

## Rapid Communication

**Balkan marsh frogs *Pelophylax kurtmuelleri* (Gayda, 1940) introduced in the Aspromonte National Park, southern Italy**Roberta Bisconti<sup>1,\*</sup>, Giuseppe Martino<sup>2</sup>, Andrea Chiochio<sup>1</sup>, Antonino Siclari<sup>2</sup> and Daniele Canestrelli<sup>1</sup><sup>1</sup>Tuscia University, Department of Ecological and Biological Science, Viterbo, 01100, Italy<sup>2</sup>Aspromonte National Park, Via Aurora 1, I-89057 Gambarie di S. Stefano in Aspromonte, ItalyAuthor e-mails: [bisconti@unitus.it](mailto:bisconti@unitus.it) (RB), [gmartino86@hotmail.com](mailto:gmartino86@hotmail.com) (GM), [a.chiochio@unitus.it](mailto:a.chiochio@unitus.it) (AC), [antonino.siclari@parcoaspromonte.gov.it](mailto:antonino.siclari@parcoaspromonte.gov.it) (AS), [canestrelli@unitus.it](mailto:canestrelli@unitus.it) (DC)

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**Abstract**

Alien marsh frogs were found during bioacoustic surveys in the Aspromonte National Park, southern Italy, during spring 2015. This is to date the southernmost finding of non-native water frogs in the Italian Peninsula. Subsequent genetic characterization clearly identified these non-native frogs as belonging to *Pelophylax kurtmuelleri*, a species of the *Pelophylax ridibundus* species group. Phylogenetic comparisons with previously published sequence data placed the collected samples in a cluster of *P. kurtmuelleri* individuals from southwest coastal areas of the Balkans. We collected further information on this introduction by interviewing local people, and by extending bioacoustic and genetic surveys to all the potential breeding sites in the area during springtime, from 2016 to 2018. Interviews revealed that a small group of Balkan people established in the area in the early '80s, suggesting that marsh frogs were introduced as a food source (frog leg consumption). We found non-native frogs at eight sites, located 10 km at most from the supposed area of introduction. Considering *i*) the invasive potential of marsh frogs, and the associated threats to native species, *ii*) their still limited spread in the study area, and *iii*) the presence in this area of a unique evolutionary lineage of native water frogs, *P. lessonae*, we urge for timely management and eradication actions of this alien frog in the area, aimed at preventing its further spread and consequent negative impact on native frogs and other components of the biological community.

**Key words:** European water frogs, *Pelophylax ridibundus* species group, non-native species, Italian Peninsula

**Introduction**

Eurasian water frogs (*Pelophylax* spp.) are a group of species distributed throughout the western Palearctic region. Frequently introduced outside their native range for commercial purposes, water frogs are emerging as an outstanding system for the study of invasion dynamics and their consequences (Vorburger et al. 2003; Schmeller et al. 2007; Paunović et al. 2010). Competition, predation, and hybridization with the native species are the interspecific interactions witnessed following the introduction of alien water frogs, and the main threats to persistence, fitness, and genetic integrity of indigenous frog populations (Razzetti et al. 2010; Holsbeek et al.

2010). Marsh frogs of the *Pelophylax ridibundus* species complex are the European water frog species most frequently involved in international trade (Holsbeek et al. 2008). Their natural range extends from eastern to south-eastern and central Europe, and their introduction has been reported in many western Europe countries (Dubey et al. 2014; Leuenberger et al. 2014; Bressi 2006; Lever 2003; Kolenda et al. 2017; Holsbeek et al. 2010; Pagano et al. 2001).

