



Far from home: tracing the origin of non-native water frogs (genus *Pelophylax*) in Malta by molecular markers

Petr Papežík · Arnold Sciberras ·
Michal Benovics · Jeffrey Sciberras ·
Alain Deidun · Peter Mikulíček

Received: 26 May 2023 / Accepted: 4 December 2023
© The Author(s) 2024

Abstract One of the most frequently translocated species outside their native range in Europe are water frogs of the genus *Pelophylax*. Recently, water frogs belonging to the same genus have also been recorded on the island of Gozo in Malta. To trace their origin, we genetically examined 17 individuals from three Gozitan localities where water frogs have been recorded recently. We analysed one mitochondrial (NADH dehydrogenase 2, *ND2*) and one nuclear (serum albumin intron 1, *SAI-1*) fragment to identify the geographic origin of the frogs and a set of microsatellite markers to determine their population-genetic structure and the predicted number of source populations. Based on the *ND2* and *SAI-1* markers, the water

frogs on the island of Gozo originate from southern Anatolia, Turkey. According to sequence variation in *ND2*, they were assigned to a *caralitanus* mtDNA clade, which is endemic to southern Anatolia and taxonomically represents either an evolutionary lineage within *P. cf. bedriagae* or a separate species *P. caralitanus*. All Gozo water frogs had only one haplotype in the *ND2* and one allele in the *SAI-1* fragment, indicating a recent and single introduction event. These results are supported by microsatellite analysis, which revealed low genetic variability and the absence of any population-genetic structure, suggesting that Gozo water frogs originate from only one source population.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10530-023-03228-8>.

P. Papežík (✉) · M. Benovics · P. Mikulíček
Department of Zoology, Faculty of Natural Sciences,
Comenius University in Bratislava, Bratislava, Slovakia
e-mail: petr.papezik.upol@gmail.com

A. Sciberras · J. Sciberras
Valleta, Malta

M. Benovics
Department of Botany and Zoology, Faculty of Natural
Sciences, Masaryk University, Brno, Czech Republic

A. Deidun
Department of Geosciences, Maths and Physics Building,
University of Malta, Msida, Malta

Keywords Invasive species · Endemic amphibians ·
Pelophylax cf. bedriagae · Central Mediterranean ·
Anatolia

Introduction

Biological invasions represent a significant threat to autochthonous fauna. Invasive species cause numerous detrimental effects, including the transmission of new pathogens, hybridisation and introgression that might compromise the genetic integrity of native populations, ecological interactions like a higher risk of predation or competition leading to population decline or even the possibility of extinction of native fauna (reviewed by Ricciardi 2013). The rate

of reported and documented invasions has grown in recent decades (Hulme 2009). Potentially invasive species are often transported out of their native range for pet trade, culinary purposes, scientific research or just accidentally along transport avenues (Keller and Lodge 2007; Keller et al. 2011; Howeth et al. 2015; Lockwood et al. 2019). The majority of these introductions are unintended and involve species that are widely distributed and common in their native range (Keller et al. 2011). However, human-mediated introductions can also involve species that are endemic, have a small native range, and are therefore more vulnerable and endangered. In these cases, introduced populations can be considered as an additional demographic and genetic resource to declining populations of the same species within their native range (e.g., Vamberger et al. 2011). On the other hand, populations of these rare species having limited distributions can hold an invasive potential in an introduced environment. These cases are well-documented in lizards (Pupins et al. 2023), snakes (Montes et al. 2022) or amphibians (Holsbeek et al. 2008; Domeneghetti et al. 2013). In this paper, we bring evidence for the geographical origin of non-native water frogs in Malta, which, in their native range, represent a unique evolutionary lineage with limited distribution.

One of the most frequently translocated taxa in Europe are water frogs of the genus *Pelophylax*. Currently, 13 sexual species and three asexual taxa of hybrid origin are recognized in the Western Palearctic (Plötner 2005; Frost 2023). They form three well-supported phylogenetic clades distributed in the western Mediterranean (*perezilsaharicus* clade), most of Europe, eastern Mediterranean and central Asia (*bedriagaelridibundus* clade), and from western to eastern Europe, the western Balkans and the Apennine Peninsula (*lessonaelshqipericus* clade) (Lymberakis et al. 2007; Akin et al. 2010a). The highest genetic diversity in *Pelophylax* water frogs is associated with the Mediterranean region, where several species and genetically diverged lineages (including disputable taxa) are found (Akin et al. 2010a; Plötner et al. 2010). In terms of ecology, water frogs are strongly bonded to water. They prefer various water bodies from slowly-flowing rivers to marshes. They are well-known as voracious predators, feeding on a large variety of invertebrate and vertebrate prey, including other amphibians and congeners (e.g., Balint et al. 2010; Paunović et al. 2010; Özcan et al.

2021; Pille et al. 2021). They also show a significant invasive potential, with numerous species introductions reported from Belgium (Holsbeek et al. 2008, 2010), Britain (García-Paris 1997; Zeisset and Beebe 2003), France (Pagano et al. 2003; Dufresnes et al. 2017a; Doniol-Valcroze et al. 2021), Italy (Domeneghetti et al. 2013; Bellati et al. 2019; Biscconti et al. 2019; Bruni et al. 2020), Portugal (García-Paris 1997), Spain (García-Paris 1997) and Switzerland (Dubey et al. 2014; Dufresnes et al. 2018). Apart from a direct impact on native fauna, non-native water frogs might cause genetic pollution of autochthonous populations via interspecific hybridization and introgression eroding the genetic integrity of local populations and disrupting reproductive modes of hybridogenetic (asexual) complexes (Holsbeek and Jooris 2010; Quilodrán et al. 2015; Dufresnes et al. 2017b).

In Malta, the only indigenous amphibian species is the painted frog, *Discoglossus pictus*. In April 2000 water frog calls were recorded at the locality Ta'Sarraflu on the island of Gozo (36°2'11.86"N, 14°11'56.74"E) for the first time (Sciberras and Schembri 2006a). However, recently, this Ta' Sarraflu population seems to be in rapid decline or might have gone extinct (A. Sciberras, J. Sciberras; pers. obs.). Subsequently, the species was recorded from another two localities in Gozo (Sciberras and Schembri 2006a, b). Moreover, the presence of eggs, tadpoles and froglets at all three introduction sites indicate their successful reproduction. Species identification based on the overall morphology and on bioacoustics assigned water frogs from Gozo to *Pelophylax bedriagae* (Sciberras and Schembri 2006a), which is native to the eastern Mediterranean (Plötner 2005; Akin et al. 2010a). *Pelophylax bedriagae* is a genetically highly variable species that involves several diverged evolutionary lineages with unresolved and debated taxonomy. Some authors (e.g., Akin et al. 2010a; Plötner et al. 2010) assign evolutionary lineages from Anatolia to the taxon named *P. cf. bedriagae*. The names *P. bedriagae* or *P. bedriagae sensu stricto* are then used for populations living in the Levant and Egypt. Some evolutionary lineages originally assigned to *P. cf. bedriagae* have been recently raised to species level, e.g., *Pelophylax cypriensis* (Plötner et al. 2012), *P. caralitanus* (Arikan 1988) and *P. cerigensis* (Beerli et al. 1994).

Several possible modes of water frog introduction to the Maltese Islands were proposed by

Sciberras and Schembri (2006a), although the geographic origin, taxonomic identification of the species based on molecular markers, and the genetic structure of introduced populations were not established at the time. Therefore, the aims of this study were (a) to identify introduced water frogs recorded from the island of Gozo using genetic markers, to reveal (b) their geographic origin and (c) the mode of introduction (the number of introduction events and source populations).

