Molecular analyses later revealed that other "*ridibundus*"-like taxa were silently introduced to the same countries, such as *P. bedriagae* from Egypt to Belgium, *P.* cf. *bedriagae* from Anatolia to Belgium and Switzerland, and *P.* cf. *bedriagae* from south-eastern Turkey to Switzerland (Holsbeek et al. 2008, 2010; Dubey et al. 2014). The Balkan frog *P. kurtmuelleri* was similarly translocated from Greece to Switzerland (Dubey et al. 2014), France (Dufresnes et al. 2017) and Poland (Kolenda et al. 2017). In northern Italy, *P. kurtmuelleri* was translocated in 1941 from Albania to western Liguria, near Imperia. Once released, the taxon became rapidly invasive, present nowadays in several north-western Italian regions (Lanza 1962; Lanza and Corti 1993; Bellati et al. 2012).

The introduction of alien water frogs has several drawbacks for native populations, mainly due to the fact that several taxa are known to readily hybridize in the wild. The main reason lies in the ability of some hybrid taxa (*kleptons*, abbreviated "kl." in nomenclature) to hemiclonally reproduce by eliminating one of the two parental genomes from the germline, a rare mode of reproduction called hybridogenesis (Schultz 1969). To date, native *kleptons* occur in the Iberian Peninsula (*P.* kl. *grafi*) and in central Europe (*P.* kl. *esculentus*), which stem from ancient hybridizations between *P. ridibundus* and *P. perezi* or *P. lessonae*, respectively. In central Italy, the endemic *P.* kl. *hispanicus* is present, carrying the parental genomes of *P. bergeri* and a new endemic Eastern-Mediterranean lineage, nowadays extinct in the wild (Dubey and Dufresnes 2018).

The *klepton* only transmits the R-type chromosomes of *P. ridibundus* during reproduction. The hybrid condition is later restored in the offspring by backcross with the non-*ridibundus* parental species. Therefore, the introduction of the Marsh frog and other "*ridibundus*"-like taxa threatens the persistence of the hybridogenetic systems via the rapid disruption of the genetic integrity of the systems, and the progressive removal of the native parental species through introgression. Introgressive hybridization also occurs between non-hybridogenetic taxa of the "*ridibundus*" group, once they are released at the same site. This is the result of labile reproductive barriers between species (e.g., Plötner et al. 2010), which mainly differentiated in allopatric glacial refugia between *ca.* 5 and 1 Ma (Akın et al. 2010a).

Table 1. List of the sampling sites considered in this study, reporting population code (PopID), number of frogs sampled at each sites (N), geographic information (Site) including Province (SS: Sassari, VS: Medio Campidano, CA: Cagliari), and geographic coordinates (Lat, Long).

Prog	PopID	N	Site (Province)	Lat	Long
1	NC1	4	Tula (SS)	40º43′56″N	8º59′08″E
2	NC2	2	Ploaghe (SS)	40º39′50″N	8º44′44″E
3	NC3	4	Pattada (SS)	40º34′55″N	9º06′38″E
4	S1	2	Pabillonis (VS)	39º35′31″N	8º43′15″E
5	S2	2	Uta (CA)	39º17′19″N	8º57′26″E

To clarify the occurrence and distribution of water frogs in Sardinia, alien populations were actively searched across the island in order to collect biological samples for diagnostic analysis. This was conducted during a herpetological field monitoring between 2016 and 2017. Our aims were to (i) ascribe allochthonous populations to the correct taxa, (ii) find concurrent taxa at the same site attesting multiple introductions, (iii) infer the presence of hybrids between "old" (i.e., the Italian pool frogs) and "new" (i.e., "*ridibundus*"-like) Sardinian invaders, (iv) update the distribution of these lineages in Sardinia, and (v) identify possible source populations of Sardinian alien water frogs, with particular reference to Italy. According to the recurrent release of several *Pelophylax* taxa in mainland Europe, in particular the Balkan frog *P. kurtmuelleri* and *P.* cf. *bedriagae* from western Anatolia, we suggested that the same could have similarly been happened in Sardinia.

Materials and methods

During 2016 and 2017, 14 individuals were sampled in the field, by collecting biological samples either from egg, muscle, or buccal swab. Water frogs were found at three sites in the North (NC1–3, N = 10) and two sites in the South (S1–2, N = 4; Table 1, Figure 1).

In northern Sardinia, water frogs were sampled in a stream and in a drinking trough among pastures and agricultural habitats characterized by the presence of dry stone walls and rocky outcrops. The habitats were at times surrounded by Mediterranean maquis (i.e., dense scrub vegetation consisting of hardy evergreen shrubs and small trees, Figure 2A). The frogs were also sampled in artificial water bodies in a mountain forested region. Sampling sites were located in the province of Sassari near Lake Coghinas (NC1), close to the town of Ploaghe (NC2), and in Mount Lerno, in the neighbourhoods of Pattada (NC3). In southern Sardinia, samples were collected in canals and small artificial water reservoirs (Figure 2B) in mixed rural habitats, near Pabillonis (S1), in the province of Cagliari, and Uta (S2), in the province of Medio Campidano. It is worth mentioning that, although rural sites also occur in northern Sardinia, no evidence attesting the presence of water frogs in those environments was found.