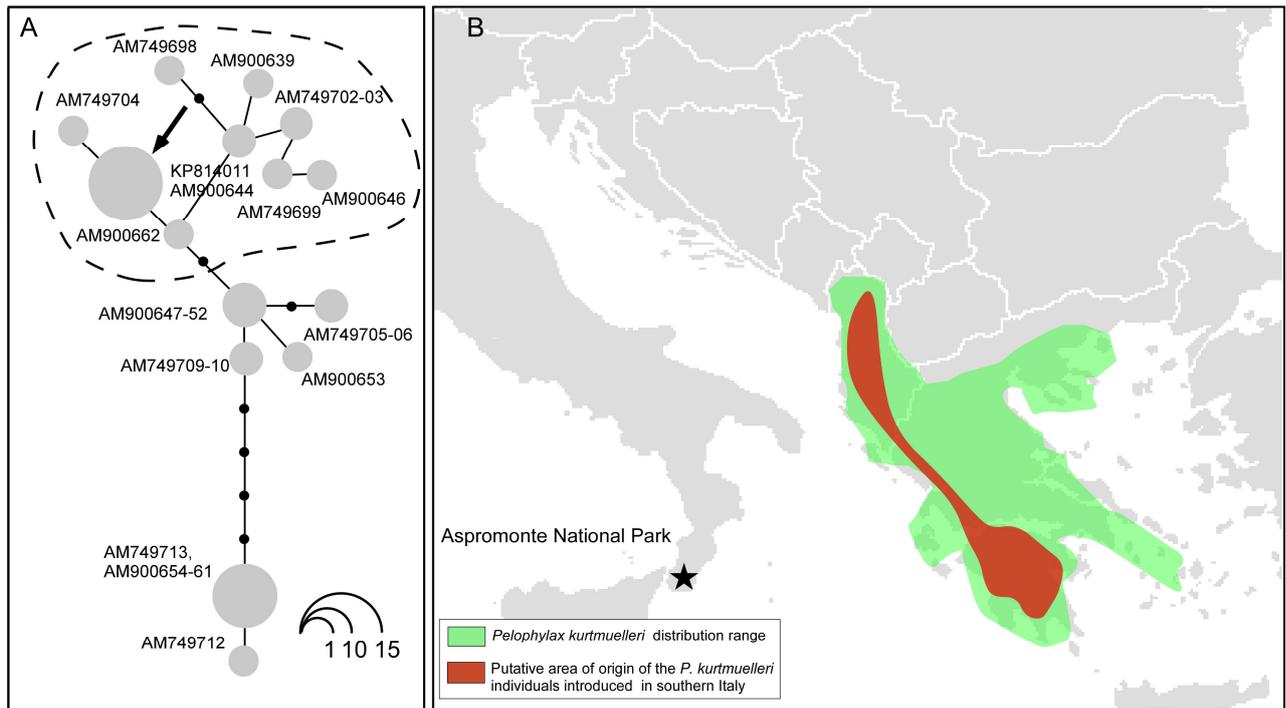
Marsh frogs traded in Italy are frequently native to the Balkan Peninsula (Ficetola and Scali 2010). The first case of introduction of Balkan water frogs in Italy was reported in northwest Italy and it dates back to 1941 (Lanza 1962; Razzetti et al. 2010). Within a few decades, the species successfully colonised many areas of north-western and north-eastern Italy (Ficetola and Scali 2010). Nowadays, alien frogs are invading many Italian wetlands, particularly in northern regions, threatening and replacing the native species, *Pelophylax lessonae* and the hybridogenetic hybrid *Pelophylax esculentus* (Andreone 1999; Razzetti et al. 2010; Lanza 1962; Lapini and Zanghellini 1993; Bressi 2007; Domeneghetti et al. 2013).

Here, we report the first case of introduction of a non-native water frog species in southern Italy. Using bioacoustic surveys, interviews with local people, as well as genetic data analysis, we assess the area of introduction and current occurrence of the alien frogs, as well as their putative area of origin. Finally, we discuss potential threats posed by this newly identified introduction.

## Materials and methods

The first occurrence of non-native water frogs within the Aspromonte National Park was documented during spring 2015, at a site located at 1350 m a.s.l. (Gambarie, municipality of Santo Stefano d'Aspromonte, Calabria region; see Figure 1, Table 1 and Supplementary material Table S1), by hearing at the typical and diagnostic mating calls of species of *P. ridibundus* species group (Wycherley et al. 2002).

