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Will there be a second extinction? Molecular identification of multiple alien water frogs (*Pelophylax ridibundus sensu lato*) in Tuscany, Central Italy, reveals genetic pollution within a unique hybridogenetic system

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The introduction of alien water frogs is perhaps one of the most underestimated herpetological conservation issues in Europe. The identification of distinct species is highly challenging at the phenotypic level, and artificial syntopy between various taxa and lineages may lead to diverse outcomes, including hybridisation and local extinction. In central Italy the native synklepton of *Pelophylax bergeri* (the parental taxon) and *P.* kl. (klepton) *hispanicus* (the hybridogenetic hybrid, which clonally transmits the genome of an extinct *ridibundus*-like taxon) is present. Until recently, data regarding the presence of alien water frogs in central Italy was scarce, and no alien taxa have been reported for Tuscany. In this study, four distinct non-native *Pelophylax* lineages have been identified via molecular analysis in the Cecina and Arno river basins and ascribed to the Marsh frog group (*P. ridibundus sensu lato*). Alien *Pelophylax ridibundus*, *P. kurtmuelleri*, and *P.* cf. *bedriagae sensu stricto* currently appear to be widespread in the Cecina basin. Furthermore, evidence of hybridisation with autochthonous taxa has been suggested by genetic analyses in four out of eight sampling localities. With a view to evaluate urgent conservation strategies, a greater sampling effort is required to assess the actual distribution and ecology of the alien lineages, and further research is necessary to measure their impact on the native hybridogenetic system of the central-southern Italian pool frogs.

Keywords: European water frogs, conservation, *Pelophylax ridibundus*, *Pelophylax* cf. *bedriagae*, *Pelophylax kurtmuelleri,* hybridisation

INTRODUCTION

The human-mediated introduction of invasive alien species is one of the main challenges facing biodiversity conservation (Meyerson & Mooney, 2007; Simberloff et al., 2013). Resulting threats to autochthonous populations may include spatial and trophic competition, direct predation, spread of pathogens, habitat modification and genetic pollution (Mooney & Cleland, 2001; Bucciarelli et al., 2014; Kraus, 2015).

One of the most fascinating examples of genetic pollution involves European water frogs of the genus *Pelophylax* (Fitzinger, 1843), a species-rich complex of taxa, whose identification could be very challenging at the phenotypic level. The genus *Pelophylax* is particularly intriguing because of the presence of different hybridogenetic systems, which originated through a sexual parasitic mechanism, in which one of the parental species is parasitised by a hybrid species, defined as klepton ("kl." in nomenclature). The hybrid species transfers only the genome of the other parental species during reproduction, generating new constitutively heterozygous hybrids via backcrossing (Berger, 1967; Spolsky & Uzzell, 1986).

Anthropogenic introductions of allochthonous water frog species or lineages have the ability to disrupt these breeding systems via hybridisation (e.g. Leuenberger et al., 2014; Dufresnes et al., 2017a), which is considered as the highest impacting mechanism of global invasive amphibians according to the Environmental Impact Classification for Alien Taxa (EICAT) scheme (Kumschick et al., 2017). The pathways of water frog introductions in Eurasia are several and varied, and the history of their release spans both in space and time. Initial translocations of some taxa, like the Italian pool frog *Pelophylax bergeri* (Günther, 1986), may perhaps date back to the Antiquity (e.g. the Roman Empire, Dufresnes et al., 2017b; Dufresnes & Dubey, 2019). Over the last centuries,frogs of greater size belonging to the *ridibundus* group, have been

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repeatedly imported to western and northern European countries from eastern and southern regions mainly for leg consumption, scientific or ornamental purposes (e.g. Lanza, 1962, 1983; Plötner et al., 2008; Schmeller et al., 2007; Holsbeek et al., 2010). Noteworthy, such releases have involved also taxa under protection, like the Albanian water frog *Pelophylax shqipericus* (Hotz, Uzzell, Günther, Tunner & Heppich, 1987), introduced in Umbria (central Italy) (Domeneghetti et al., 2013). In addition to intentional releases, the unintentional dissemination of eggs and/or tadpoles along with the release of fish stocks for sport fishing and/or the importation of aquatic vegetation for plant nursery may have further promoted the establishment of alien populations of these frogs, as recently assumed for the alien taxa introduced in islands like Sardinia (Bellati et al., 2019).

Based on the current state of knowledge, alien populations of the Marsh frog *Pelophylax ridibundus* (Pallas, 1771) are established in many European countries including Belgium, Germany, Luxemburg, France, Switzerland and Italy (Plötner, 2005; Schmeller et al., 2007; Holsbeek et al., 2010; Bellati et al., 2012; Laghi et al., 2013; Dufresnes et al., 2017a). As previously stated, most of the introduced populations of this species originated from southern and eastern European countries (hereafter the "Eastern" lineage), but alien populations from central Europe (hereafter the "Western" lineage) may also occur. Several invasive populations also include the Balkan frog *Pelophylax kurtmuelleri* (Gayda, 1940), native to Greece and Macedonia, and/or several cryptic taxa previously ascribed to the Levantine frog *Pelophylax bedriagae* (Camerano, 1882) whose natural range extends from Anatolia (in Turkey) and Eastern Europe to the Middle East (Pasqualini, 2013; Laghi et al., 2013; Dufresnes et al., 2017a; Dufresnes et al., 2018; Bellati et al., 2019).

Noteworthy, *P. ridibundus* (including *P. kurtmuelleri*) and *P. bedriagae* are assessed as having Massive (MV) impacts, which is the maximum impact score (Kumschick et al., 2017). The impact of alien water frogs on native populations is strongly affected through their geographical origin, on which the capacity to induce hybridogenesis depends (Hotz et al., 1985; Holsbeek & Jooris, 2010; Leuenberger et al., 2014). For instance, the introduction of Western *P. ridibundus* in the hybridogenic system composed by *Pelophylax lessonae* (Camerano, 1882) and *Pelophylax* kl. *esculentus* (Linnaeus, 1758) may lead to a rapid loss of the genome of the former species due to hemiclonal exclusion (e.g. Plötner et al., 2008; Holsbeek & Jooris, 2010). The occurrence of hemiclones carrying the alien genome of the Balkan frog has been recently demonstrated in the wild, suggesting that crosses between *Pelophylax perezi* López-Seoane, 1885, the parental taxon of *Pelophylax* kl. *grafi* (Crochet, Dubois, Ohler, Tunner 1995), and the former may result in the differentiation of a new klepton (Dufresnes et al., 2017a). By contrast, Eastern *P. ridibundus*, *P. bedriagae* and its relatives are incapable of producing hybridogenetic hybrids (Hotz et al., 1985; Plötner, 2005). However, the situation is even more complicated given the fact that introductions can frequently involve more allochthonous taxa simultaneously, so that the evolutionary trajectories are difficult to predict (Dufresnes et al., 2017a). Finally, the impact of European water frog introductions is also driven by ecological preferences. These can limit interspecific mating to some extent and later originate a stable coexistence of the native and alien taxa in sufficiently heterogeneous environments (Leuenberger et al., 2014).

In Italy, native populations of water frogs belong to two distinct hybridogenetic systems (i.e. synklepton), namely the *lessonae-esculentus* system (widespread across central Europe and inhabiting northern Italy, from the Po Plain up to the border of northern Apennines, Lanza et al., 2007), and the *bergeri-hispanicus* system occurring southwards, from northern Apennines up to Sicily (Canestrelli & Nascetti, 2008). As widely demonstrated for *P.* kl. *esculentus*, *Pelophylax* kl. *hispanicus* (Fitzinger, 1826) should similarly reproduce via hybridogenesis with its parental taxon *P. bergeri*, although a recent molecular study has shown that the clonal genome transmitted by the hybridogen originated through the hybridisation with a species of the *ridibundus* group related to the Cyprian and Western Anatolian lineages, nowadays extinct in the wild (i.e. P. n.t. 1, Dubey & Dufresnes, 2017). According to this recent insight, a high conservation value should be conferred to this endemic hybridogenetic system (Dubey & Dufresnes, 2018). To date, the presence of alien water frogs has been widely documented in the northern part of the Italian Peninsula, where the most widespread alien taxon is *P. kurtmuelleri*, initially introduced in western Liguria in 1941 (Lanza, 1962), and now present in regions of Piedmont, Lombardy and Emilia-Romagna (Bellati et al., 2012; Laghi et al., 2013). In the latter region at least, mixed populations of *P. kurtmuelleri*, *P. ridibundus* and *P.* cf. *bedriagae* occur together with the native synklepton of *P. bergeri*/*P.* kl. *hispanicus* (Pasqualini, 2013). A recent study also revealed the occurrence of both *P. kurtmuelleri* and *P.* cf. *bedriagae* in the island of Sardinia (western Mediterranean), where also *P. bergeri* has been introduced in historical times (Bellati et al., 2019). It is noteworthy that reports of allochthonous water frogs are scarce within the range of the endemic synklepton, and limited to Umbria (central Italy: *P. ridibundus* and *P. shqipericus*, Domeneghetti et al., 2013), Lazio (central Italy: unidentified frogs of the *ridibundus* group) and Calabria (southern Italy: *P. kurtmuelleri*, Bisconti et al., 2019). To date, no evidence of genetic pollution for the native system has been reported in the literature. Nonetheless, early detection of alien water frogs is difficult because related species are often impossible to discriminate according to morphology and hybrids are not easy to detect visually (Domeneghetti et al., 2013; Dufresnes et al., 2017a). Therefore, the risk of silent introduction and an unobserved spread of alien taxa within the range of the endemic synklepton is real, with unknown effects on the persistence of both native populations and the unique genome retained by *P.* kl. *hispanicus* in its clonal germline.