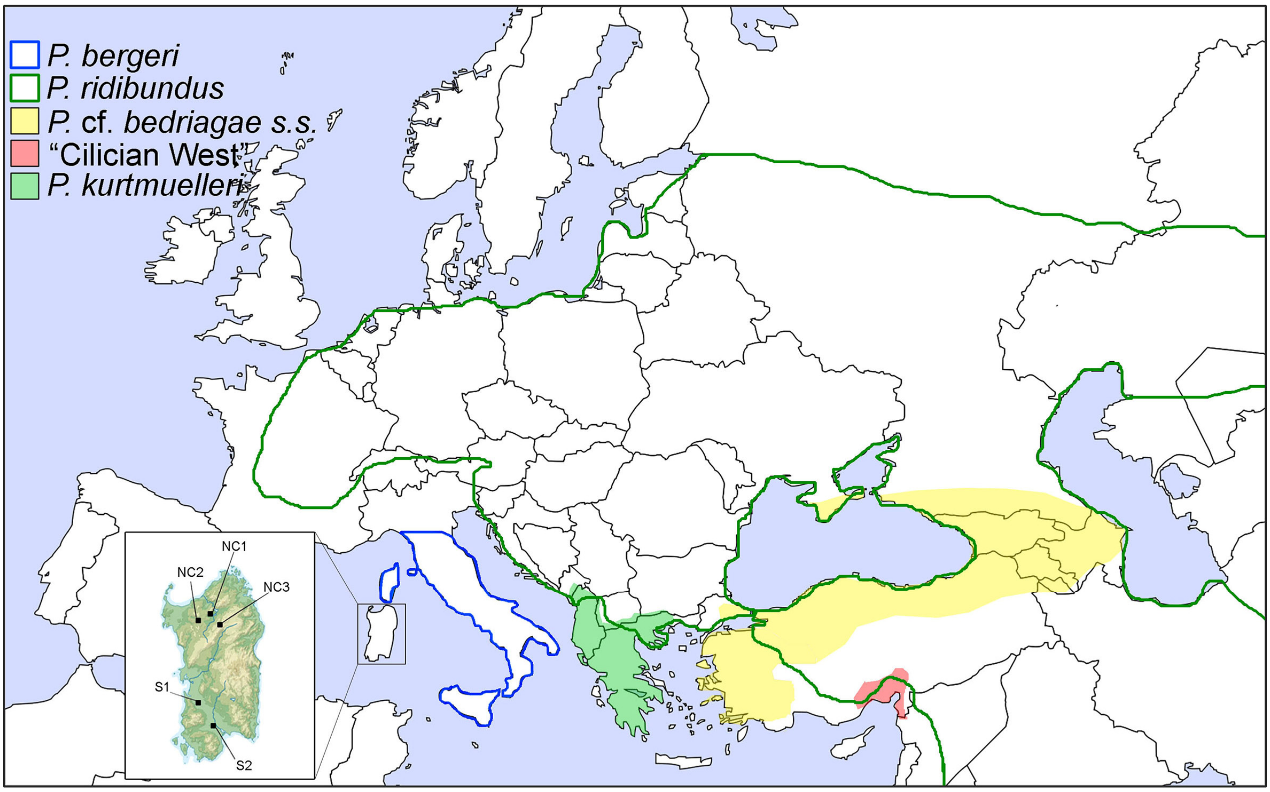

Figure 1. Map of Europe showing the geographic location of Sardinia (western Mediterranean) and the location of sampling sites (see Table 1 for definition of population codes). The native ranges of the taxa introduced in Sardinia is also depicted on the map using different colours, while coloured lines delimited the distributional range of the European Marsh frog (*P. ridibundus*) and the Italian pool frog (*P. bergeri*).

Animals were captured in the field using small mesh nets and classified as “*ridibundus*”-like taxa (Figure 2C, D, E); after each sampling session the equipment was disinfected following the instructions reported in: *Societas Herpetologica Italica*, http://www-3.unipv.it/webshi/conserv/monitanf.htm. It should be noted that the occurrence of individuals at sampling sites was very low compared to the high densities often reported for introduced mainland populations (e.g., Lanza et al. 2007; A. Bellati, *unpublished results*). Despite active search on the island, the animals were only detected at the five surveyed sites. Our sampling therefore corresponded to the most updated distribution of the species in Sardinia.