As a part of an ongoing bioacoustic survey of amphibian species in the area, we recorded calls belonging to both the native (*P. lessonae*) and the alien water frogs. Mating calls were recorded using a handheld Sennheiser ME66 directional microphone coupled with a K6 powering module (Sennheiser Electronic Corporation, Old Lyme, CT, USA) and a ZOOM H4N PRO recorder (ZOOM Corporation, Tokyo, Japan). Calls were digitalised at a sample rate of 44.1 kHz with a 16-bit resolution. Recordings were checked for track quality using the software Adobe Audition 3.0 (Adobe Systems, San Jose, CA, USA). Oscillograms and spectrograms were generated with the R package Seewave (Sueur et al. 2008), with a Fast Fourier transform of 1024 points and a Hamming's sampling window.



**Figure 1.** A) Phylogenetic network showing the affinity of the alien marsh frog in southern Italy (black arrow) with the *P. ridibundus* s.l. ND2 sequences available in GenBank. B) Approximate geographic distribution of *Pelophylax kurtmuelleri* (green area) within the Balkan peninsula, and of the haplotypes showing the closest phylogenetic relationships with those sampled in southern Italy (red area, and dotted circle in panel A).

**Table 1.** Geographic location and altitude of the eight sites within the Aspromonte National Park (southern Italy) where *Pelophylax kurtmuelleri* individuals have been documented.

Site	Latitude (N)	Longitude (E)	Altitude (m a.s.l.)
1	38.179689	15.848186	1321
2	38.189086	15.828275	1168
3	38.192531	15.828589	1158
4	38.194842	15.831886	1131
5	38.197994	15.842197	1148
6	38.199850	15.819314	1122
7	38.200700	15.799008	1184
8	38.210686	15.842761	1106

In order to address the most likely site and tempo of alien frog introduction, we carried out interviews with local people in the area. The site of first identification of alien frogs is a small, high-altitude hamlet, mostly devoted to winter recreational activities. We asked elders for present or past foreign settlements in the area, without specifying nationality or a specific temporal frame.

We assessed the extent of occurrence of alien frogs by inspecting all water frog breeding sites within the area surrounding the site of first finding. Starting from this site, night sessions of bioacoustic survey were carried out by progressively extending the search area towards the four cardinal points, with three visits per season (from spring 2016 to spring 2018) of each potential breeding site. The progressive broadening of the searching area was stopped when alien individuals were not recorded in the last five kilometres.