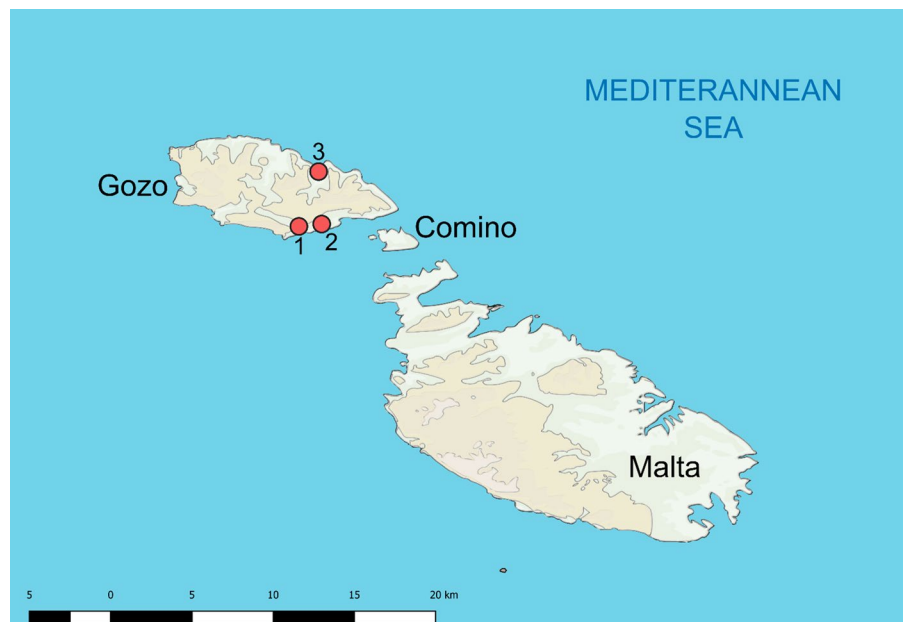
Material and methods

Sampling and localities

In the summer and autumn 2022, we collected 17 samples of *Pelophylax* spp. from three localities on the island of Gozo, Maltese archipelago, in the central Mediterranean (Fig. 1, Table 1). Mgarr ix-Xini is a deeply-incised, tightly meandering gorge located along the south-western coast of the island (36°1'13.77"N, 14°16'15.75"E). Although it does not feature a permanent watercourse, its steep flanking rocky walls do provide substantial shade and shelter from direct solar exposure, such

that vestigial bodies of freshwater and mud persist throughout most of the year along the valley floor. Ghajn il-Papri is a sizeable freshwater pond excavated in clay in the 1990s as a water reservoir, along the southern coast of Gozo (36°1'21.85"N, 14°17'16.49"E). The contiguous area is dominated by the Giant Reed, *Arundo donax* L., and the African tamarisk, *Tamarix africana* Poir., whilst the water itself is dominated by the southern reed-mace, *Typha domingensis* Pers. The pond is also a haunt for the painted frog. Ramla valley runs from the village of Nadur to the sand dune remnants at Ramla l-Hamra, along the north coast of Gozo (36°3'41.85"N, 14°17'1.64"E). During the rainy season, the watercourse within the valley reaches the sea by meandering through the contiguous supralittoral dune remnants and sandy beach. The watercourse is dominated by growths of the Giant Reed and is strongly eutrophic, especially during the dry season, as a result of the intense agricultural activity supported within the same valley. These localities are isolated from each other and are not connected by any water source. The straight geographic distances between the sites are 1.5 km (Mgarr ix-Xini—Ghajn il-Papri), 4.4 km (Ghajn il-Papri—Ramla valley) and 4.7 km (Mgarr ix-Xini—Ramla valley).

Fig. 1 A map of sampled localities in the Gozo Island. 1—Mgarr ix-Xini, 2—Ghajn il-Papri and 3—Ramla valley



For genetic analyses, a toe clip was collected from each individual. Tissue samples were preserved in 96% ethanol and stored at -25°C .

Lab work and sequence alignment

Genomic DNA was extracted from toe clips using NucleoSpin® Tissue kit (Macherey–Nagel, Düren, Germany) following the manufacturer's protocol.

One mitochondrial (NADH dehydrogenase 2, *ND2*) and one nuclear (serum albumin intron 1, *SAI-1*) marker were used to find out the origin of water frogs in Gozo. These markers were chosen because their sequences are the most represented in the GenBank and are therefore accessible for comparison and cover presumed geographical origin of Maltese individuals.

Complete *ND2* gene, 1,038 bp, was amplified using a combination of primers ND2-L1 and ND2-H2 (Plötner et al. 2008). PCR was carried out in a total volume of 10 μl with 5 μl of VWR Red Taq 2 \times mix (VWR, Radnor, PA, USA), 0.2 μl of each primer (10 μM), 3.6 μl of ddH₂O, and 1 μl of DNA. The following PCR program was used: initial denaturation for 2 min at 94°C , followed by 35 cycles consisting of denaturation for 30 s at 94°C , primer annealing for 20 s at 63°C and elongation for 1 min at 72°C , with

a final elongation step for 10 min at 72°C (modified from Plötner et al. 2008).

For amplification of the *SAI-1* fragment, the primers *sai_Intron_1_F* and *sai_Intron_1_R* were used according to Tecker et al. (2017). PCR was carried out in a total volume of 10 μl with 5 μl of VWR Red Taq 2 \times mix, 0.3 μl of each primer (10 μM), 3.4 μl of ddH₂O, and 1 μl of DNA. PCR program was as follows: 3 min of initial denaturation at 96°C , followed by 35 cycles of denaturation for 60 s at 94°C , primer annealing for 60 s at 50°C , and elongation for 2 min at 72°C , with a final elongation for 5 min at 72°C .

Purification of PCR products was performed using the EPPiC Fast (A&A Biotechnology, Gdansk, Poland) following the manufacturer's protocol. Amplicons were sequenced commercially in Macrogen Europe (Amsterdam, Netherlands) with the same primers as for PCR. Newly obtained sequences were combined with those deposited in GenBank (Tables S1, S2). Sequences were manually aligned, checked and translated to detect stop codons using Geneious Prime 2020.2.4 (Biomatters, Auckland, New Zealand). The newly generated sequences were deposited in GenBank under accession numbers OR786315 and OR786316.

Three different datasets of sequences were analysed. First, a *ND2* dataset of Maltese individuals and

Table 1 A list of sampled *Pelophylax* individuals in three localities in the Gozo Island

ID	Sex	Age*	Locality ID	Locality number	Longitude	Latitude
MIK3957	Male	Subadult	Mgarr ix-Xini	1	36.0206	14.2708
MIK3958	Male	Subadult	Mgarr ix-Xini	1	36.0206	14.2708
MIK3959	Male	Subadult	Mgarr ix-Xini	1	36.0206	14.2708
MIK3960	Male	Subadult	Mgarr ix-Xini	1	36.0206	14.2708
MIK3961	Female	Juvenile	Mgarr ix-Xini	1	36.0206	14.2708
MIK3962	Male	Subadult	Mgarr ix-Xini	1	36.0206	14.2708
MIK3963	Male	Subadult	Ghajn il-Papri	2	36.0222	14.2863
MIK3964	Male	Subadult	Ghajn il-Papri	2	36.0222	14.2863
MIK3965	Male	Adult	Ghajn il-Papri	2	36.0222	14.2863
MIK3966	Female	Subadult	Ghajn il-Papri	2	36.0222	14.2863
MIK3967	Female	Adult	Ghajn il-Papri	2	36.0222	14.2863
MIK3968	Male	Subadult	Ghajn il-Papri	2	36.0222	14.2863
MIK3969	Male	Adult	Ramla valley	3	36.0578	14.2842
MIK3970	Male	Subadult	Ramla valley	3	36.0578	14.2842
MIK3971	Female	Adult	Ramla valley	3	36.0578	14.2842
MIK3972	Male	Adult	Ramla valley	3	36.0578	14.2842
MIK3973	Female	Adult	Ramla valley	3	36.0578	14.2842

Locality numbers correspond with those given in Fig. 1

*Age was estimated based on the snout-vent length (SVL) with juveniles < 50 mm, 50 mm < subadults < 100 mm, and adults > 100 mm

western Palaearctic water frog species (*P. perezii*, *P. saharicus*, *P. lessonae*, *P. bergeri*, *P. shqipericus*, *P. epeiroticus*, *P. cretensis*, *P. cerigensis*, *P. caralitanus*, *P. cf. bedriagae*, *P. bedriagae*, *P. kurtmuelleri*, *P. ridibundus*) was used to confirm species identification of water frogs from Gozo. An eastern Palearctic species *P. plancyi* were used as an outgroup. Next, a *ND2* dataset comprising only Maltese individuals and GenBank sequences of *P. bedriagae*, *P. cf. bedriagae*, *P. caralitanus* and *P. cerigensis* was used to infer the geographic origin of the Gozo frogs. Finally, a similar dataset was prepared also for *SAI-1* sequences. However, we did not use *SAI-1* marker to reconstruct phylogenetic relationships in the whole genus since the species-specific variation in the intron resulted in conflicting topologies (see Dubey and Dufresnes 2017; Vucić et al. 2018).