Swabs were taken from adults by gently rubbing the cotton tip on the inner oral membrane (buccal) and placed in 95% ethanol (Pidancier et al. 2003). Tissue samples obtained both from eggs and tadpoles were stored in absolute ethanol (95–100%). To exclude maternal effect on mtDNA variation, the tadpoles were sampled at different developmental stages at site NC3 during the same field session. Furthermore, buccal swabs were obtained only from adult males at NC1, where an egg was sampled. All samples were kept at −20 °C in the laboratory until extraction. Genomic DNA was purified using the commercial kit *GenElute Mammalian Genomic DNA Miniprep Kit* (Sigma-Aldrich, Saint Louis, USA) and following the manufacturer’s instructions. The NADH dehydrogenase subunit 3 (*ND3*)

Figure 2. A) Typical northern Sardinian landscape where alien *Pelophylax* populations were sampled (NC1); B) an example of the microhabitat where frogs were collected, in the South (S1) of Sardinia; C, D, E) Pictures of alien water frogs sampled at NC2, S1 and NC3, respectively.

was selected as an informative marker for both species identification and putative assignment of alien frogs to their native geographic range. Indeed, previous studies showed that this marker can clearly discriminate between main European *Pelophylax* clades (e.g., the "*lessonae*" clade, comprising the pool frogs *P. lessonae* and *P. bergeri*, and the "*ridibundus*" clade, which is composed of several differentiated lineages including *P. kurtmuelleri*, *P. bedriagae* and its relatives, Holsbeek et al. 2008; Plötner et al. 2008, 2010; Akın et al. 2010a).

Polymerase chain reactions were set up in 20 µl with available primers (Plötner et al. 2008) and 0.05 U of *MasterTaq DNA polymerase* (biotechrabbit GbmH, Hennigsdorf, Germany). Amplification conditions consisted of 35 cycles of 30 s denaturation at 94 °C, 20 s annealing at 50 °C and 60 s extension at 72 °C. PCR fragments were then purified by use of the *GenElute Gel Extraction Kit* (Sigma-Aldrich, Saint Louis, USA) and sequenced at Eurofins Genomics (Ebersberg, Germany).

Mitochondrial sequences were aligned using Geneious 11 (Biomatters Ltd.). Haplotypes were extracted using the online *DNA to haplotypes collapse and converter* function available at Fabox (http://users-birc.au.dk/biopv/php/fabox/) and compared to homologous public records in NCBI (https://www.ncbi.nlm.nih.gov/) using the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

A Neighbor-Joining (NJ) tree implementing the pairwise (*p*) distance model was built in MEGA 6 (Tamura et al. 2013) to infer the occurrence of distinct mtDNA lineages in our dataset and to assign them to the correct taxon. For this purpose, homologous sequences of European *P. ridibundus*, *P. kurtmuelleri*, *P. bedriagae* and its relatives, for which geographical data was available, were included (N = 217, please refer to Figure 3 for all the accession numbers and corresponding references). Sequences of *P. lessonae* (AM749726: Italy, AM749727: Romania, Plötner et al. 2008), *P. bergeri* (GU812231-2: Italy, Akın et al. 2010a), *P. saharicus* (GU812241: Algeria, GU812243: Tunisia, Akın et al. 2010a) and *P. nigromaculatus* (GU812246: North Korea, Sumida et al. 2001) were selected as outgroups. Node support was assessed through 1000 bootstrap replicates. A parsimony network was built in TCS 1.21 (Clement et al. 2000) to examine the extent of haplotype sharing among Sardinian populations and their relationships with already available haplotypes of the Main Haplo Groups (MHG, Akın et al. 2010a) matching alien lineages identified in this study (see *Results*). The 95% default parsimony threshold was chosen. Networks were visualized using the online web tool tcsBU (Santos et al. 2015).

To exclude the presence of hybrid genotypes, two types of nuclear markers were further isolated in all the samples, i.e., *SAI-1* (serum-albumin intron-1, Hauswaldt et al. 2012) and a set of four diagnostic microsatellite loci (Garner et al. 2000; Zeisset et al. 2000; Arioli 2007; Christiansen 2009). Analysis of the first marker (*SAI-1*) allows the detection of distinct genome sets (L- or R-type) in pure vs. hybrid individuals by returning genome-specific banding patterns (Hauswaldt et al. 2012). Assuming possible hybridization between "old" Sardinian invaders (the Italian pool frogs) and the newly discovered taxa of the "*ridibundus*" group, we hypothesized to find a fragment of *ca*. 300 bp (base pair) diagnostic for the L-type genome of *P. bergeri* co-amplified with a longer fragment diagnostic for the R-type genome of *ca*. 800 bp in hybrid individuals (Hauswaldt et al. 2012). Amplifications were performed in 10 µl with the available primer pair Pel-SA-F1 and Pel-SA-R2 (Hauswaldt et al. 2012), adding 0.05 U of *Hot-StartTaq DNA polymerase* (biotechrabbit GbmH). The thermocycling profile was provided for 35 cycles of 40 s denaturation at 94 °C, 40 s annealing at 59 °C and and 90 s extension at 72 °C, and a final elongation step at 72 °C for 10 min. PCR products (5 µl) were visualized on a 2% agarose gel.

Four microsatellites diagnostic for the detection of L- and R-type genomes were further screened. Locus RICA1a27 (Christiansen et al. 2009) and RICA18 (Garner et al. 2000) were chosen to selectively amplify L-genome of *P. bergeri* and *P.* kl. *hispanicus*. Although these loci were initially isolated in the sister species of *P. bergeri* (*P. lessonae*), their efficiency was tested for

Table 2. Samples of water frogs collected in Sardinia. We provided sample name (SampleID), sampling sites as in Table 1 (PopID), sample type (Type: 1-swab, 2-tadpole, 3-egg), Taxon ID according to mtDNA (*ND3*) analysis, and haplotype (HaploID). Accession numbers of sequences produced in this study are also reported (Acc. N°).