Given that a proper identification of species and hybrid individuals can only be achieved via molecular tools (Cristescu, 2015; Dufresnes et al., 2017a, b), a molecular-based approach in this work was accordingly adopted, involving both mitochondrial (mtDNA) and nuclear (nuDNA) markers to detail the species composition of alien *Pelophylax* taxa in two distinct river basins in Tuscany. The

main goals of our research were to: i) ascribe each alien frogs to the correct taxon and/or evolutionary lineage; ii) determine the possible occurrence of hybridisation events between alien and native frogs; iii) establish the scale of expansion of the allochthonous taxa in the Cecina river.

METHODS

We became aware about the presence of frogs of the *ridibundus* group in the Cecina basin from a picture taken in a private natural swimming pool in June 2015 in the Municipality of Pomarance, in the province of Pisa. In addition, a dead female was collected in the Arno basin on the road in Signa, in the province of Florence, in May 2016. From then, further confirmations were made in both areas according to mating calls, that appear clearly distinct for the non-native taxa of the *ridibundus*-like group (Schneider & Sinsch, 1992).

In order to assess the species identity and the spread of alien taxa, sampling sessions were conducted in 8 localities in the Cecina basin between the province of Pisa and Siena (5 in the Cecina river [San Martino, SM; Casino di Terra, CDT; Masso delle Fanciulle, MF; Monteguidi, MG; Anqua, CN], 2 in two tributaries [Trossa, TR; Possera, POS] and one in the natural swimming pool [Il Cerreto, CER]), while in the Arno basin, only an artificial water body (Lago Troscio, Chiari del Padule dei Colli Alti) was sampled in the Municipality of Signa (hereafter SI), about 2 km far from the point where the dead specimen was previously found (Table 1; Fig. 1).

All the sampling localities were selected based on allochthonous male calls and accessibility, with the only exception of CN where only typical calls from native taxa were recorded. In the field, individuals were classified as allochthonous via the detection of diagnostic phenotypic traits, like the shape of metatarsal tubercle and the coloration of male vocal sacs, that appeared well-differentiated in taxa of the *ridibundus* group compared to native taxa (Berger, 1973). All the captured frogs showed morphological traits attributable to *ridibundus*-like species, except for two individuals from locality CDT and all the samples from CN. Particularly noteworthy is that in these two localities, tadpoles were also found and sampled. 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).

Tissue samples consisted of phalanges clipped from adult frogs and small pieces of tadpole tail fins, which were immediately stored in 96 % ethanol. A total of 51 and 4 samples were collected in the Cecina and Arno basins, respectively. Genomic DNA was purified using the commercial kit GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, Saint Louis, USA). The entire mitochondrial genes ND2 (1038 bp) and ND3 (340 bp) were PCR-amplified following Plötner et al. (2008), then amplicons were purified with GenElute Gel Extraction Kit (Sigma-Aldrich) and sequenced at Eurofins Genomics (Ebersberg, Germany). Obtained sequences were aligned using Geneious 11 (Biomatters Ltd., New Zealand) and compared with all the homologous sequences available in GenBank (/https://www.ncbi.nlm.nih.gov/, accessed

Table 1. List of sampling sites, reporting the locality code (ID_{LOC}), coordinates (Lat, Long), the number of samples per population (n) and the taxa identified according to mtDNA analysis (ID_{TAXA}: Re = Eastern *P. ridibundus*, Rw = Western *P. ridibundus*, Bd = *P.* cf. *bedriagae s.s.*, K = *P. kurtmuelleri*, Ber/His = *P. bergeri*/*P.* kl. *hispanicus*).

ID_{Loc}	Lat	Long	n	ID_{TAXA}
Cecina basin				
CN	43°13'39.5"N	11°00'55.5"E	6	Ber/His
CDT	43°19'24.0"N	10°40'13.4"E	8	Ber/His; Re
CER	43°20'20.9"N	10°49'49.5"E	8	Re; Bd; Rw
MF	43°18'36.8"N	10°55'22.5"E	8	Ber/His; Re; Bd
MG	43°17'21.7"N	10°58'51.3"E	8	Ber/His; Re; Bd
POS	43°16'03.2"N	10°54'45.5"E	2	Re; Bd
SM	43°20'19.3"N	10°34'27.1"E	4	Re; Bd
TR	43°19'28.8"N	10°44'48.2"E	7	Ber/His; Re; Bd; K
Arno basin				
SI	43°48'08.3"N	11°05'38.3"E	4	Rw

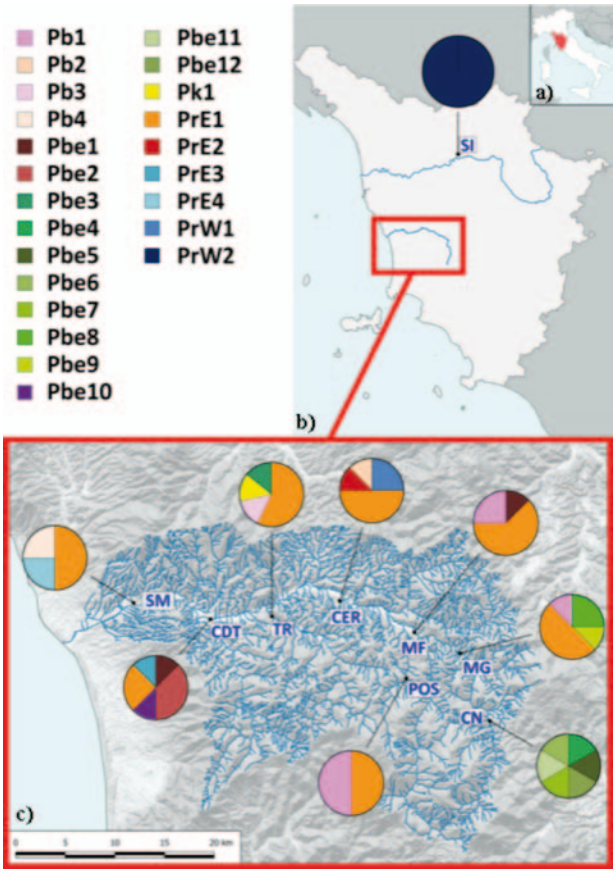

Figure 1. Distribution of water frog lineages detected in Tuscany. Circles refer to sampled populations and indicate relative frequencies of mtDNA haplotypes, which are coloured as reported in the legend. (a) Location of Tuscany region; (b) Arno and Cecina river basins; (c) detail of the Cecina river basin.

on August 24th, 2018) using the Basic Local Alignment Search Tool (BLAST). Haplotypes were extracted in FaBox 1.41 (http://users-birc.au.dk/biopv/php/fabox/, Villesen, 2007) using the DNA to haplotype collapser and converter tool.