Phylogenetic analyses

Phylogenetic relationships were then inferred using maximum-likelihood (ML) and Bayesian interference (BI) by RAxML v. 8.1.12 (Stamatakis 2014) and MrBayes v. 3.2 (Ronquist et al. 2012), respectively. For both analyses, all parameters were a priori set free to simulate a general time reversible (GTR) evolutionary model and not reduce the robustness of the heuristic search. This allowed respective algorithms to select the optimal model for DNA evolution over the initial search period. For coding regions (i.e., *ND2*) the sequence data were treated as partitioned, computing the optimal substitution model individually for each position within a codon. The best ML tree with the highest likelihood was selected from 100 generated trees. The clade support in the ML was assessed by 1,000 bootstrap pseudoreplicates. The BI analysis was set as follows: two separate runs with four chains in each run, 10 million generations sampled every 100 generations. The convergence of both runs was confirmed by the average standard deviation of split frequencies and potential scale reduction factor diagnostics ($P < 0.01$). The first 30% of trees were discarded as the burn-in after inspection for stationarity of log-likelihood scores of sampled trees in Tracer v. 1.7 (Rambaut et al. 2018). A consensus tree was constructed from the post-burn-in samples.

Analyses of microsatellites

Altogether, 20 microsatellite loci were tested to infer the population-genetic structure of Gozo water frogs. Selected loci, originally designed for *P. ridibundus*, *P. lessonae* and *P. perezii*, were amplified in three multiplex PCRs. Multiplex 1: RICA5, RICA18 (Garner et al. 2000); Rrid059A, Rrid082A (Hotz et al. 2001); Res22 (Zeisset et al. 2000) and Re1Caga10, Re2Caga3, CA1b6 (Arioli 2007). Multiplex 2: RICA2a34, Rrid064A, ReGA1a23, RICA1a27, Rrid135A (Christiansen and Reyer 2009); Rrid013A (Hotz et al. 2001) and Pper3.22, Pper4.7 (Sánchez-Montes et al. 2016). Multiplex 3: RICA1b5 (Garner et al. 2000); Ga1a19 (Arioli 2007); Res17 (Zeisset et al. 2000) and Rrid169A (Christiansen and Reyer 2009).

PCR reactions were performed in a total volume of 10 μ l and consist of 5 μ l of Qiagen Microsatellite PCR Master mix (Qiagen, Hilden, Germany), 0.1 μ l of each primer (10 μ M), 3 μ l of ddH₂O and 1 μ l of DNA. PCR program was modified from Christiansen and Reyer (2009): 5 min of initial denaturation at 95 °C followed by 30 cycles of denaturation for 30 s at 95 °C, 58 °C for 90 s and 72 °C for 1 min, with a final extension at 60 °C for 30 min. Microsatellite fragments were run on an automated ABI genetic analyser.

The Bayesian clustering implemented in the program Structure 2.3.4 (Pritchard et al. 2000) was used to infer the genetic structure of water frog populations. The analysis was based on runs of 10⁶ iterations, following a burn-in period of 10⁵ iterations. An admixture and allele-frequencies correlated models were used. The expected number of inferred clusters (K) varied from one to five; for each K five independent runs were carried out to test the accuracy of the results.

A number of different alleles, observed (H_O) and expected (H_E) heterozygosity and the genetic differentiation among populations using F_{ST} statistics were calculated in GenAlEx 6.5 (Peakall and Smouse 2006, 2012). The presence of null alleles in microsatellite data was estimated using the program Micro-Checker 2.2.3 (van Oosterhout et al. 2004).

Results

Phylogenetic analyses

Phylogenetic relationships in the western Palearctic water frogs reconstructed based on 216 sequences of mitochondrial *ND2* gene (alignment length 1,038 bp, $\ln L = -7099.546$ for ML) confirmed species affiliation of Maltese individuals to a clade comprising *P. cf. bedriagae*, *P. caralitanus* and *P. cerigensis* with a branch support 96/1 (bootstrap support value from ML/posterior probability from BI; Fig. 2). These taxa were used in subsequent phylogenetic analyses based on both *ND2* and *SAI-1* to identify the geographic origin of water frogs introduced to Gozo.

By combining newly produced Maltese sequences and available GenBank data of *P. bedriagae*, *P. cf. bedriagae*, *P. caralitanus* and *P. cerigensis*, we obtained a dataset of 114 *ND2* sequences (alignment length 1,038 bp). Both ML and BI trees had identical topologies regarding the major clades with $\ln L = -3788.296$. Maltese individuals formed a clade with GenBank sequences from southern Anatolia and the Island of Ikaria (Fig. 3, Fig. S1) with a branch support 88/1. The clade represents sequences of *P. caralitanus* sensu Akin et al. (2010a) and Plötner et al. (2010). All individuals from the Gozo Island possessed a single *ND2* haplotype.

In the *SAI-1* dataset, we analysed 85 sequences (alignment length 1,055 bp). Major clades had identical topologies in both ML and BI trees with $\ln L = -1766.168$. In concordance with mtDNA, water frogs from Gozo were assigned to a clade with sequences from southern Anatolia and Cyprus (Fig. 4, Fig. S2) with a branch support 60/0.85. Unlike the mtDNA, the *SAI-1* clade comprising sequences from Malta and sequences of *P. caralitanus* from southern Anatolia was paraphyletic to other Anatolian sequences assigned to *P. caralitanus*. In the *SAI-1* fragment, all Gozo water frogs possessed a single allele.

Population-genetic structure

Of the 20 microsatellite loci tested, eight did not amplify or produced difficult-to-interpret allelic patterns (RICA5, Rrid059A, Res22, Rrid064A, ReGA1a23, RICA1a27, Rrid135A, Re1Caga10). Of the remaining 12 loci, five were monomorphic

(RICA18, Rrid013A, Rrid169A, RICA1b5, Res17) and seven were polymorphic (Rrid082A, CA1b6, Re2Caga3, Pper3.22, Pper4.7, RICA2a34, Gal1a19). Null alleles were not detected in any of these polymorphic loci.

To investigate the population-genetic structure of water frogs from Gozo, we performed Bayesian clustering implemented in Structure, calculated F_{ST} statistics, compared allele frequencies and observed (H_O) and expected (H_E) heterozygosity. Posterior probabilities (log likelihood, L_n) for K from one to five were virtually identical (ranging from -167.1 to -168.8). When $K=2, 3, 4$, and 5 were assumed, the proportion of the individual assigned to each cluster was always symmetrical (Fig. S3). This symmetrical proportion shows the absence of a real population structure, suggesting that the Gozo water frogs originate from the same source population. F_{ST} statistics between the Gozo populations were zero (Table S3), corresponding to the absence of population-genetic structure estimated in Structure.

The values of genetic variability were relatively low. The mean number of different alleles in 12 amplified microsatellite loci ranged from 1.667 to 1.750, H_O from 0.317 to 0.347, and H_E from 0.252 to 0.282 (Table 2).

Discussion

The geographic origin of Maltese water frogs

In this study, we analysed the genetic variability of mitochondrial gene *ND2* and a nuclear intron *SAI-1* in 17 individuals of introduced water frogs sampled from three populations on the island of Gozo in the Maltese archipelago (Fig. 5). By comparing with additional *ND2* and *SAI-1* sequences from GenBank, we elucidated the geographic origin of these non-native frogs and their taxonomic position. Using microsatellite markers, we determined their population-genetic structure and thus revealed the number of source populations.