SampleID	PopID	Type	TaxonID	HaploID	Acc. N°
Tul-1	NC1	1	*P. kurtmuelleri*	Pk1	MK032135
Tul-2	NC1	1	*P. kurtmuelleri*	Pk1	MK032136
Tul-3	NC1	1	*P.*cf. *bedriagae sensu stricto*	Pb1	MK032137
Tul-4	NC1	3	*P.*cf. *bedriagae sensu stricto*	Pb1	MK032138
Plo-1	NC2	1	*P.*cf. *bedriagae sensu stricto*	Pb2	MK032139
Plo-2	NC2	1	*P.*cf. *bedriagae sensu stricto*	Pb1	MK032140
Mle-1	NC3	2	*P. kurtmuelleri*	Pk2	MK032141
Mle-2	NC3	2	*P. kurtmuelleri*	Pk2	MK032142
Mle-3	NC3	2	*P. kurtmuelleri*	Pk2	MK032143
Mle-4	NC3	2	*P. kurtmuelleri*	Pk2	MK032144
Pab-1	S1	1	*P.* cf. *bedriagae* “Cilician West”	Pb3	MK032145
Pab-2	S1	1	*P.* cf. *bedriagae* “Cilician West”	Pb3	MK032146
Uta-1	S2	1	*P.* cf. *bedriagae* “Cilician West”	Pb3	MK032147
Uta-2	S2	1	*P.* cf. *bedriagae* “Cilician West”	Pb3	MK032148

an ongoing population genetics study on pure populations of the Italian pool frogs from Central Italy (A. Bellati, *unpublished results*). To detect R-specific alleles, Rrid169A (Christiansen and Reyer 2009) and Re2CAGA3 (Arioli 2007) were chosen. Loci were amplified in 10 μl with previously published primer pairs and 0.05 U of *Hot-StartTaq DNA polymerase* (biotechrabbit GbmH), under the following thermal cycling conditions: initial denaturation of 5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 45 s at the locus-specific annealing temperature (55 °C for Re2CAGA3, 60 °C for the remaining loci), and 60 s at 72 °C. The forward primer of each pair was fluorescently-labeled for detection on an ABI3130 capillary sequencer. Allele scoring and dimensioning were conducted in Geneious 11.

Results

All the samples were successfully amplified at mtDNA, returning high sequence polymorphism in Sardinian frogs. The final *ND3* alignment (340 bp long) did not return gaps or premature stop codons. Overall, five distinct haplotypes were detected in the surveyed populations (Table 2).

Specifically, two haplotypes (hereafter Pk1 and Pk2) were found in two out of three sites in the North, matching at 99% probability sequences AM749695-696-698-699-700-701 from Greece. It is noteworthy that these sequences refer to the *P. kurtmuelleri* lineage—haplotypes R1,2,4-7—according to Plötner et al. (2008). In our dataset, each Balkan haplotype was private of a different population (Table 2): Pk1 was found only at site NC1 (N = 2), while all the samples from site NC3 (N = 4) shared Pk2. Haplotypes Pb1 and Pb2 were also detected in the North and match (100%) sequences GU812195 and GQ902107 from Western Turkey, ascribed to *P.* cf. *bedriagae sensu stricto* (Akın et al. 2010a, b). The remaining haplotype (Pb3) was only found in southern populations and was identical to the reference sequence GU812162. This sequence was reported in a localized area of south-eastern Turkey by previous authors, where a distinct clade of *P.* cf. *bedriagae* named “Cilician West” occurs (Akın et al. 2010a). Accession numbers of newly analyzed Sardinian samples are listed in Table 2.

The final alignment including our sequences and reference *Pelophylax* sequences (N = 231, excluding the outgroup) returned 128 unique haplotypes. The NJ tree based on *p*-distances showed the presence within the whole dataset of 12 differentiated and highly supported clades, corresponding to the Main Haplo Group (MHG) previously identified in the literature (Akın et al. 2010a, Figure 3). According to our results, the Sardinian samples belong to: the *P. kurtmuelleri* subclade within MHG1, corresponding to European *P. ridibundus* (haplotypes Pk1 and Pk2); the *P.* cf. *bedriagae sensu stricto* clade (MHG6c in Akın et al. 2010a) widespread in Anatolia, but also present in Russia and Greece (haplotypes Pb1 and Pb2); and the *P.* cf. *bedriagae* “Cilician West” clade (MHG4 in Akın et al. 2010a) present in south-eastern Turkey (haplotypes Pb3, Figure 3). The haplotype network reconstruction, based on *ND3* polymorphism of our samples and reference samples of European *P. ridibundus* (MHG1, 17 haplotypes), *P.* cf. *bedriagae sensu stricto* (MHG6c, 29 haplotypes), and *P. bedriagae* “Cilician West” (MHG4, 7 haplotypes), further assigned the Sardinian samples to three distinct and highly differentiated networks (Figure 4).