The concatenated alignment of ND2 and ND3 was used to infer phylogenetic relationships with Bayesian Inference (BI) using MrBayes 3.2 (Ronquist et al., 2012). Previously published ND2 and ND3 sequences of *P. ridibundus*, *P. kurtmuelleri*, *P. bedriagae* and its relatives (referred as *P.* cf. *bedriagae* Main Haplo Groups [MHG] 2-9 according to the classification proposed by Akın et al., 2010), *P. bergeri*, *P. lessonae*, and *P.* kl. *esculentus* were included in the analysis (n = 252). Sequences of *P. nigromaculatus* (n = 1), *P. saharicus* (n = 3) and *P. perezi* (n = 2) were used as outgroup (see Table S1 for all the accession numbers). The software Partitionfinder 1.1.0 (Lanfear et al., 2012) was used to test for the best partitioning scheme of the concatenated dataset. The input configuration file contained 6 partitions, corresponding to individual codon positions for the two mtDNA genes. The "greedy" algorithm (heuristic search) was used with branch lengths estimated as "unlinked" to search for the best scheme. A total of 24 a priori schemes with varying degrees of complexity were statistically compared in Partitionfinder using the BIC (Bayesian Information Criterium, Schwarz 1978).

Bayesian analysis was performed sampling two runs and four chains for each run for 10×10^6 generations (started on random trees) and four incrementally heated Markov chains (using default heating values) and sampling every 100th tree. After checking the distribution of log-likelihoods and parameter values using Tracer 1.5 (available at http://beast.bio.ed.ac.uk/Tracer) the burn-in threshold was set to 25 %. The resulting phylogram and posterior probabilities were visualised in FigTree 1.3 (Rambaut, 2009). Posterior probabilities ≥ 0.95 were considered significant.

Network reconstruction of concatenated ND2+ND3 haplotypes was performed using the software TCS 1.21 (Clement et al., 2000) calculating the minimum number of mutational steps required to connect different haplogroups, under the parsimony informative model by Templeton et al. (1992). To this end, only reference sequences belonging to the phylogenetic clades (MHG) including our samples were considered. The 95 % parsimony threshold was chosen to infer disconnections between deeply differentiated mitochondrial haplogroups corresponding to distinct *Pelophylax* taxa. Results were visualised and edited using the online web-tool tcsBU, available at https://cibio.up.pt/software/tcsBU/index.html (Múrias dos Santos et al., 2015).

Number of variable sites, haplotype diversity (hd), nucleotide diversity (pi) and the average number of nucleotide differences (k) were calculated using DNAsp 5 (Librado & Rozas, 2009) to describe the genetic variation of the major mtDNA lineages found in the Cecina basin. The same indexes were calculated for each locality (including SI from the Arno basin) to describe genetic variation of water frog populations at the spatial level.

To test for the presence of kleptons in the dataset, the protocol provided by Hauswaldt et al. (2012) was adopted. This PCR-based method detects fragments of genome-specific sequence length of the nuclear serum albumin intron-1 (SAI-1), i.e. shorter PCR products in water frogs of the *lessonae* group (~300 bp) compared to the ones from the *ridibundus* group (>700 bp). Consequently, genome-specific banding patterns can be visualised via standard electrophoresis on agarose gel as distinct bands. In hybrids carrying both genome types both small and large bands are displayed (as both PCR products are generated). These types of hybrids can be either native kleptons possessing both the L-type genome of *P. bergeri* as well as the R-type genome of the extinct species, or hybrids stemming from the hybridisation between *P. bergeri* and an alien taxon. The thermal conditions and the primer used were the same as Hauswaldt et al. (2012).

Nine nuclear microsatellite markers were further screened in the samples collected in the Cecina basin (n = 51) to detect population genetic structure and potential admixed genotypes resulting from the cross between alien taxa carrying the R-type genome and *P.* kl. *hispanicus*, which similarly only transmits a clonal R-genome set during reproduction. Precisely, according to the literature six of these markers were thought to be diagnostic for the detection of L- and R-type genomes respectively (i.e. they selectively amplify only one genome set). Loci RlCA1a27 (Christiansen, 2009), RlCA 5 and RlCA18 (Garner et al., 2000) were chosen to selectively amplify the L-genome in *P. bergeri* and *P.* kl*. hispanicus*. To detect R-specific alleles, Rrid169A (Christiansen, 2009), Re2CAGA3 (Arioli, 2007) and Res22 (Zeisset et al., 2000) loci were chosen. Three more loci that amplify both the L-type and the R-type genomes were amplified in all the samples: Ca1b6 (Arioli, 2007), Ga1a19 (Arioli, 2007; Christiansen, 2009) and RlCA1b5 (Garner et al., 2000).

It is noteworthy that all the selected loci have been previously isolated in taxa of the *lessonae-esculentus* system. We therefore assumed that they should provide similar information about the selective amplification of one or the other genome set as a result of the close phylogenetic relationships of the taxa involved in our target study system. Indeed, *P. bergeri* has been considered a subspecies of *P. lessonae* (both appearing morphologically indistinguishable to each other until recent molecular achievements [Canestrelli & Nascetti, 2008]), whereas the R-type genome of *P.* kl. *hispanicus* is phylogenetically close to the R-type genome of at least one taxon detected in our study area, i.e. *P.* cf. *bedriagae sensu stricto* (Dubey & Dufresnes, 2018, see Results).

All the loci were PCR-amplified in 10 µl with previously published primer pairs (see Table S2) 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 (see Table S2 for locus-specific amplification conditions), and 60 s at 72 °C. The forward primer of each pair was fluorescently-labelled for detection on an ABI3130 capillary sequencer. Sequencing was performed after setting 3 post-PCR multiplexes set up by combining 2 to 4 loci (see Table S2). Allele scoring and dimensioning were conducted in Geneious 11 (Biomatters Ltd.).

Assignment of individual genotypes to distinctive

gene pools was performed by using a Bayesian algorithm implemented in the software Structure 2.3.4 (Pritchard et al., 2000), thus adopting an iterative approach to test the occurrence of K clusters without a prior knowledge of the geographic origin of individuals. Hardy-Weinberg and linkage equilibrium within the inferred clusters was assumed (Pritchard et al., 2000). These assumptions are unlikely to be met in populations of hybrid and clonal organisms because of fixed heterozygosity and linkage of multilocus genotypes. Nevertheless, several studies demonstrated that the Bayesian models implemented are robust to deviations from these assumptions and provide biologically meaningful structuring supported by other independent analyses (e.g. Halkett et al., 2005; Schmidt et al., 2011). Analyses were carried out on the 8 nuclear loci using an admixture model without prior species information, with 50,000 burn-in steps followed by 100,000 iterations. All runs were replicated 8 times with K ranging from 1 to 8. Following simulations, the most likely number of clusters (K) was calculated from the ΔK method (Evanno et al., 2005) implemented in StructureHarvester 0.6.93 (Earl & vonHoldt 2012, available at http://taylor0.biology.ucla.edu/structureHarvester/). Graphical representations of the Structure results were depicted using an online application
CLUMPAK (Kopelman et al., 2015, available at http://clumpak.tau.ac.il).

RESULTS

Mitochondrial ND2 and ND3 sequences revealed the occurrence of at least four alien lineages in the Cecina basin, besides the native Italian pool frogs *P. bergeri* and *P.* kl. *hispanicus* (Tables 1,2). A total of 23 distinct mtDNA haplotypes were detected in the concatenated dataset, 11 (47.8 %) belonging to the allochthonous taxa (Table 2). Eastern *P. ridibundus* and *P.* cf. *bedriagae sensu stricto* (*sensu* Akın et al., 2010) exhibited four distinct haplotypes each (PrE1-4 and Pb1-4), while haplotypes PrW1 and PrW2 belong to Western *P. ridibundus*, each one being private of a different river basin. Haplotype Pk was the only one assigned to *P. kurtmuelleri* (Table 2). The remaining 12 mtDNA haplotypes belonged to the native lineage of *P. bergeri* (Pbe1-12, Table 2). Matching probabilities between our sequences and reference sequences in Genbank ranged from 99 % to 100 % according to BLAST search (Table 2). The most widespread alien lineage within the studied area was the Eastern *P. ridibundus* (n = 25), whose mtDNA was detected in all the surveyed localities apart from the one closest to the river source (CN). Furthermore, the mtDNA of *P.* cf. *bedriagae sensu stricto* (n = 7) was found in six localities (CER, MF, MG, POS, SM, TR). Finally, Western *P. ridibundus* (n = 2) and *P. kurtmuelleri* (n = 1) were found in only one locality, respectively CER and TR. The four individuals sampled in the Arno basin were all assigned to the Western *P. ridibundus* lineage.