Our phylogenetic analyses assigned water frogs recorded from Gozo to the same cluster as samples from southern Anatolia, Cyprus and the Aegean Greek Island of Ikaria. However, the *ND2* sequence from Ikaria represents a different haplotype than the Maltese samples, and therefore we hypothesize that

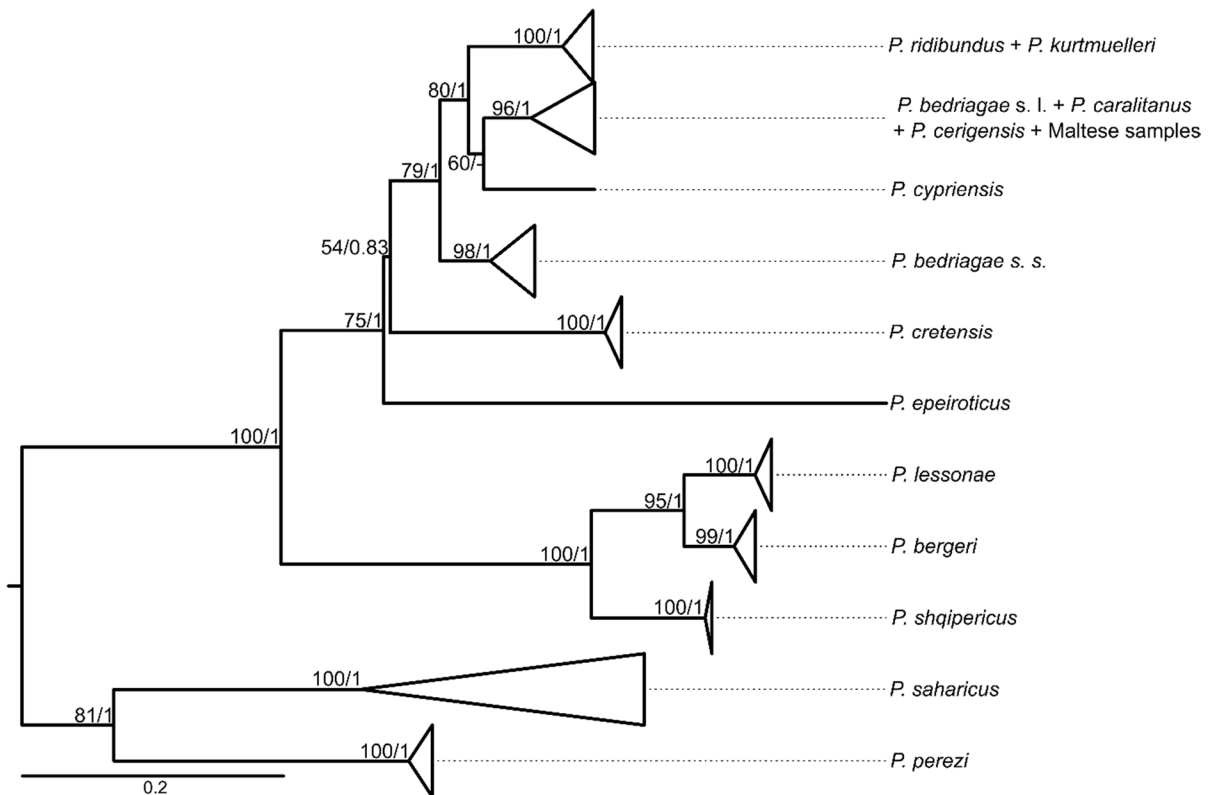


Fig. 2 Maximum likelihood (ML) tree and phylogenetic relationships of the *Pelophylax* species. A sequence of *P. plancyi* was included as an outgroup (not shown). A position of Maltese samples is indicated. The Bayesian interference (BI) tree

showed the same topology of the major clades as the ML tree. Numbers above the branches indicate bootstrap values (ML) and the posterior probabilities values (BI)

Ikaria can be ruled out as a source population for the introduction of water frogs to Gozo. In the *SAI-1* marker, Gozitan water frogs shared the same allele with individuals from both Cyprus and southern Anatolia. Therefore, both regions may be a likely source of introduction. However, the occurrence of Anatolian water frogs in Cyprus strongly suggests that these frogs were introduced to Cyprus unintentionally and recently by humans, and thus are not native (Plötner et al. 2015). Considering this hypothesis, we cannot, therefore, decide whether *Pelophylax* frogs were introduced to Gozo directly from southern Anatolia or via Cyprus.

The occurrence of water frogs on the island of Gozo appears to be the result of a recent and single introduction. This hypothesis is supported by the zero genetic variability of the sampled individuals at *ND2* and *SAI-1*, and low genetic variability in

microsatellites. Specifically, all the samples from the three Gozo sites had one haplotype in *ND2* and one allele at *SAI-1*, suggesting that the introduced frogs may have originated from a small group of a few individuals, from one or several geographically interconnected sites, and may have been introduced to Gozo only once and recently. This hypothesis is further supported by results from the analysis of microsatellites, which revealed the absence of any population-genetic structure and any genetic differentiation within the Gozo sites. If the introduction from the East had involved many individuals, from several localities and over repeated occasions, we would expect a higher genetic variability of Maltese frogs for all the genetic markers and also a significant differentiation amongst the populations (Allendorf and Lundquist 2003).

According to published data, water frogs were introduced to Ta'Saraflu on the island of Gozo

Table 2 Genetic variability in 12 microsatellite loci in water frog populations from the Gozo Island

Population	N	N_A	H_O	H_E
Mgarr ix-Xini	6	1.750 ± 0.218	0.319 ± 0.097	0.282 ± 0.080
Ghajn il-Papri	6	1.667 ± 0.188	0.347 ± 0.099	0.277 ± 0.076
Ramla valley	5	1.667 ± 0.188	0.317 ± 0.097	0.252 ± 0.072

N number of analysed individuals, N_A mean number of different alleles, H_O observed heterozygosity, H_E unbiased expected heterozygosity

probably in the early 1990's and they were first recorded in April 2000 (Sciberras and Schembri 2006a, b). In addition, mating calls were also recorded in hotel resorts on the island of Malta (Sciberras and Schembri 2006a) and at several other localities in both Gozo and Malta. However, the status of these populations remains questionable as they were located in private properties with limited access to the sites (A. Sciberras, J. Sciberras; pers. obs.). The exact mode of their introduction, as well as the individual abundance within the same populations and their survival strategies remained unclear at the time. However, several possible hypotheses were proposed, including the introduction by local farmers or through deliberate releases from purchases made at Maltese street markets (Sciberras and Schembri 2006a).

No official importation data for *Pelophylax* species to the Maltese islands could be retrieved from archives held by Maltese national environmental authorities. However, there are anecdotal reports from local farmers based within at the Ta' Sarraflu area in Gozo suggesting a deliberate introduction of tadpoles of the species imported from Turkey. This 'deliberate release' hypothesis is further supported by the fact that the three locations inhabited by introduced water frogs are not hydrodynamically connected to each other, with one of them consisting of a man-made reservoir, with water frogs, being aquatic amphibians, not being able, unlike the native painted frogs, to disperse across arid areas without human intervention. If confirmed, this deliberate introduction is similar to the one observed for a number of non-indigenous crustaceans (e.g., *Procambarus clarkii*) within freshwater sites around the Maltese Islands (Deidun et al. 2018).

Which species of water frogs live in Malta?

Water frogs of the genus *Pelophylax* represent morphologically a uniform group and their precise species identification often relies on an analysis of bioacoustics (mating calls of males) and of molecular markers (Plötner et al. 2005). Bioacoustics are commonly used also for the description of new species (Schneider et al. 1984, 1993; Schneider and Sinsch 1992) or even for hybrid identification (Schneider et al. 1984). However, using solely bioacoustic traits might not be powerful enough to reconstruct species evolutionary histories or to distinguish between genetically diverged but closely related species (e.g., *P. lessonae* and *P. shqipericus* in Sinsch and Schneider 1996). On the other hand, molecular markers can easily distinguish among various water frog species or can resolve phylogenetic relationships amongst them (e.g., Lymberakis et al. 2007; Akin et al. 2010a).

Based on our results, Maltese individuals were assigned to the mitochondrial clade comprising *P. cf. bedriagae*, supporting their eastern Mediterranean origin proposed on the basis of bioacoustics by Prof. U. Sinsch (Sciberras and Schembri 2006a). *Pelophylax cf. bedriagae* comprises several diverged evolutionary lineages. Some of these lineages were recently described as separate species, e.g., *P. cypriensis*, *P. cerigensis* or *P. caralitanus* (Akin et al. 2010a; Plötner et al. 2010, 2015), but their taxonomic status is still doubtful and contested. In mtDNA phylogeny, Maltese samples could be unequivocally assigned to the clade *P. caralitanus* (or *P. cf. caralitanus* sensu Akin et al. 2010a and Plötner et al. 2010) (see Fig. 3). *Pelophylax caralitanus* was originally described as a subspecies of *P. ridibundus* (Arikan 1988) and is distributed predominantly in Göller Yöresi (Lake District) in Anatolia, Turkey. Apart from differences in morphometry (Jdeidi et al. 2001), allozymes (Beerli 1994), bioacoustics (Schneider and Sinsch 1999) and mtDNA (Akin et al. 2010a,b), the main trait distinguishing *P. caralitanus* from other water frog taxa living in Anatolia is the presence of prominent orange-coloured mottling of the ventral side of the body (Arikan 1988). Maltese water frogs, however, do not show any sign of this mottling pattern (Fig. 6). A discrepancy between the colour variation of the belly and the *caralitanus*-specific mtDNA haplotypes was also observed in Anatolian populations by Akin et al. (2010b).