The analysis of *SAI-1* polymorphism returned the typical banding pattern of “*P. ridibundus*” (Table 3). Shorter fragments typical of the pool frogs (*ca*. 300 bp) were not detected in our dataset, excluding the presence of introgressed *P. bergeri*, *P.* kl. *hispanicus*, as well as the “new” RL hybrids. The latter may have resulted from the crossbreed between the newly identified taxa and the Italian pool frogs previously introduced to the island in historical times. According to Hauswaldt and coauthors (2012), the R fragment appears slightly shorter (*ca*. 700 bp) in *P. kurtmuelleri*, compared to *P. ridibundus* and *P. bedriagae* (*ca*. 850 bp). As a result, species identification based on nuclear polymorphism agreed with the inferred mtDNA lineage in all the samples collected in southern Sardinia and at site NC2, where exclusively the “*bedriagae*” mitogenome was detected. Nuclear DNA of the only egg collected in this study (Tul-4) showed correspondence with mtDNA and was therefore assigned to *P. kurtmuelleri*. Similarly, fragments typical of the Balkan frog were found in samples Tul-2 (NC1), Mle-2 and Mle-3 (NC3), carrying *P. kurtmuelleri* mitogenome. Interestingly, the remaining samples showed a combination of the two fragments (Tul-1, Tul-3, and Mle-4), or cyto-nuclear mismatch (Mle-1, Table 3).

All the samples failed to amplify the L-specific microsatellites RICA18 and RICA1a27, further confirming the absence of both *P. bergeri* and *P.* kl. *hispanicus* alleles in the dataset (Table 3).