The phylogenetic analysis assigned the samples from Tuscany to different clusters according to the taxa and lineages occurring in our dataset (Fig. 2). Overall, the tree topology recovered in our analysis resembles the one previously detected by Akın et al. (2010). Native haplotypes were assigned to the Italian *P. bergeri*/*P.* kl. *hispanicus* clade, well-differentiated from the sister-clade including *P. lessonae* and *P.* kl. *esculentus* from northern Italy and central Europe (bpp = 1.00), and deeply phylogenetically divergent from the identified alien taxa. Haplotypes PrE1-4, PrW1-2 and Pk fell within the MHG1, which corresponds to the European *P. ridibundus* clade (bpp = 1.00). Within this clade, only haplotypes PrW1 and PrW2 clustered in a well-supported subclade corresponding to the Western lineage according to previous authors (Plötner et al., 2008). Haplotypes Pb1-4 clustered within the monophyletic MHG6c clade (*P.* cf. *bedriagae sensu stricto*, Akın et al., 2010; bpp = 1.00), native to Turkey, Greece and Russia (Fig. 3). According to the phylogenetic tree inference, our haplotypes were assigned to distinct networks in the haplotype network analysis (Fig. 3). Within *P. ridibundus*, haplotypes PrE and PrW were deeply differentiated from each other (min 14 steps). However, the exact geographic origin of the haplotypes recovered in alien populations from central Italy was difficult to assess, as they match or were close to reference haplotypes widespread in most of the European countries. On the other hand, haplotype Pk appeared closer to the Greek haplogroup generally ascribed to *P. kurtmuelleri*. The haplotype network of *P.* cf. *bedriagae s.s.* similarly revealed high structuring within this eastern haplogroup, but haplotypes Pb1-4 were clearly close to the Turkish reference haplotypes, although none of the haplotypes recovered match those already available in Genbank. Regarding the native taxa, the haplotypes detected in this study greatly improved the current knowledge concerning mtDNA variation of the endemic hybridogenetic system, returning a complex scenario of molecular variation in the Cecina basin, with private mitochondrial haplotypes occurring in *P. bergeri* (Pbe2, Pbe4, Pbe7) or *P.* kl. *hispanicus* (Pbe1, Pbe3, Pbe5-6, Pbe8-12), respectively (Fig. 3).

As expected, the autochthonous taxa were characterised by higher diversity indices compared to alien species (hd = 0.958 ± 0.036, pi = 0.00291 ± 0.00029, k = 4.017, Table 3a). Nonetheless at the population level, mixed localities where both alien and native mtDNA lineages were detected showed higher pi (from 0.08802 in MG to 0.01957 in CER, Table 3b) and k (from 121.286 to 26.964 in the same localities, Table 3b) than the others.

The analysis of nuDNA polymorphism based on SAI-1 sequence polymorphism revealed the presence of mixed L-/R- genotypes in the dataset (n = 11), all recovered within the Cecina basin. None of these samples returned a clear mismatch between mtDNA clade and nuDNA banding pattern, all having *P. bergeri* mtDNA.

R-specific diagnostic loci successfully amplified alien genomes (i.e. those carrying alien mtDNA, n = 39) and samples showing the double-banding pattern in SAI-1 analysis (putatively assigned to *P.* kl. *hispanicus* according to mtDNA). All the samples assigned to the endemic klepton shared the same allele at both locus Res22 (109) and Rrid169A (199), whereas the same loci turned out to be polymorphic in the other samples. Locus Re2CAGA3 failed to amplify in most of the samples from locality CDT, as well as from all those collected in CN. We therefore chose to exclude this locus from downstream analyses.

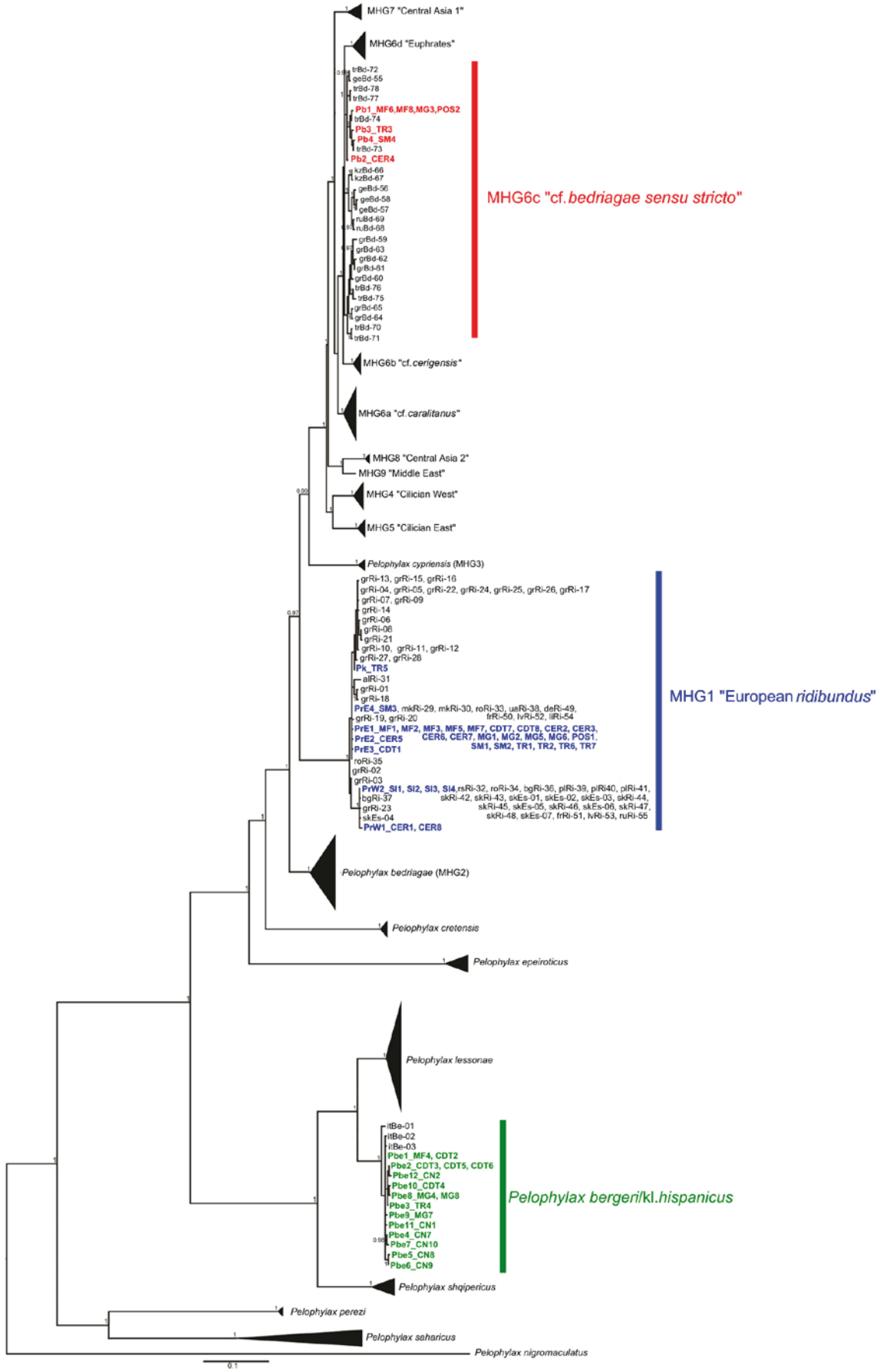

Figure 2. Phylogenetic tree based on ND2+ND3 mtDNA of *Pelophylax* spp. according to Bayesian Inference. Samples from this study are coloured according to the clades to which the sequences belong, along with haplotype code (see Table 2). Reference sequences are named as in Table S1. Posterior probability values ≥ 0.95 are reported at nodes.