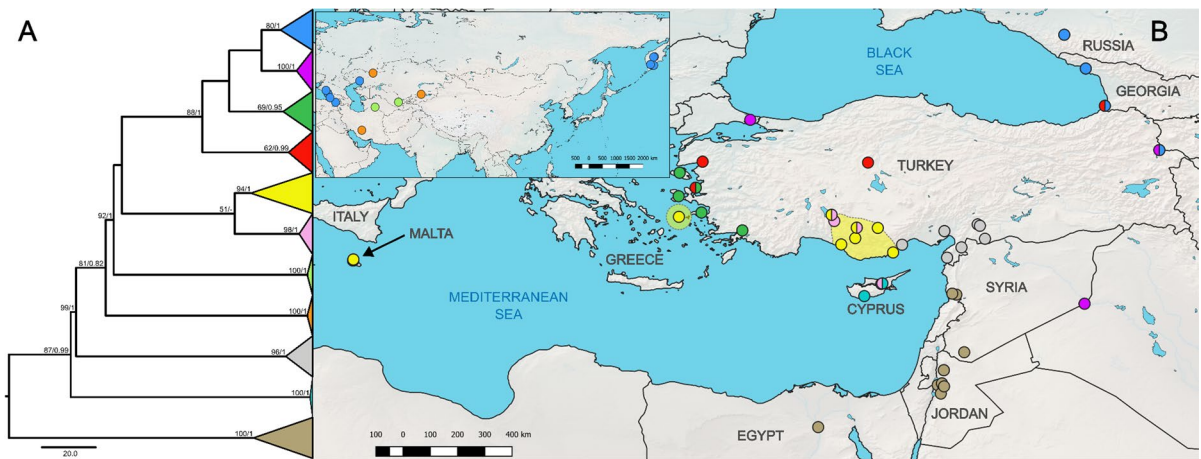


Fig. 3 Phylogenetic relationships (A) and geographic origin (B) of newly obtained *ND2* sequences from the Gozo Island and those already published in GenBank. Maximum likelihood (ML) tree and Bayesian interference (BI) trees showed the same topology of the major clades. Numbers above the branches indicate bootstrap values (ML) and the posterior

probabilities values (BI). The Maltese archipelago is highlighted by an arrow. The presumed geographic origin indicated by the yellow area was created by connecting border localities of the same clade. An inserted map shows the distribution of other clades in Central Asia

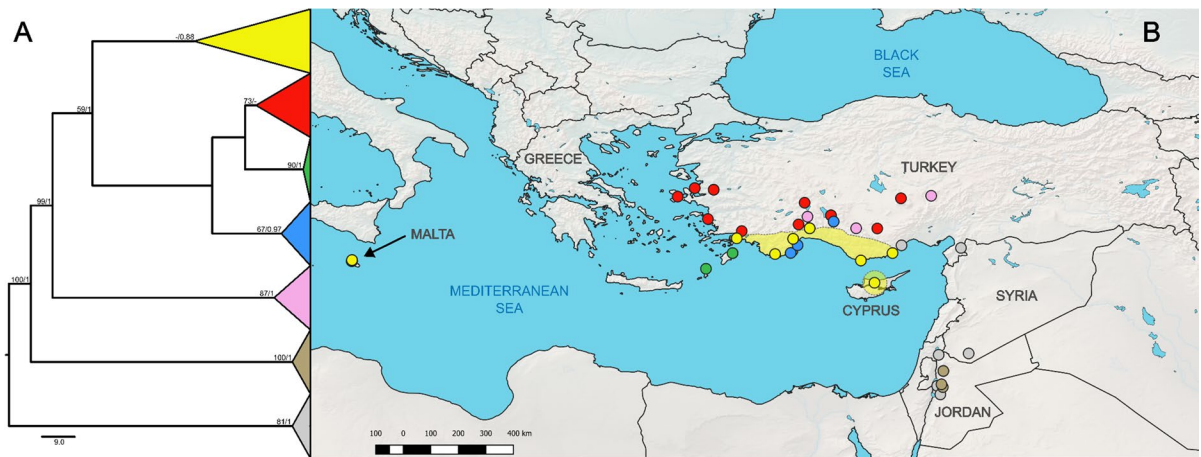


Fig. 4 Phylogenetic relationships (A) and geographic origin (B) of newly obtained *SAI-1* sequences from the Gozo Island and those already published in GenBank. Maximum likelihood (ML) tree and Bayesian interference (BI) trees showed the same topology of the major clades. Numbers above the

branches indicate bootstrap value (ML) and posterior probabilities values (BI). The Maltese archipelago is highlighted by an arrow. The presumed geographic origin indicated by the yellow area was created by connecting border localities of the same clade

In the case of *SAI-1*, the position of Maltese samples in a phylogenetic tree did not correspond to mtDNA. Although Maltese *SAI-1* sequences formed a clade with populations from southern Anatolia and Cyprus, they were phylogenetically different from individuals probably representing *P. caralitanus*

(Fig. 4, Plötner et al. 2015). The observed discrepancy between *ND2* and *SAI-1* phylogenies might be attributed to rapid radiation in a group of eastern Mediterranean water frogs that makes a resolution of phylogenetic relationships based on *SAI-1* sequences difficult (Plötner et al. 2009). It could be

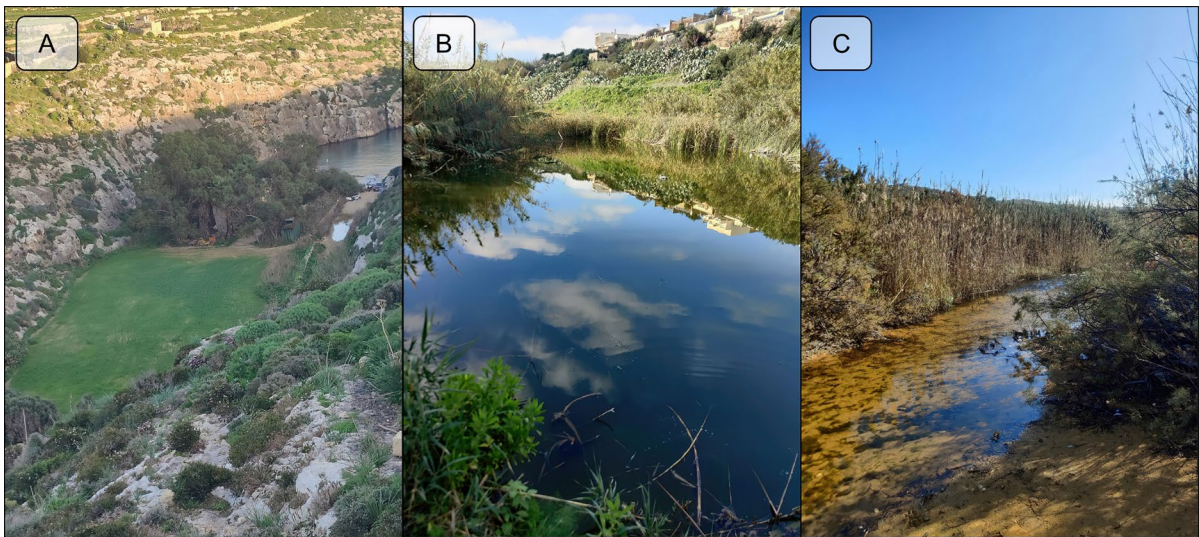


Fig. 5 Sampled localities in the Gozo Island, Malta. **A** Mgarr ix-Xini, **B** Ghajn il-Papri, **C** Ramla valley. Photos by A. Sciberras

also assumed that the discrepancy between mtDNA and nuDNA phylogeny (as well as between mtDNA and morphology as pointed out above) might be the result of shared interspecific polymorphism caused by hybridization or an incomplete lineage sorting (Toews and Brelsford 2012).

Thus, the unresolved phylogenetic relationships of Anatolian water frog populations (or *P. cf. bedriagae* in general) and the questionable taxonomic status of various evolutionary lineages in Anatolia make the definitive taxonomic identification of introduced water frogs in Gozo difficult. Based on mtDNA (*ND2*) and nuDNA (*SAI-1*) markers, as well as on bioacoustics (Sciberras and Schembri 2006a,b), it is clear that the water frogs in Gozo belong to *P. cf. bedriagae* group. Considering only the variation in the mitochondrial gene *ND2*, these frogs are included in the *caralitanus* cluster, regardless of whether we consider this clade as a separate species *P. caralitanus* or just an evolutionary lineage within *P. cf. bedriagae*.