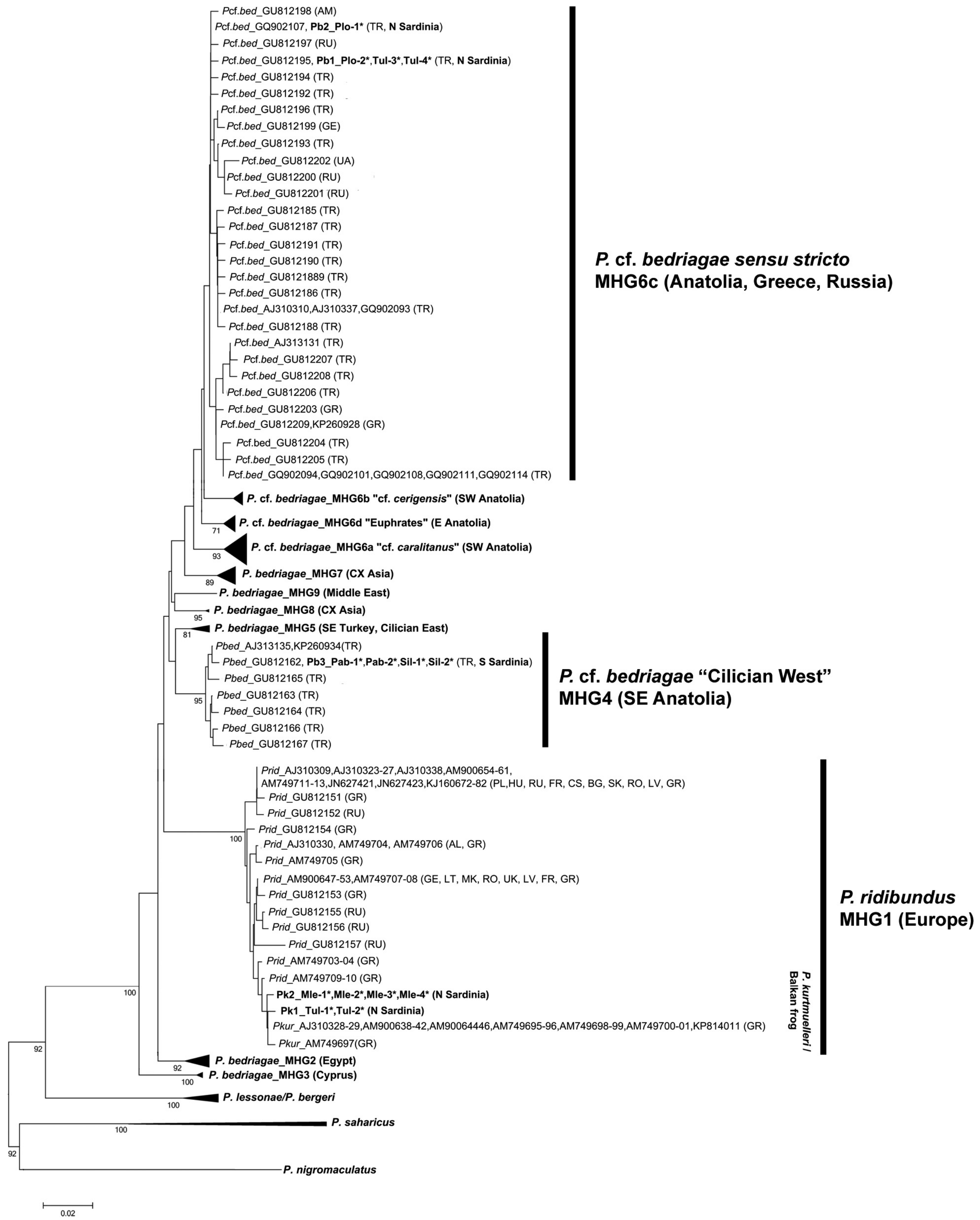

Figure 3. Neighbor-Joining tree (*p*-distance based) of mitochondrial *ND3* showing the relationships between Sardinian samples and reference sequences of the "*ridibundus*" complex, downloaded from GenBank (only bootstrap > 75 have been reported). * denotes samples analysed in the study (in bold). Vertical bars indicate Main Haplo Groups (MHG) identified by Akın et al. (2010a). The geographic origin of samples and reference sequences is given within brackets (for country codes, please refer to Figure 4). Reference sequences were published by: AJ) Plötner et al. 2001, AM) Plötner et al. 2008, GU) Akın et al. 2010a, GQ) Akın et al. 2010b, JN) Hofman et al. 2012, LN) Plötner et al. 2015, KP) Hofman et al. 2016, HE) Plötner et al. 2012, KJ) Mikulíček et al. 2014.

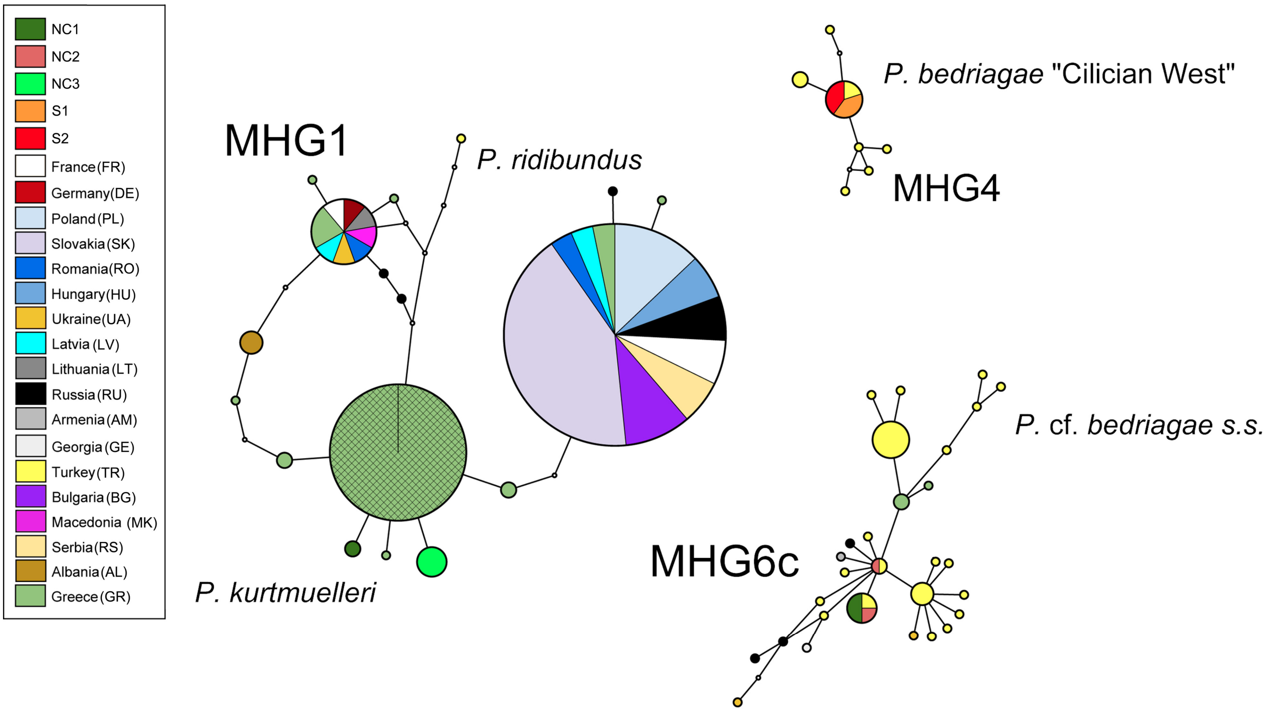

Figure 4. 95% parsimony network of *ND3* haplotypes including Sardinian samples and reference haplotypes ascribed to MHG1 (European *P. ridibundus*), MHG4 (*P.* cf. *bedriagae* "Cilician West"), and MHG6c (*P.* cf. *bedriagae sensu stricto*): each circle corresponds to a distinct haplotype, whose size is proportional to the number of samples sharing the same haplotype; bars indicate single mutational steps to connect each haplotype; white small dots represent absent haplotypes (not sampled or extinct); colours denote different countries of origin, while texture identifies the *P. kurtmuelleri* private haplotype identified in MHG1 by Akın et al. 2010a.

Table 3. nuDNA polymorphism of alien Sardinian water frogs analysed in the study. For each sample (SampleID), aside from information regarding the population (PopID) and the species (assessed by mtDNA analysis), size (in bp) of the *SAI-1* fragment(s) and the two diagnostic microsatellites loci are reported.