Table 2. List of the mtDNA haplotypes found in this study, with relative Accession Numbers.

mtDNA species	ID_{HAPLO} (Acc. N.)	ID_{LOC} [ID_{SAMPLE}]	$ND2_{HAPLO}$	ND2 % (Acc. N./country)	$ND3_{HAPLO}$	ND3 % (Acc. N./country)
Eastern *P. ridibundus*	PrE1 (MN864866)	CDT[7-8], CER[2-3-6-7], MF[1-2-3-5-7], MG[1-2-5-6], POS[1], SM[1-2], TR[1-2-6-7]	Re1n2	99 % *P. ridibundus* (AM749707/France)	Re1n3	100 % *P. ridibundus* (AM749708/Greece)
	PrE2 (MN864867)	CER[5]	Re2n2	99 % *P. ridibundus* (AM749707/France)	Re1n3	100 % *P. ridibundus* (AM749708/Greece)
	PrE3 (MN864868)	CDT[1]	Re3n2	99 % *P. ridibundus* (AM749707/France)	Re1n3	100 % *P. ridibundus* (AM749708/Greece)
	PrE4 (MN864869)	SM[3]	Re4n2	99 % *P. ridibundus* (AM749707/France)	Re1n3	100 % *P. ridibundus* (AM749708/Greece)
Western *P. ridibundus*	PrW1 (MN864870)	CER[1-8]	Rw1n2	99 % *P. ridibundus* (JN627423/Poland)	Rw1n3	100 % *P. ridibundus* (JN627423/Poland)
	PrW2 (MN864871)	SI[1-2-3-4]	Rw2n2	100 % *P. ridibundus* (JN627423/Poland)	Rw1n3	100 % *P. ridibundus* (JN627423/Poland)
P. cf. *bedriagae sensu stricto*	Pb1 (MN864872)	MF[6-8], MG[3], POS[2]	Bd1n2	99% *P.* cf. *bedriagae* (GU812110/Turkey)	Bd1n3	99 % *P.* cf. *bedriagae* (KP260928/Greece)
	Pb2 (MN864873)	CER[4]	Bd2n2	99% *P.* cf. *bedriagae* (GU812111/Turkey)	Bd1n3	99 % *P.* cf. *bedriagae* (KP260928/Greece)
	Pb3 (MN864874)	TR[3]	Bd3n2	99% *P.* cf. *bedriagae* (GU812111/Turkey)	Bd2n3	100 % *P.* cf. *bedriagae* (KP260928/Greece)
	Pb4 (MN864875)	SM[4]	Bd4n2	99% *P.* cf. *bedriagae* (GU812109/Turkey)	Bd2n3	100 % *P.* cf. *bedriagae* (KP260928/Greece)
P. kurtmuelleri	Pk (MN864876)	TR[5]	Ku1n2	99 % *P. kurtmuelleri* (KP814011/Greece)	Ku1n3	100 % *P. kurtmuelleri* (KP814011/Greece)
P. bergeri/ *P.* kl. *hispanicus*	Pbe1 (MN864877)	MF[4], CDT[2]	Be1n2	99 % *P. bergeri* (GU812135/France)	Be1n3	100% *P. bergeri* (HG763869/Italy)
	Pbe2 (MN864878)	CDT[3-5-6]	Be2n2	99 % *P. bergeri* (GU812135/France)	Be1n3	100% *P. bergeri* (HG763869/Italy)
	Pbe3 (MN864879)	TR[4]	Be3n2	99 % *P. bergeri* (GU812135/France)	Be1n3	100% *P. bergeri* (HG763869/Italy)
	Pbe4 (MN864880)	CN[7]	Be4n2	99 % *P. bergeri* (GU812135/France)	Be1n3	100% *P. bergeri* (HG763869/Italy)
	Pbe5 (MN864881)	CN[8]	Be5n2	99 % *P. bergeri* (GU812135/France)	Be1n3	100% *P. bergeri* (HG763869/Italy)
	Pbe6 (MN864882)	CN[9]	Be6n2	99 % *P. bergeri* (GU812135/France)	Be1n3	100% *P. bergeri* (HG763869/Italy)
	Pbe7 (MN864883)	CN[10]	Be7n2	99 % *P. bergeri* (GU812135/France)	Be1n3	100% *P. bergeri* (HG763869/Italy)
	Pbe8 (MN864884)	MG[4-8]	Be8n2	99 % *P. bergeri* (GU812135/France)	Be2n3	100% *P. bergeri* (HG763869/Italy)
	Pbe9 (MN864885)	MG[7]	Be9n2	99 % *P. bergeri* (GU812135/France)	Be2n3	100% *P. bergeri* (HG763869/Italy)
	Pbe10 (MN864886)	CDT[4]	Be10n2	99 % *P. bergeri* (GU812135/France)	Be2n3	100% *P. bergeri* (HG763869/Italy)
	Pbe11 (MN864887)	CN[1]	Be11n2	99 % *P. bergeri* (GU812135/France)	Be3n3	100% *P. bergeri* (HG763869/Italy)
	Pbe12 (MN864888)	CN[2]	Be12n2	99% *P. bergeri* (GU812135/France)	Be4n3	100% *P. bergeri* (HG763869/Italy)

L-specific loci were positively amplified only in samples carrying the *P. bergeri* mtDNA (n = 16), apart from locus RlCA5, which was similarly amplified in samples carrying only the R genome. As this locus also showed a high occurrence of null alleles, we decided to further exclude it. The three aspecific loci were successfully amplified in the entire dataset, only locus RlCA1b5 showing missing data in two alien (SM4, TR1) and three putative native (CDT3-5, MG7) samples, probably due to the presence of null alleles.

The Structure analysis performed on the 7 selected loci identified K = 2 as the most likely number of clusters occurring in the dataset, each one clustering with high likelihoods (Fig. 4). The majority of the samples classified as aliens or natives respectively according to both mtDNA and SAI-1 analyses, were assigned to alternative clusters. However, several exceptions were observed, i.e. 8 alien individuals with "pure" native or a mix of alien and native alleles (from left to right in Fig. 4: MF1-CDT1-CDT7-MG2-MG6-TR7-SM3 carrying Eastern *P. ridibundus* mtDNA and MG3 assigned to *P.* cf. *bedriagae s.s.*).

DISCUSSION

The results presented in this study reveal for the first time the presence of several alien lineages of water frogs in Tuscany, at least in two distinct river basins. While only Western *P. ridibundus* was found in the Arno basin, four alien lineages (Eastern and Western *P. ridibundus*, *P.* cf. *bedriagae s.s.*, and *P. kurtmuelleri*) were found present

in the Cecina basin. According to the distribution of distinct alien taxa, while alien populations probably originated from a single introduction event in the Arno basin, multiple introductions most likely have occurred in the Cecina basin. At the time of our sampling, alien frogs have already invaded the Cecina basin - particularly Eastern *P. ridibundus*, whose haplotype PrE1 was detected in all the surveyed localities, along about 45 km from SM to MG. Nonetheless, considering the high dispersal ability of alien water frogs, we assume that the first introduction probably took place in recent times, as alien genotypes are known to replace native populations very