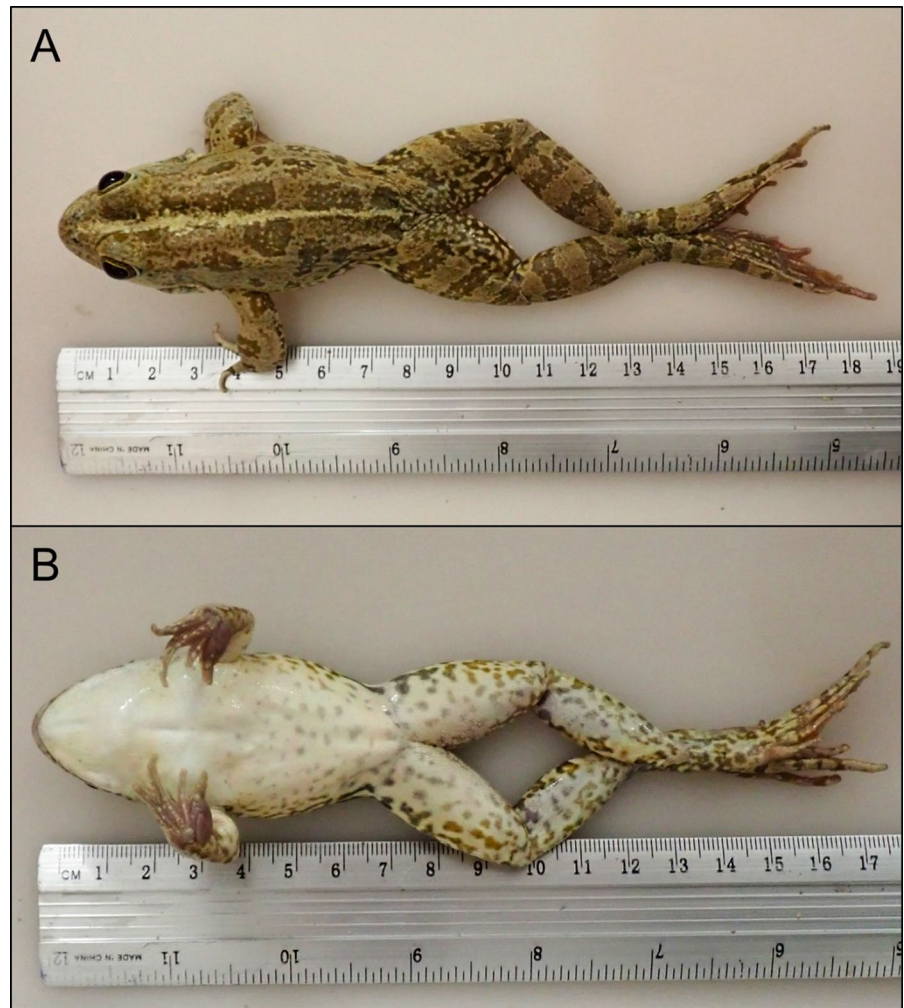
Anatolian endemic as an invasive species

There are numerous reports of the introduction of water frogs of the genus *Pelophylax* in Europe (see e.g., Holsbeek and Jooris 2010; Bellati et al. 2019). These species exhibit, in fact, a potential to act as invasive species in regions where they have been

introduced (Kumschick et al. 2017). Several detrimental effects on autochthonous (batracho)fauna were described, including a direct predatory pressure (Pille et al. 2021), the transfer of parasites, a competition for food sources and habitats (Schmeller et al. 2007; Quilodrán et al. 2015) or hybridization with local *Pelophylax* species leading to introgression and disruption of unique reproductive modes associated with asexual (hybridogenetic) reproduction (Vorburger and Reyer 2003; Holsbeek and Jooris 2010; Quilodrán et al. 2015; Dufresnes and Dubey 2017). However, most of these cases included species with vast native distributions (Holsbeek et al. 2010), while introductions of endemic species having restricted distributions to new regions are scarce. A rare example of such a type of introduction is represented by the introduction of *P. shqipericus* (endemic to southern Montenegro and an Adriatic part of Albania) to Umbria, Italy, with the ecological impacts of such an introduction on native fauna being as yet unknown (Domeneghetti et al. 2013).

Introduced populations of endemic and rare species might be viewed as a back-up for declining populations in their original range (e.g., Vamberger et al. 2011). In Malta, however, water frogs have a detrimental ecological impact on local (batracho)fauna as was already documented by Sciberras and Schembri (2006a), who reported a predatory

Fig. 6 Overall habitus of water frogs from the Gozo Island, Malta. A dorsal (A) and ventral (B) side is shown. Photos by A. Sciberras



behaviour of these frogs upon *Discoglossus pictus*, the only amphibian species native to Malta, or upon endemic shrew subspecies, *Crocidura sicula calypso*. Described feeding ecology and evidence of successful reproduction underline the invasive potential of the whole *Pelophylax* genus and should be a starting point for effective species management to reduce further range expansion of introduced water frogs across the Maltese islands.

Aquatic ecosystems have a highly restricted distribution on the Maltese Islands, a semi-arid archipelago, with this dearth being compounded further by anthropogenic impacts, including an over-extraction of fluvial deposits for agricultural purposes, trampling and vehicular access and contamination of the same

deposits through pesticides, herbicides and other pollutants. Consequently, any heightened competition for space and trophic resources from allochthonous species is an added stressor which is expected to further impinge on the native aquatic ecosystems.

Acknowledgements We are grateful to The Environment and Resources Authority (ERA) of Malta for their effort to find any note concerning possible route of introduction. We would like to thank to Adam Javorčík for editing photographs taken in the field with phone camera and Andrej Ficek for his help in the laboratory with fragmentation analysis. This study was supported by the Slovak Research and Development Agency under the contract APVV-19-0076.

Author contributions PP and PM contributed to the conception and design of the study. AS and JS provided information about the species distribution in Malta and collected biological

material. MB, PM and PP carried out laboratory and statistical analyses. AD contributed to the contextualization of the results in relation to non-native species introduced to Malta and held communication with local authorities. The first draft of the manuscript was written by PP and PM and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding Open access funding provided by The Ministry of Education, Science, Research and Sport of the Slovak Republic in cooperation with Centre for Scientific and Technical Information of the Slovak Republic. This work was supported by the Slovak Research and Development Agency under contract APVV-19-0076.

Data availability The newly generated sequences were deposited in GenBank under accession numbers OR786315 (ND2) and OR786316 (SAI-1).