SampleID	PopID	TaxonID	*SAI-1*	Rrid169A	Re2CAGA3
Tul-1	NC1	*P. kurtmuelleri*	700–850	236/236	113/125
Tul-2	NC1	*P. kurtmuelleri*	700	236/236	113/125
Tul-3	NC1	*P.*cf. *bedriagae sensu stricto*	700–850	258/266	113/113
Tul-4	NC1	*P.*cf. *bedriagae sensu stricto*	700	266/266	113/125
Plo-1	NC2	*P.*cf. *bedriagae sensu stricto*	850	236/236	125/125
Plo-2	NC2	*P.*cf. *bedriagae sensu stricto*	850	236/236	113/125
Mle-1	NC3	*P. kurtmuelleri*	850	246/246	113/113
Mle-2	NC3	*P. kurtmuelleri*	700	246/258	113/113
Mle-3	NC3	*P. kurtmuelleri*	700	258/258	113/113
Mle-4	NC3	*P. kurtmuelleri*	700–850	258/258	125/125
Pab-1	S1	*P.* cf. *bedriagae* "Cilician West"	850	258/258	85/89
Pab-2	S1	*P.* cf. *bedriagae* "Cilician West"	850	258/258	85/89
Uta-1	S2	*P.* cf. *bedriagae* "Cilician West"	850	246/246	85/89
Uta-2	S2	*P.* cf. *bedriagae* "Cilician West"	850	246/246	85/89

By contrast, diagnostic "*ridibundus*" alleles were detected in all the samples analyzed. Overall, 4 alleles at locus Rrid169A were scored, all but one—allele 266—were shared between distinct mtDNA lineages (Table 3). Locus Re2CAGA3 also returned 4 alleles, two—alleles 85 and 89—being exclusive of southern populations where members of the "Cilician West" clade were found (Table 3).

Discussion

In this work, the occurrence of at least three distinct and previously unreported *Pelophylax* taxa for Sardinia was detected: the Balkan frog (*P. kurtmuelleri*) from Greece; the Anatolian frog (*P.* cf. *bedriagae sensu stricto*) native to western Turkey, and widespread from Anatolia to central Russia and Thrace, where it overlaps with the European *P. ridibundus* populations (corresponding to MHG6c in Akın et al. 2010a); and *P.* cf. *bedriagae* "Cilician West", whose native range corresponds to a very localized region, i.e., the Cilician plain of southern Turkey and the eastern side of the Amanos Mountains (Akın et al. 2010a).

Alien *P. bedriagae* has already been reported on the islands of Malta (southern Mediterranean, Schembri 2010) and Cyprus (eastern Mediterranean, Plötner et al. 2015), where hybridization with genome introgression between the alien frogs and the endemic *P. cypriensis* has been demonstrated. Our work reports for the first time the occurrence of alien members of the *P. bedriagae* complex and the alien Balkan frog on the western Mediterranean island.

According to the occurrence and distribution of distinct *Pelophylax* taxa in Sardinia, the introduction of alien water frogs seems to be the result of multiple independent releases on the island. The Balkan frog *P. kurtmuelleri* has been widely translocated across mainland Europe (see Holsbeek et al. 2008, 2010 for Belgium; Dubey et al. 2014 for Switzerland; Pagano et al. 2003; Dufresnes e al. 2017 for France; and Kolenda et al. 2017 for Poland). In Italy, the taxon was introduced into western Liguria from Albania for food consumption at the beginning of 1940s (Lanza 1962). Therefore, it is unlikely that individuals released in Sardinia, whose mtDNA haplotype match reference sequences from Greece, were translocated from northern Italy where populations share a single mtDNA haplotype typical of the native source population in Albania (A. Bellati, *unpublished results*).

A more reliable scenario involves translocations of alien *P. kurtmuelleri* and *P.* cf. *bedriagae* from recently detected populations in Central Italy. Indeed, haplotype Pb2 present in northern Sardinia has been detected in Tuscany (Bruni et al., *in press*), suggesting that this region could correspond to the source area of the Sardinian invaders. At least for northern Sardinia, we therefore assume that multiple introductions may have been linked to the import from the central Italian regions for commercial activities (e.g., plant nursery, game fishing, and food). A similar scenario of human-mediated introduction has been proposed for the recently detected Common toad *Bufo bufo* in north-central Sardinia (Cossu et al. 2018).

Conversely, members of the "Cilician West" clade have not yet been detected in mainland Italy, but frogs belonging to this lineage have already been introduced in Switzerland (Dubey et al. 2014). This suggests that the animals are actively collected for exportation in the area of the Cilician plain (the Ceyhan area), in south-eastern Turkey. Therefore, we could hypothesize that they have been imported to Sardinia via alternative routes.

Several studies have shown that the release of alien frogs in mainland Europe have dramatic consequences on the persistence of native populations threatened by local competition and hybridization with alien genotypes. This poses at serious risk the persistence of hybridogenetic systems (e.g., Holsbeek et al. 2010; Dufresnes et al. 2017). According to the Environmental Impact Classification for Alien Taxa (EICAT) scheme, both *P. ridibundus*—including the Balkan *P. kurtmuelleri*—and *P. bedriagae* have been recognized as the most highly impacting alien amphibians (maximum impact score: MV = Massive), due to their ability to hybridise with native taxa, thus producing viable and fertile hybrids in most cases (Kumschick et al. 2017).