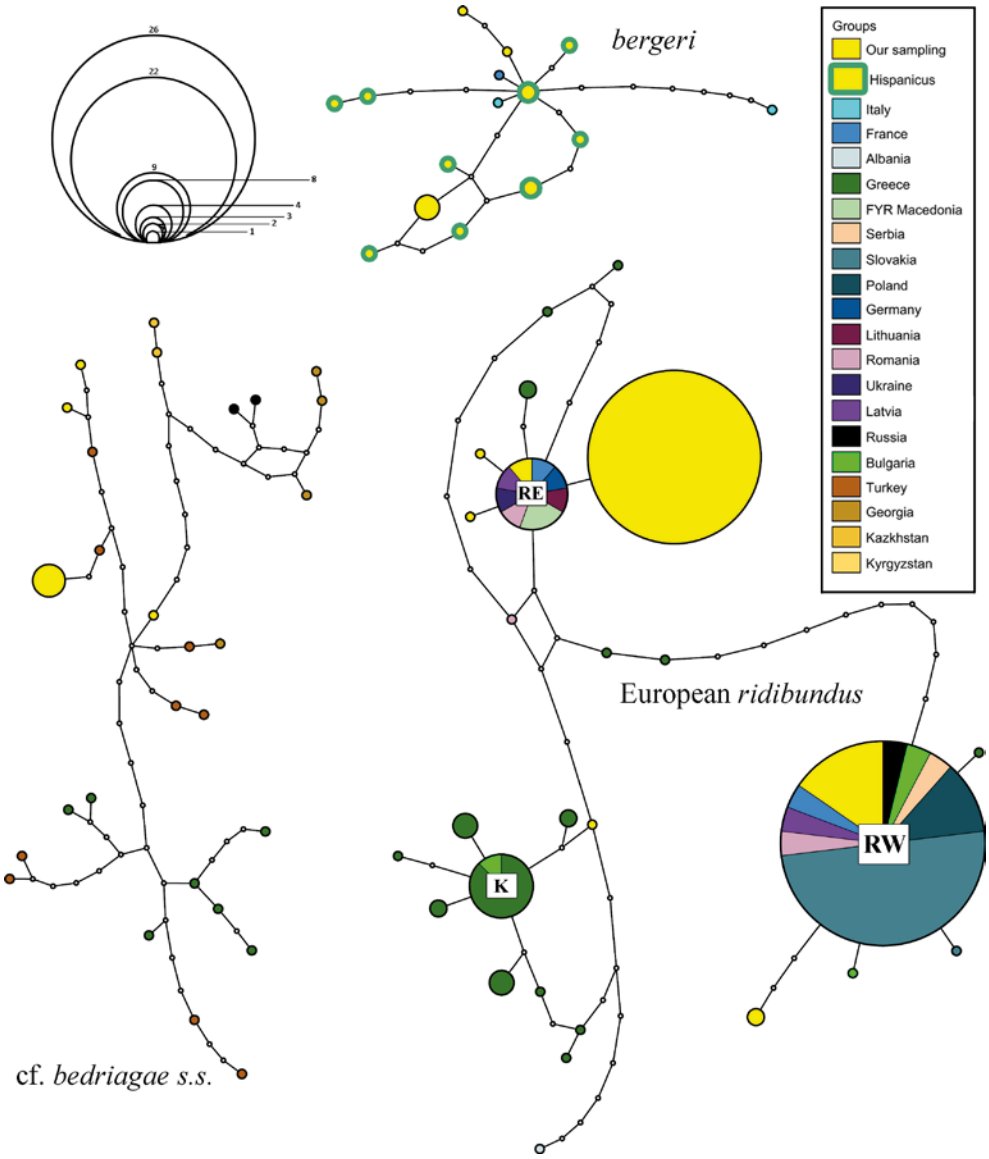

Figure 3. 95 % parsimony networks of mtDNA haplotypes (ND2+ND3) including our samples and reference sequences of corresponding taxa. Haplotypes are indicated by circles whose areas correspond to the haplotype frequency. Distinct colours denote different countries.

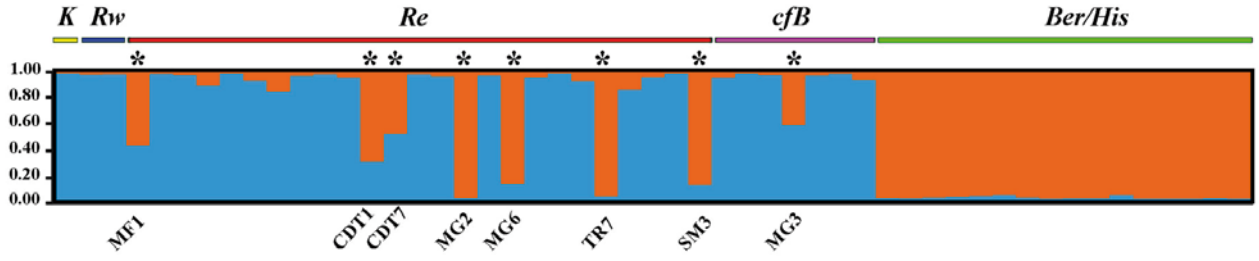

Figure 4. Bayesian clustering analysis (STRUCTURE) for the most likely number of clusters (K = 2), with no prior information on taxonomic identity. Individuals from the Cecina basin are assigned with high likelihood to either the blue (alien), or the orange (native) cluster, with however 8 exceptions (7 Eastern *P. ridibundus*, 1 *P.* cf. *bedriagae s.s.*).

Table 3. Haplotype diversity (hd), nucleotide diversity (pi) and average number of nucleotide differences (k) calculated for species (a) and localities (b). N: number of samples, S: number of segregating sites, H: number of haplotypes, ± Sd: standard deviation.

	N	S	H	hd±Sd	pi±Sd	k
a) Species						
Re	25	3	4	0.230±0.110	0.00028±0.00014	0.380
Rw	6	3	2	0.533±0.172	0.0016 ± 0.00037	1.600
K	1	n.c.	1	n.c.	n.c.	n.c.
Bd	7	11	4	0.714±0.181	0.00311±0.00088	4.286
Ber/His	16	14	12	0.958±0.036	0.00291±0.00029	4.017
b) Localities						
MF	8	237	3	0.607±0.164	0.05865±0.02284	80.821
CER	8	90	4	0.750±0.139	0.01957±0.00969	26.964
CDT	8	211	5	0.857±0.108	0.08154±0.01840	112.357
MG	8	236	4	0.750±0.139	0.08802±0.01571	121.286
TR	7	240	4	0.714±0.181	0.05812±0.02694	80.095
POS	2	84	2	1.000±0.500	0.06096±0.03048	84.000
SM	4	82	3	0.833±0.222	0.02987±0.01562	41.167
CN	6	11	6	1.000±0.096	0.00348±0.00055	4.800
SI	4	0	1	0	0	0
Overall	55	23	250	0.832±0.049	0.7312±0.00639	100.764

quickly once introduced - as a result of hybridogenetic mechanisms and genome exclusion (e.g. Dufresnes et al., 2017a). Nevertheless, pure native lineages were still found coexisting in mixed populations together with the alien taxa in the sampling area.

Although data on the release of alien water frogs is not available for the investigated basins, it seems quite reasonable to date back the introduction of at least some taxa in the Cecina basin to early 2000's in an area close to MF, where local fishermen released fish from the Balkans for game fishing (Fabio Ilio Fineschi, pers. comm.). The time-window we propose for the initial introduction of aliens is in line with the information reported in the Herpetological Atlas of Tuscany, published in 2006, in which allochthonous frogs are not mentioned (Vanni & Nistri, 2006).

Given the greater size compared to the native ones, adult alien frogs were also likely imported in the region for human consumption, as already happened in northern Italian regions like Piedmont and Lombardy (Bellati A., unpublished data). Gastronomic festivals of frogs are also quite common in Tuscany, particularly in the area of SI in the Arno basin, where traditional cuisine includes a typical frog-based dish.

The lack of alien alleles and polluted hybrids in the easternmost locality CN, which is closer to the Cecina water source, in the province of Siena, coupled with the lack of alien frogs in the westernmost area of the basin, particularly at the mouth of the same river in the province of Livorno (based on calls, G. Bruni pers. obs.), corroborates the hypothesis that introductions took place within the Province of Pisa, where only allochthonous individuals have been detected in several localities (CER, MF, POS). According to this scenario, following introduction, allochthonous lineages may have quickly spread in both directions.

The alien species assemblages detected in the Cecina basin resemble the one in the northern side of the Appennines but the distribution pattern of the alien lineages points to a wider abundance and expansion of the Eastern *P. ridibundus* in the region, instead of *P. kurtmuelleri* (Bellati et al., 2012; Pasqualini, 2013). Whether this trend is the result of an older introduction or the consequence of a more effective dispersal ability of this invader cannot be assessed at present. Indeed, the detection of newly metamorphosed individuals of *P. ridibundus* (carrying PrE1 and PrW1 mtDNA haplotypes) in locality CER, and the occurrence of a tadpole carrying haplotype PrE1 and clearly assigned to the alien gene pool by the Structure analysis in CDT, attest the successful reproduction of at least some lineages in the area. The detection of *P. kurtmuelleri* and Western *P. ridibundus* at single localities could be both the consequence of local independent releases, species-specific ecological preference or sampling bias.

According to the results obtained in our study, the main threat for the persistence of the *bergeri-hispanicus* synklepton lies in the ability of alien lineages to hybridise with native taxa, thus producing viable and fertile hybrids in most cases (Dufresnes et al., 2017a; Kumschick et al., 2017). For instance, a tadpole (CDT7) carrying PrE1 sampled in locality CDT turned out to be a hybrid carrying both native and alien R-type genomes.

Mechanisms underlying hybridisation and hybridogenesis involving *P. bergeri* and members of the *ridibundus* group still deserve investigation (Holsbeek & Jooris, 2010). Therefore, we can infer several alternative scenarios promoted by the co-occurrence of different alien taxa in the range of native ones (Dubey et al., 2014). In Western Europe, introgression of *P. bedriagae* genes in *P. ridibundus* and *P.* kl. *esculentus* gene pools is recurrent (Holsbeek et al., 2008), thus the impact of this introduced taxon in Tuscany could be highly negative on the native hybridogenetic complex, potentially leading to genome replacement through hemiclonal exclusion or recombination processes. The recent discovery that

P. kl. *hispanicus* retains the genome of an extinct species further poses important conservation implications for the safeguard of the parental *P. bergeri* and its klepton (Dubey & Dufresnes, 2017). In our analysis, 11 individuals were assigned to the native *P.* kl. *hispanicus* according to the observed length of polymorphism at the diagnostic SAI-1 marker (Hauswaldt et al., 2012), indeed bearing alleles associated to the native R-genome and *P. bergeri* mtDNA. On the other hand, introgression of native alleles have been detected at the microsatellite loci in 8 samples carrying alien mtDNA, further suggesting effective gene flow by hybridisation between the taxa considered. This conclusion is supported by the detection of alien individuals in all the localities (CDT, MG, MF, TR, SM) where non-hybridogenetic hybrids were detected.