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Akin Ç, Bilgin CC, Beerli P, Westaway R, Ohst T, Litvinchuk SN, Uzzell T, Bilgin M, Hotz H, Guex G-D, Plötner J (2010a) Phylogeographic patterns of genetic diversity in eastern Mediterranean water frogs were determined by geological processes and climate change in the Late Cenozoic. *J Biogeogr* 37:2111–2124. <https://doi.org/10.1111/j.1365-2699.2010.02368.x>
- Akin Ç, Bilgin M, Bilgin CC (2010b) Discordance between ventral colour and mtDNA haplotype in the water frogs *Rana (ridibunda) caralitana*, 1988 Arikian. *Amphibia-Reptilia* 31:9–20. <https://doi.org/10.1163/156853810790457867>
- Allendorf FW, Lundquist LL (2003) Introduction: population biology, evolution, and control of invasive species. *Conserv Biol* 17:24–30. <https://doi.org/10.1046/j.1523-1739.2003.02365.x>
- Arikian H (1988) On a new form *Rana ridibunda* (Anura: Ranidae) from Turkey. *Istanbul Universitesi Fen Fakültesi Mecmuası* 53:81–87
- Arioli, M (2007): Reproductive patterns and population genetics in pure hybridogenetic water frog populations. Erlangung der naturwissenschaftlichen Doktorwürde (Dr. sc. nat.) vorgelegt der Mathematisch-naturwissenschaftlichen Fakultät der Universität Zürich.
- Balint N, Andrei C, Ianc R, Ursuț A (2010) On the diet of the *Pelophylax ridibundus* (Anura, Ranidae) in Țicleni, Romania. *South Western J Horticult Biol Environ* 1:56–66
- Beerli P, Hotz H, Tunner H, Heppich S, Uzzell T (1994) Two new water frog species from the Aegean islands Crete and Karpathos (Amphibia, Salientia, Ranidae). *Notulae Naturae* 470:1–9
- Bellati A, Bassu L, Nulchis V, Corti C (2019) Detection of alien *Pelophylax* species in Sardinia (western Mediterranean, Italy). *BioInvasions Records* 8:8–25. <https://doi.org/10.3391/bir.2019.8.1.02>
- Bisconti R, Martino G, Chiocchio A, Siclari A, Canestrelli D (2019) Balkan marsh frog *Pelophylax kurtmuelleri* (Gayda, 1940) introduced in the Aspromonte National Park, southern Italy. *BioInvasions Records* 8:26–33. <https://doi.org/10.3391/bir.2019.8.1.03>
- Bruni G, Mirabella I, Domeneghetti D, Fasola M, Bellati A (2020) Will there be a second extinction? Molecular identification of multiple alien water frog (*Pelophylax ridibundus* sensu lato) in Tuscany, Central Italy, reveals genetic pollution within a unique hybridogenetic system. *Herpetol J* 30:147–158. <https://doi.org/10.33256/hj30.3.147158>
- Christiansen D, Reyer H-U (2009) From clonal to sexual hybrids: genetic recombination via triploids in all-hybrid populations of water frogs. *Evolution* 63:1754–1768. <https://doi.org/10.1111/j.1558-5646.2009.00673.x>
- Deidun A, Sciberras A, Formosa J, Zava B, Insacco G, Corsini-Foka M, Crandall KA (2018) Invasion by non-indigenous freshwater decapods of Malta and Sicily, central Mediterranean Sea. *J Crustac Biol* 38:748–753. <https://doi.org/10.1093/jcobiol/ruy076>
- 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
- Doniol-Valcroze P, Mazepa G, Grimal F, Sourrouille P, Perrin N, Litvinchuk SN, Crochet P-A (2021) Discovery of a *Pelophylax saharicus* (Anura, Ranidae) population in Southern France: a new potentially invasive species of water frogs in Europe. *Amphibia-Reptilia* 42:427–442. <https://doi.org/10.1163/15685381-bja10066>
- Dubey S, Dufresnes C (2017) An extinct vertebrate preserved by its living hybridogenetic descendant. *Sci Rep* 7:12768. <https://doi.org/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. *Biol J Lin Soc* 112:442–449. <https://doi.org/10.1111/bij.12283>
- Dufresnes C, Denoël M, di Santo L, Dubey S (2017a) Multiple uprising invasions of *Pelophylax* water frogs, potentially inducing a new hybridogenetic complex. *Sci Rep* 6:6506. <https://doi.org/10.1038/s41598-017-06655-5>

- 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 frog (*Pelophylax bergeri*) across Western Europe unraveled by multilocus phylogeography. *Biol Invasions* 19:1407–1420. <https://doi.org/10.1007/s10530-016-1359-z>
- Dufresnes C, Leuenberger J, Amrhein V, Bühler C, Thiébaud J, Bohnenstengel T, Dubey S (2018) Invasion genetics of marsh frog (*Pelophylax ridibundus* sensu lato) in Switzerland. *Biol J Lin Soc* 123:402–410. <https://doi.org/10.1093/biolinnean/blx140>
- Frost DR (2023) Amphibian Species of the World: An Online Reference. Version 6.2 (December 26, 2023). Electronic Database accessible at <https://amphibiansoftheworld.amnh.org/index.php>. American Museum of Natural History, New York, USA. <https://doi.org/10.5531/db.vz.0001>
- García-París M, Rana perezi Seoane (1885). In: Gasc JP, Cabela A, Crnobrnja-Isailovic J, Dolmen D, Grossenbacher K, Haffner P, Lescure P, Martens H, Martínez-Rica JP, Maurin H, Oliveira ME, Sofianidou TS, Veith M, Zuiderwijk A (eds) Atlas of amphibians and reptiles in Europe. Societas Europaea Herpetologica et Muséum National d'Histoire Naturelle, Paris, pp 152–153
- Garner TWJ, Gautschi B, Röthlisberger S, Reyer H-U (2000) A set of CA repeat microsatellite markers derived from the pool frog, *Rana lessonae*. *Mol Ecol* 9:2173–2174. <https://doi.org/10.1046/j.1365-294X.2000.105311.x>
- Holsbeek G, Jooris R (2010) Potential impact of genome exclusion by alien species in the hybridogenetic water frogs (*Pelophylax esculentus* complex). *Biol Invasions* 12:1–13. <https://doi.org/10.1007/s10530-009-9427-2>
- Holsbeek G, Mergeay J, Hotz H, Plötner J, Volckaert FAM, 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. *Mol Ecol* 17:5023–5035. <https://doi.org/10.1111/j.1365-294X.2008.03984.x>
- Holsbeek G, Mergeay J, Volckaert FAM, De Meester L (2010) Genetic detection of multiple exotic water frog species in Belgium illustrates the need for monitoring and immediate action. *Biol Invasions* 12:1459–1463. <https://doi.org/10.1007/s10530-009-9570-9>
- Hotz H, Uzzell T, Guex G-D, Alpers D, Semlitsch RD, Beerli P (2001) Microsatellites: a tool for evolutionary genetic studies of western Palearctic water frogs. *Mitteilung Aus Dem Museum Für Naturkunde Berlin, Zoologische Reihe* 77:43–50. <https://doi.org/10.1002/mmzn.20010770108>
- Howeth JG, Gantz CA, Angermeier PL, Frimpong EA, Hoff MH, Keller RP, Mandrak NE, Marchetti MP, Olden JD, Romagosa CM, Lodge DM (2015) Predicting invasiveness of species in trade: climate match, trophic guild and fecundity influence establishment and impact of non-native freshwater fishes. *Divers Distrib* 22:148–160. <https://doi.org/10.1111/ddi.12391>
- Hulme PE (2009) Trade, transport and trouble: managing invasive species pathways in an era of globalization. *J Appl Ecol* 46:10–18. <https://doi.org/10.1111/j.1365-2664.2008.01600.x>
- Jdeidi T, Bilgin CC, Kence M (2001) New localities extend the range of *Rana bedriagae* caralitana Arikani, 1988 (Anura: Ranidae) further west and suggest specific status. *Turkish J Zool* 25:152–158
- Keller RP, Lodge DM (2007) Species invasions from commerce in live aquatic organisms: problem and possible solutions. *Bioscience* 57:428–436. <https://doi.org/10.1641/B570509>
- Keller RP, Geist J, Jeschke JM, Kühn I (2011) Invasive species in Europe: ecology, status, and policy. *Environ Sci Europe* 23:23. <https://doi.org/10.1186/2190-4715-23-23>
- Kumschick S, Vimercati G, de Villiers FA, Mokhatla MM, Davies SJ, Thorp CJ, Rebelo AD, Measey GJ (2017) Impact assessment with different scoring tools: how well do alien amphibian assessment match? *NeoBiota* 33:52–66. <https://doi.org/10.3897/neobiota.33.10376>
- Lockwood JL, Welbourne DJ, Romagosa CM, Cassey P, Mandrak NE, Strecker A, Leung B, Stringham OC, Udell B, Episcopio-Sturgeon DJ, Tlustý MF, Sinclair J, Springborn RM, Pienaar EF, Rhyne AL, Keller RP (2019) When pets become pests: the role of the exotic pet trade in producing invasive vertebrate animals. *Front Ecol Environ* 17:323–330. <https://doi.org/10.1002/fee.2059>
- Lymberakis P, Poulakakis N, Manthou G, Tsigonopoulos CS, Magoulas A, Mylonas M (2007) Mitochondrial phylogeography of *Rana* (*Pelophylax*) populations in the Eastern Mediterranean region. *Mol Phylogenet Evol* 44:115–125. <https://doi.org/10.1016/j.ympev.2007.03.009>
- Montes E, Kraus F, Chergui B, Pleguezuelos JM (2022) Collapse of the endemic lizard *Podarcis pityusensis* on the island of Ibiza mediated by an invasive snake. *Cur Zool* 68:295–303. <https://doi.org/10.1093/cz/zoab022>
- Özcan L, Yorulmaz B, Ayaz D (2021) Data on food composition of the Levant marsh frog (*Pelophylax bedriagae*) in Southwestern Anatolia. *North-Western J Zool* 17:227–231
- Pagano A, Dubois A, Lesbarrères D, Lodé T (2003) Frog alien species: a way for genetic invasion? *C R Biol* 326:85–92. [https://doi.org/10.1016/S1631-0691\(03\)00043-X](https://doi.org/10.1016/S1631-0691(03)00043-X)
- Paunović A, Bjelić-Čabrilo O, Šimić S (2010) The diet of water frogs (*Pelophylax esculentus* "complex") from the Petrovaradinski Rit marsh (Serbia). *Archiv Biol Sci* 62:799–806. <https://doi.org/10.2298/ABS1003797P>
- Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Mol Ecol Notes* 6:288–295
- Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. *Bioinformatics* 28:2537–2539
- Pille F, Pinto L, Denoël M (2021) Predation pressure of invasive marsh frogs: a threat to native amphibians? *Diversity* 13:595. <https://doi.org/10.3390/d13110595>
- Plötner J (2005) Die westpaläarktischen Wasserfrösche: von Märtyrern der Wissenschaft zur biologischen Sensation. Bielfeld, Laurenti
- Plötner J, Uzzell T, Beerli P, Spolsky C, Ohst T, Litvinchuk SN, Guex G-D, Reyer H-U, Hotz H (2008) Widespread unidirectional transfer of mitochondrial DNA: a case in western Palearctic water frogs. *J Evol Biol* 21:668–681. <https://doi.org/10.1111/j.1420-9101.2008.01527.x>
- Plötner J, Köhler F, Uzzell T, Beerli P, Schreiber R, Guex G-D, Hotz H (2009) Evolution of serum albumin intron-1 shaped by a 5' truncated non-long terminal repeat retrotransposon in western Palearctic water frogs