In Sardinia, the establishment and spread of water frogs might pose a threat to the persistence of the native amphibian community, mainly via competitive interactions, and predation. Marsh frogs are particularly strong competitors in terms of food resources and breeding sites (Semlitsch and Reyer 1992) and they can also prey on larvae and smaller adults of other amphibians (see Measey et al. 2015 and reference therein). Moreover, they could be a vector of the chytrid fungus, *Batrachochytrium dendrobatidis* (Fisher and Garner 2007) which is currently spreading worldwide.

Although we did not find any evidence in this work of genetic introgression with the Italian pool frogs that should similarly be present on the island, it cannot be ruled out that hybridization with these “old” invaders could occur in the future. Regarding this, it is important to point out that although the Italian pool frogs are considered allochthonous to the Sardinian herpetofauna, the absence of genetic data poses a conservation concern to the possible evolutionary uniqueness of these insular populations, which may represent a biodiversity reservoir for mainland populations of the *P. bergeri*/*P.* kl. *hispanicus* hybridogenetic system. Interestingly, although these anurans were absent from Sardinia until recently, few fossil records of green frogs have been reported (Delfino 2006), suggesting that the island could potentially host these species in vacant niches. Indeed, the Italian pool frogs (and particularly, *P. bergeri*) are Mediterranean elements already naturally present on other western Mediterranean islands, such as Corsica.

At present, only possible signs of genotype admixture between the taxa identified in this study was detected, at least in population NC1 and NC3, both at *SAI-1* and microsatellite loci.

Although a relatively small number of samples were screened, it could be suggested that hybridization between the Balkan frog and the Anatolian frog may occur in the North of Sardinia. Indeed, the *SAI-1* marker returned two fragments, each one typical of the species detected by mtDNA analysis, in three of the analyzed samples, while a mismatch between nuDNA and mtDNA species assignment was observed in sample Mle-4 from NC3. Allele sharing was further observed at microsatellite loci among samples ascribed to distinct mtDNA lineages, and private alleles were mainly detected in southern populations.

The presence of both *P.* cf. *bedriagae sensu stricto* and *P. kurtmuelleri* mtDNA haplotypes in population NC1 further supports the hypothesis that hybrid genotypes could be present in the region. In population NC3, all the samples analyzed (only tadpoles) were assigned to *P. kurtmuelleri* according to mtDNA polymorphism. Assuming a failure in sampling *P.* cf. *bedriagae sensu stricto* in population NC3, the two larvae showing double banding pattern at *SAI-1* could have originated from the crossbreed between males of this taxon and *P. kurtmuelleri* females. The scenario of interspecific crossbreeds is in accordance with the biology of the involved species, as larger females (like *P. kurtmuelleri*) are selected for reproduction by *Pelophylax* males (Lanza et al. 2007). We can further assume that putative reproductive barriers between species (particularly pre-zygotic mechanisms such as selection according to the male call) could be altered in new environments.

Interspecific viable crossings between distinct water frog species have been widely tested by previous authors (e.g., Günther 1973, 1982; Berger et al. 1982; Plötner et al. 2010; Bellati A., *unpublished results*), and although information concerning the outcome of the crossbreed between the Balkan frog and the Anatolian frog has not been found in the literature, we can assume that the joint release of different taxa can favor the production of new hybrid genotypes, whose invasion may be potential even higher than those of the parental species. It has been largely shown that hybrids could have additional advantages compared to the parental species, a phenomenon known as the “hybrid strength”: for example, they can bear higher genetic diversity, resulting in an increased potential for local adaptation to a broad range of environmental conditions (e.g., Dlugosch and Parker 2008; Liebhold and Tobin 2008), higher survival, growth and development rates, lower metabolic demand, and increased resistance to diseases (Grant and Grant 1992; Frankham et al. 2002; Seehausen 2004).

At present, the assignment of both eggs and tadpoles to alien lineages attests that both the Balkan and the Anatolian frogs are able to reproduce in Sardinia. Moreover, in the surveyed areas, all the ecological requirements of these amphibians (e.g., presence of streams and various types of water bodies, favorable climate, and trophic availability) seem to be fulfilled. Therefore, all the species should be considered well-established on the island. Once introduced, it could be assumed that *Pelophylax* may have occupied the available vacant niches. According to the spatial distribution of mtDNA haplotypes recovered in this study, we can hypothesize that natural dispersal of individuals along irrigation canals (due to intensive agricultural practices) could have likely occurred in southern Sardinia after the first release. Indeed, the southern populations share the same haplotype (Pb3), despite being ca. 50 km far from each other. Conversely, connection among the three northern populations appears less obvious according to the orography and the xeric climate of the island, although natural dispersal cannot be ruled out, and anthropic intervention may facilitate dispersal as well.

In conclusion, effort should be directed to monitor the population dynamics, in order to prevent them from spreading and further translocations. According to the high diversity of alien water frogs detected in this study, further ecological, distributional, and population genetic studies are deserved in order to clarify possible interactions within the *Pelophylax* species, as well as the latter and the native Sardinian amphibians.

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