The lack of alien frogs at the site closer to the river source could suggest that the area is still preserved from invaders at present. Alternatively, it could be the result of different ecological preferences in native and alien taxa, which might lead to spatial isolation limiting interspecific mating events (Plénet et al., 2005; Leuenberger et al., 2014). Indeed, native taxa show high degree of population structuring and high haplotype diversity at molecular markers, compatible with the occurrence of several stable populations in suitable slow running water or satellite stagnant waterbody habitats, besides those sampled along the river course. On the other hand, the Cecina River perfectly meets the ecological requirements of the alien taxa, including oxygenated shallow running water with riparian vegetation (Pagano et al., 2001; Leuenberger et al., 2014). Recently, alien populations of at least the Balkan frog and the Anatolian frog have been detected in Sardinia (Bellati et al., 2019) and it is noteworthy that the ND3 haplotype Pb2 (*P.* cf. *bedriagae s.s.*) found in the north of Sardinia match the haplotype Bd1n3 occurring in MF, CER, MG and POS, suggesting that at least this species could have been recently introduced to Sardinia from Tuscany, as a result of commercial activities (e.g. plant nursery, game fishing, and food; Bellati et al., 2019).

In conclusion, this study genetically characterises alien water frogs in Tuscany for the first time, with the detection of all the allochthonous species already reported for northern Italy, but at different frequencies, thus suggesting independent histories of release. More interestingly, our research provides the first evidence of hybridisation events with native *P. bergeri*/*P.* kl. *hispanicus* complex, posing major concern about the conservation of this unique hybridogenetic system. Since alien frogs are difficult to eradicate, it is pivotal to preserve source habitats and create new suitable breeding sites for native taxa (e.g. small ponds with aquatic vegetation), to favour spatial isolation. In Tuscany the distribution of alien water frogs directly concerns the Sites of Community Importance IT5170007 "Fiume Cecina da Ponteginori a Berignone" and IT5140011 "Stagni della Piana Fiorentina e Pratese", in which conservation actions should be implemented and tested. Further research is highly desirable to establish the current extent of alien water frogs' distribution in Tuscany and other regions in central-southern Italy. Moreover, a greater sampling effort is required to assess the effective level of admixture between native and alien taxa in the *P. bergeri*/*P.* kl. *hispanicus* system's range.

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REFERENCES

Akın, Ç., Can Bilgin, C., Beerli, P., Westaway, R., Ohst, T., Litvinchuk, S.N., Uzzell, T., Metin, B., Hansjürg, H., Gaston-Denis, G. & Plötner, J. (2010). Phylogeographic patterns of genetic diversity in eastern Mediterranean water frogs were determined by geological processes and climate change in the Late Cenozoic. *Journal of Biogeography* 37, 2111-2124. Doi: 10.1111/j.1365-2699.2010.02368.x

Arioli, M. (2007). Reproductive patterns and population genetics in pure hybridogenetic water frog populations of *Rana esculenta*. Dissertation. University of Zurich.

Bellati. A., Bassu, L., Nulchis, V. & Corti. C. (2019). Detection of alien *Pelophylax* species in Sardinia (western Mediterranean, Italy). *BioInvasions Records* 8, 8-25. Doi: 10.3391/bir.2019.8.1.02

Bellati, A., Razzetti, E., Resteghini, M., Sacchi, R., Pellitteri-Rosa, D., Casiraghi, M., Bernini, F., Galeotti, P. & Fasola, M. (2012). First molecular characterization of invasive alien populations of *Pelophylax kurtmuelleri* (Gayda, 1940) and new records from Italy. In *Atti IX Congresso Nazionale della Societas Herpetologica Italica*, pp 287-288.

Berger, L. (1967). Embrional and larval development of F1 generation of green frogs different combinations. *Acta Zoologica Cracoviensia* 12, 123-162.

Berger, L. (1973). Systematics and hybridisation in European green frogs of *Rana esculenta* complex. *Journal of Herpetology* 7, 1-10.

Bisconti, R., Martino, G., Chiocchio, A., Siclari, A. & Canestrelli, D. (2019). Balkan marsh frogs *Pelophylax kurtmuelleri* (Gayda, 1940) introduced in the Aspromonte National Park, southern Italy. *Bioinvasion Records* 8, 26-33. Doi: 10.3391/bir.2019.8.1.03

Bucciarelli, G.M., Blaustein, A.R., Garcia, T.S. & Kats, L.B. (2014). Invasion complexities: the diverse impacts of nonnative species on amphibians. *Copeia* 2014, 611-632.

Canestrelli, D. & Nascetti, G. (2008). Phylogeography of the pool frog *Rana* (*Pelophylax*) *lessonae* in the Italian peninsula and Sicily: multiple refugia, glacial expansions and nuclear–mitochondrial discordance. *Journal of Biogeography* 35, 1923-1936.

Christiansen, D.G. (2009). Gamete types, sex determination and stable equilibria of all-hybrid populations of diploid

and triploid edible frogs (*Pelophylax esculentus*). *BMC Evolutionary Biology* 9, 135. Doi: 10.1186/1471-2148-9-135

Clement, M., Posada, D.C.K.A. & Crandall, K.A. (2000). TCS: a computer program to estimate gene genealogies. *Molecular Ecology* 9, 1657-1659. Doi: 10.1046/j.1365-294x.2000.01020.x

Cristescu, M.E. (2015). Genetic reconstructions of invasion history. *Molecular Ecology* 24, 2212-2225. Doi: 10.1111/mec.13117

Domeneghetti, D., Bruni, G., Fasola, M. & Bellati, A. (2013). Discovery of alien water frogs (gen. *Pelophylax*) in Umbria, with first report of *P. shqipericus* for Italy. *Acta Herpetologica* 8, 171-176. Doi: 10.13128/Acta_Herpetol-13338

Dubey, S. & Dufresnes, C. (2017). An extinct vertebrate preserved by its living hybridogenetic descendant. *Scientific Reports* 7, 12768. Doi: 10.1038/s41598-017-12942-y

Dubey, S., Leuenberger, J. & Perrin, N. (2014). Multiple origins of invasive and 'native' water frogs (*Pelophylax* spp.) in Switzerland. *Biological Journal of the Linnean Society* 112, 442-449. Doi: 10.1111/bij.12283

Dufresnes, C., Di Santo, L., Leuenberger, J., Schuerch, J., Mazepa, G., Grandjean, N., Canestrelli, D., Perrin, N. & Dubey, S. (2017b). Cryptic invasion of Italian pool frogs (*Pelophylax bergeri*) across Western Europe unraveled by multilocus phylogeography. *Biological Invasions* 19, 1407-1420. Doi: 10.1007/s10530-016-1359-z

Dufresnes, C., Denoël, M., Di Santo, L, & Dubey, S. (2017a). Multiple uprising invasions of *Pelophylax* water frogs, potentially inducing a new hybridogenetic complex. *Scientific Reports* 7, 6506. Doi: 10.1038/s41598-017-06655-5

Dufresnes, C., Leuenberger, J., Amrhein, V., Bühler, C., Thiébaud, J., Bohnenstengel, T. & Dubey, S. (2018). Invasion genetics of marsh frogs (*Pelophylax ridibundus sensu lato*) in Switzerland. *Biological Journal of the Linnean Society* 123, 402-410. Doi: 10.1093/biolinnean/blx140

Earl, D.A. & VonHoldt, B.M. (2012). STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetic Resources* 4, 359-361. Doi: 10.1007/s12686-011-9548-7

Evanno, G., Regnaut, S. & Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* 14, 2611-2620. Doi: 10.1111/j.1365-294X.2005.02553.x

Garner, T.W., Gautschi, B., Röthlisberger, S. & Reyer, H.U. (2000). A set of CA repeat microsatellite markers derived from the pool frog, *Rana lessonae*. *Molecular Ecology* 9, 2173-2175. Doi: 10.1046/j.1365-294X.2000.105311.x

Hauswaldt, J.S., Hoer, M., Ogielska, M., Christiansen, D.G., Dziewulska-Szwajkowska, D., Czernicka, E. & Vences, M. (2012). A simplified molecular method for distinguishing among species and ploidy levels in European water frogs (*Pelophylax*). *Molecular Ecology Resources* 12, 797-805. Doi: 10.1111/j.1755-0998.2012.03160.x

Halkett, F., Simon, J.C. & Balloux, F. (2005). Tackling the population genetics of clonal and partially clonal organisms. *Trends in Ecology and Evolution* 20, 194-201. Doi: 10.1016/j.tree.2005.01.001