- (Neobatrachia). *Mol Phylogenet Evol* 53:784–791. <https://doi.org/10.1016/j.ympev.2009.07.037>
- Plötner J, Uzzell T, Beerli P, Akin Ç, Bilgin CC, Haefeli C, Ohst T, Köhler F, Schreiber R, Guex G-D, Litvinchuk SN, Westaway R, Reyer H-U, Pruvost N, Hotz H (2010) Genetic divergence and evolution of reproductive isolation in eastern mediterranean water frogs. In: Glaubrecht M (ed) *Evolution in action. Case studies in adaptive radiation, speciation and the origin of biodiversity*. Springer, Heidelberg, pp 373–403
- Plötner J, Baier F, Akin Ç, Mazepa G, Schreiber R, Beerli P, Litvinchuk SN, Bilgin CC, Borkin L, Uzzell T (2012) Genetic data reveal that water frogs of Cyprus (genus *Pelophylax*) are an endemic species of Messinian origin. *Zoosyst Evolut* 88:261–283. <https://doi.org/10.1002/zoos.201200021>
- Plötner J, Akin Pekşen Ç, Baier F, Uzzell T, Bilgin CC (2015) Genetic evidence for human-mediated introduction of Anatolian water frogs (*Pelophylax* cf. *bedriagae*) to Cyprus (Amphibia: Ranidae). *Zool Middle East* 61:125–132. <https://doi.org/10.1080/09397140.2015.1027495>
- Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics* 155:945–959. <https://doi.org/10.1093/genetics/155.2.945>
- Pupins M, Martinez-Silvestre A, Arribas O, Čeiņans A, Kirjulina M (2023) First record of *Scinax ruber*, *Podarcis siculus*, *Podarcis ionicus* and their parasites in Latvia: fruit trade is an intercontinental alien herpetofauna and parasitofauna invasion vector into Europe. *BioInvasions Records* 12:321–329. <https://doi.org/10.3391/bir.2023.12.1.29>
- Quilodrán CS, Montoya-Burgos JI, Currat M (2015) Modelling interspecific hybridization with genome exclusion to identify conservation actions: the case of native and invasive *Pelophylax* waterfrogs. *Evol Appl* 8:199–210. <https://doi.org/10.1111/eva.12245>
- Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA (2018) Posterior summarization in bayesian phylogenetics using tracer 1.7. *Syst Biol* 67:901–904. <https://doi.org/10.1093/sysbio/syy032>
- Ricciardi A (2013) Invasive Species. In: Leemans R (ed) *Ecological systems*. Springer, New York, pp 161–168. https://doi.org/10.1007/978-1-4614-5755-8_10
- Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst Biol* 61:539–542. <https://doi.org/10.1093/sysbio/sys029>
- Sánchez-Montes G, Recuero E, Gutiérrez-Rodríguez J, Gomez-Mestre I, Martínez-Solano I (2016) Species assignment in the *Pelophylax ridibundus* x *P. perezi* hybridogenetic complex based on 16 newly characterised microsatellite markers. *Herpetol J* 33:99–108
- Schmeller DS, Pagano A, Plénet S, Veith M (2007) Introducing water frogs—Is there a risk for indigenous species in France? – Y a-t-il un risque pour les espèces indigènes en France ? *CR Biol* 330:684–690. <https://doi.org/10.1016/j.crv.2007.04.005>
- Schneider H, Sinsch U (1992) Mating call variation in lake frogs referred to as *Rana ridibunda* Pallas, 1771. Taxonomic implications. *Zeitschrift Für Zoologische Systematik Und Evolutionsforschung* 30:297–315. <https://doi.org/10.1111/j.1439-0469.1992.tb00179.x>
- Schneider H, Sinsch U (1999) Taxonomic reassessment of Middle Eastern water frogs: bioacoustic variation among populations considered as *Rana ridibunda*, *R. bedriagae* or *R. levantina*. *J Zool Syst Evol Res* 37:57–66. <https://doi.org/10.1046/j.1439-0469.1999.372098.x>
- Schneider H, Sofianidou TS, Kyriakopoulou-Sklavounou P (1984) Bioacoustic and morphometric studies in water frogs (genus *Rana*) of the lake Ioannina in Greece, and description of a new species (Anura, Amphibia). *Zeitschrift Für Zoologische Systematik Und Evolutionsforschung* 22:349–366. <https://doi.org/10.1111/j.1439-0469.1984.tb00669.x>
- Schneider H, Sinsch U, Sofianidou TS (1993) The water frogs of Greece: bioacoustic evidence for a new species. *J Zool Evolut Res* 31(1):47–63. <https://doi.org/10.1111/j.1439-0469.1993.tb00178.x>
- Sciberras A, Schembri P (2006a) Occurrence of the alien Bedriaga's frog (*Rana bedriagae* Camerano, 1882) in the Maltese islands, and implications for conservation. *Herpetol Bull* 95:2–5
- Sciberras A, Schembri P (2006b) *Rana Bedriagae*. *Herpetol Rev* 37:102
- Sinsch U, Schneider H (1996) Bioacoustic assessment of the taxonomic status of pool frog populations (*Rana lessonae*) with reference to a topotypical population. *J Zool Syst Evol Res* 34:63–73. <https://doi.org/10.1111/j.1439-0469.1996.tb00811.x>
- Stamatakis A (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30:1312–1313. <https://doi.org/10.1093/bioinformatics/btu033>
- Tecker A, Göcking C, Menke N, Schreiber R, Plötner J (2017) Neue Daten zur Morphologie, Genetik und Verbreitung der Wasserfrösche (*Pelophylax* spp.) im Münsterland (NRW) unter besonderer Berücksichtigung des Kleinen Wassefrosch (*Pelophylax lessonae*). *Zeitschrift Für Herpetologie* 24:19–44
- Toews DPL, Brelsford A (2012) The biogeography of mitochondrial and nuclear discordance in animals. *Mol Ecol* 21:3907–3930. <https://doi.org/10.1111/j.1365-294X.2012.05664.x>
- Vamberger M, Corti C, Stuckas H, Fritz U (2011) Is the imperilled spur-thighed tortoise (*Testudo graeca*) native in Sardinia? Implications from population genetics and for conservation. *Amphibia-Reptilia* 32:9–25. <https://doi.org/10.1163/017353710X541869>
- van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Mol Ecol Notes* 4:535–538
- Vorburger C, Reyer H-U (2003) A genetic mechanism of species replacement in European waterfrogs? *Conserv Genet* 4:141–155. <https://doi.org/10.1023/A:1023346824722>
- Vucic M, Jelić D, Klobučar GIV, Prkljačić B, Jelić M (2018) Molecular identification of species and hybrids of water frogs (genus *Pelophylax*) from Lake Skadar, Southeast Adriatic drainages (Amphibia: Ranidae). *Salamandra* 54:147–157

Zeisset I, Beebee TJC (2003) Population genetics of a successful invader: the marsh frog *Rana ridibunda* in Briatrain. *Mol Ecol* 12:639–646. <https://doi.org/10.1046/j.1365-294X.2003.01775.x>

Zeisset I, Rowe G, Beebee TJC (2000) Polymerase chain reaction primers for microsatellite loci in the North European water frogs *Rana ridibunda* and *R. lessonae*. *Mol Ecol*

9:1173–1174. <https://doi.org/10.1046/j.1365-294x.2000.00954-2.x>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.