Holsbeek, G. & Jooris, R. (2010). Potential impact of genome exclusion by alien species in the hybridogenetic water frogs (*Pelophylax esculentus* complex). *Biological Invasions* 12, 1. Doi: 10.1007/s10530-009-9427-2

Holsbeek, G., Mergeay, J., Hotz, H., Plötner, J., Volckaert, F.A.M. & De Meester, L. (2008). A cryptic invasion within an invasion and widespread introgression in the European water frog complex: consequences of uncontrolled commercial trade and weak international legislation. *Molecular Ecology* 17, 5023-5035. Doi: 10.1111/j.1365-294X.2008.03984.x

Holsbeek, G., Mergeay, J., Volckaert, F.A.M. & De Meester, L. (2010). Genetic detection of multiple exotic water frog species in Belgium illustrates the need for monitoring and immediate action. *Biological Invasions* 12, 1459-1463. Doi: 10.1007/s10530-009-9570-9

Hotz, H., Mancino, G., Bucci-innocenti, S., Ragghianti, M., Berger, L. & Uzzell, T. (1985). *Rana ridibunda* varies geographically in inducing clonal gametogenesis in interspecies hybrids. *Journal of Experimental Zoology* 236, 199-210.Doi: 10.1002/jez.1402360210

Kopelman, N.M., Mayzel, J., Jakobsson, M., Rosenberg, N.A. & Mayrose, I. (2015). Clumpak: a program for identifying clustering modes and packaging population structure inferences across K. *Molecular Ecology Resources* 15, 1179-1191. Doi: 10.1111/1755-0998.12387

Kraus, F. (2015). Impacts from invasive reptiles and amphibians. *Annual Review of Ecology, Evolution, and Systematics* 46, 75-97. Doi: 10.1146/annurev-ecolsys-112414-054450

Kumschick, S., Vimercati, G., de Villiers, F.A., Mokhatla, M.M., Davies, S.J., Thorp, C.J., Rebelo, A.D. & Measey, G.J. (2017). Impact assessment with different scoring tools: How well do alien amphibian assessments match? *Neobiota* 33, 53.

Lanfear, R., Calcott, B., Ho, S.Y., Guindon, S. (2012). PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution* 29, 1695-1701. Doi: 10.1093/molbev/mss020

Lanza, B. (1962). On the introduction of *Rana ridibunda* Pallas and *Rana catesbeiana* Shaw in Italy. *Copeia* 1962, 642-643.

Lanza, B. (1983). Anfibi, Rettili (Amphibia, Reptilia). Guide per il riconoscimento delle specie animali nelle acque interne italiane. 27. Consiglio Nazionale delle Ricerche, Roma, Italy.

Laghi, P., Miserocchi, D. & Valli, M. (2013). Determinazione genetica della presenza delle rane verdi alloctone *Pelophylax ridibundus* e *Pelophylax kurtmuelleri* (Amphibia, Anura, Ranidae) in due località della Romagna. *Quaderni del Museo di Storia Naturale di Ferrara* 1, 75-78.

Leuenberger, J., Gander, A., Schmidt, B.R. & Perrin, N. (2014). Are invasive marsh frogs (*Pelophylax ridibundus*) replacing the native *P. lessonae*/*P. esculentus* hybridogenetic complex in Western Europe? Genetic evidence from a field study. *Conservation Genetics* 15, 869-878. Doi: 10.1007/s10592-014-0585-0

Librado, P. & Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25, 1451-1452. Doi: 10.1093/bioinformatics/btp187

Meyerson, L.A. & Mooney, H.A. (2007). Invasive alien species in an era of globalization. *Frontiers in Ecology and the Environment* 5, 199-208. DOI: 10.1890/1540-9295(2007)5[199:IASIAE]2.0.CO;2

Mooney, H.A. & Cleland. E.E. (2001). The evolutionary impact of invasive species. *Proceeding of the National Academy of Sciences* 9, 5446-5451. Doi: 10.1073/pnas.091093398

Múrias dos Santos, A., Cabezas, M.P., Tavares, A.I., Xavier, R. & Branco, M. (2015). tcsBU: a tool to extend TCS network layout and visualization. *Bioinformatics* 32, 627-628. Doi: 10.1093/bioinformatics/btv636

Pagano, A., Joly, P., Plénet, S., Lehman, A. & Grolet, O. (2001b). Breeding habitat partitioning in the *Rana esculenta* complex: the intermediate niche hypothesis supported. *Ecoscience* 8, 294-300. Doi: 10.1080/11956860.2001.11682656

Pasqualini, V. (2013). Implicazioni eco-evolutive e conservazionistiche dell'introduzione di rane verdi del complesso *Rana* (*Pelophylax*) *esculenta* in Italia. Dissertation, University of Tuscia.

Plénet, S., Joly, P., Hervant, F., Fromont, E. & Grolet, O. (2005). Are hybridogenetic complexes structured by habitat in water frogs? *Journal of Evolutionary Biology* 18, 1575-1586. Doi: 10.1111/j.1420-9101.2005.00961.x

Plötner, J. (2005). Die westpaläarktischen Wasserfrösche-Von Märtyrern der Wissenschaft zur biologischen Sensation. Laurenti Verlag, Bielefeld, Germany.

Plötner, J., Uzzell, T., Beerli, P., Spolsky, C., Ohst, T., Litvinchuk, S.N., Guex, G.D., Reyer, H.U. & Hotz, H. (2008). Widespread unidirectional transfer of mitochondrial DNA: a case in western Palaearctic water frogs. *Journal of Evolutionary Biology* 21, 668-681. Doi: 10.1111/j.1420-9101.2008.01527.x

Pritchard, J.K., Stephens, M. & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics* 155, 945-959.

Rambaut, A. (2009). Molecular evolution, phylogenetics and epidemiology, FigTree v.1.3.1.

Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Huelsenbeck, J.P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* 61, 539-542. Doi: 10.1093/sysbio/sys029

Schmeller, D.S., Pagano, A., Plénet, S. & Veith, M. (2007). Introducing water frogs–Is there a risk for indigenous species in France? *Comptes Rendus Biologies* 330, 684-690. Doi: 10.1016/j.crvi.2007.04.005

Schmidt, D.S., Bond, N.R., Adams, M. & Hughes, J.M. (2011). Cytonuclear evidence for hybridogenetic reproduction in natural populations of the Australian carp gudgeon (Hypseleotris: Eleotridae). *Molecular Ecology* 20, 3367-3380. Doi: 10.1111/j.1365-294X.2011.05206.x

Schneider, H. & Sinsch U. (1992). Mating call variation in lake frogs referred to as *Rana ridibunda* Pallas, 1771. *Journal of Zoological Systematics and Evolutionary Research* 30, 297-315. Doi: 10.1111/j.1439-0469.1992.tb00179.x

Schwarz, G.E. (1978). Estimating the dimension of a models. *The Annals of Statistics* 6, 461-464.

Simberloff, D., Martin, J.L., Genovesi, P., Maris, V., Wardle, D.A., Aronson, J., Courchamp, F., Galil, B., Garcìa-Berthou, E., Pascal, M., Pyšek, P., Sousa, R., Tabacchi. E. & Vilà, M. (2013). Impacts of biological invasions: what's what and the way forward. *Trends in Ecology and Evolution* 28, 58-66. Doi: 10.1016/j.tree.2012.07.013

Spolsky, C. & Uzzell, T. (1986). Evolutionary history of the hybridogenetic hybrid frog *Rana esculenta* as deduced from mtDNA analyses. *Molecular Biology and Evolution* 3, 44-56. Doi: 10.1093/oxfordjournals.molbev.a040376

Templeton, A.R., Crandall, K.A. & Sing, C.F. (1992). A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics* 132, 619-633.

Vanni, S. & Nistri, A. (2006). Atlante degli Anfibi e dei Rettili della Toscana. Regione Toscana, Università degli Studi di Firenze, Museo di Storia Naturale. Sezione Zoologica "La Specola", Firenze, 379 pp.

Villesen, P. (2007). FaBox: an online toolbox for fasta sequences. *Molecular Ecology Resources* 7, 965-968. Doi: 10.1111/j.1471-8286.2007.01821.x

Zeisset, I., Rowe, G. & Beebee, T.J. (2000). Polymerase chain reaction primers for microsatellite loci in the north European water frogs *Rana ridibunda* and *R. lessonae*. *Molecular Ecology* 9, 1173-1174. Doi: 10.1046/j.1365-294x.2000.00954-2.x

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