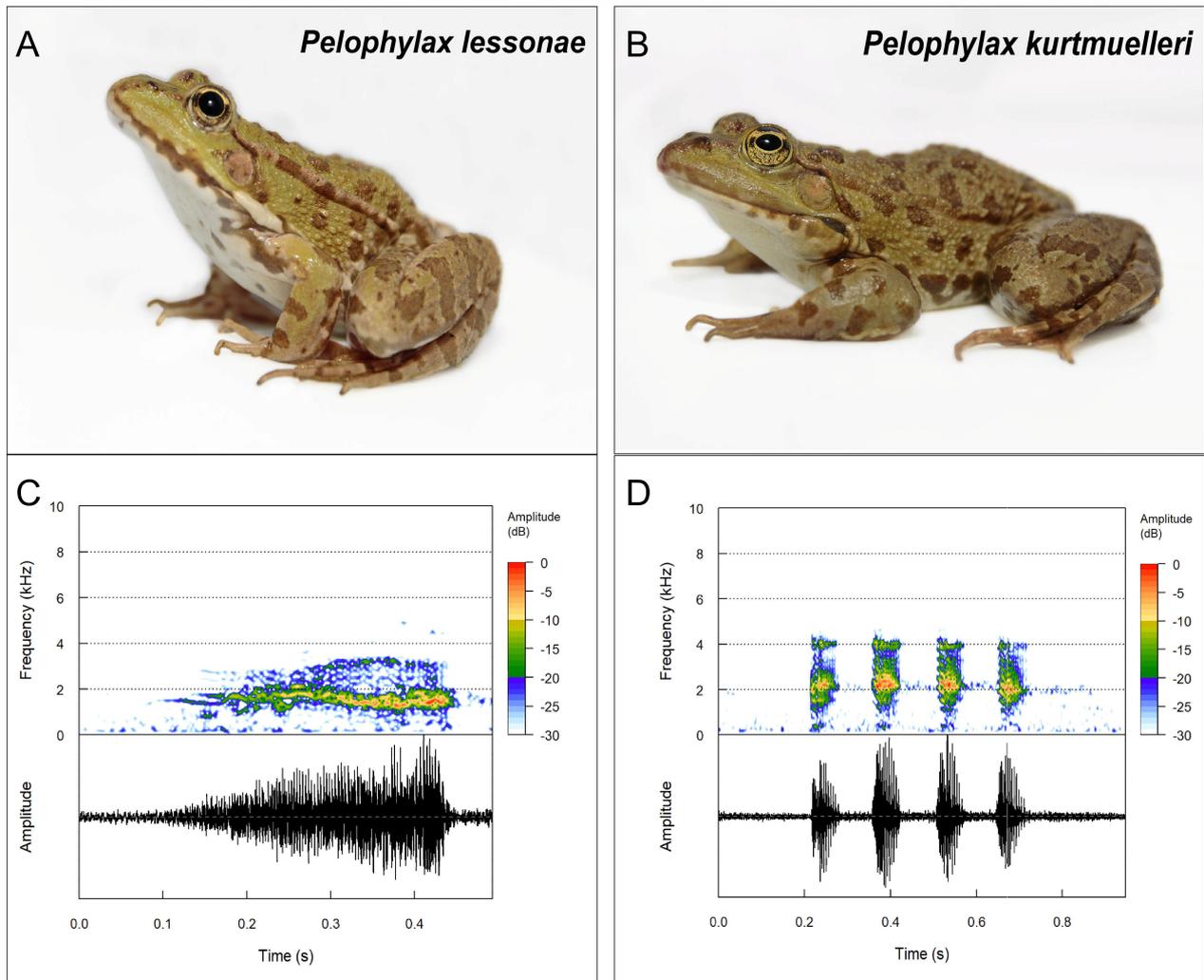
The correct taxonomic assignment and putative geographic origin of the alien frogs, and their putative geographic origin, were assessed by sequencing a diagnostic mitochondrial gene fragment. Tissue samples of putatively alien water frogs were retrieved from each site of occurrence as toe-clips. All the individuals were then released back to the collection site. Tissues were stored in 95% ethanol until DNA extraction.

Genomic DNA was extracted following the standard cetyltrimethylammonium-bromide protocol (Doyle and Doyle 1987). A fragment of the mitochondrial NADH dehydrogenase subunit 2 (ND2) gene was amplified by polymerase chain reaction (PCR) using the primers ND2L2 (GGACTCGCCCCYCTACTTCTG) and ND2H2 (CTCCGCTTAAGGCTTTG-AAGGC) (Plötner et al. 2008; Akin et al. 2010). Amplifications were carried out in 25 µl volume containing MgCl<sub>2</sub> (2.5 mM), the reaction buffer (2x), the four dNTPs (0.2 mM each), the two primers (0.2 mM each), the enzyme Taq polymerase (GoTaqPromega) and 20–50 ng of DNA template. PCR reactions were conducted with an initial step of 94 °C for 5 minutes, 39 cycles at 94 °C for 45 seconds, 48 °C for 1 minute, 72 °C for 90 seconds, and a final step of 72 °C for 10 minutes. Sequencing of PCR products was conducted on both strands by Macrogen Inc. (<http://www.macrogen.com>), using an ABI PRISM® 3730 sequencing system (Applied Biosystems).

Chromatograms were checked by eye using FinchTV 1.4 (Geospiza Inc.) and the sequences were aligned using ClustalX 2.1 (Larkin et al. 2007). To identify the collected samples, sequences were aligned against all the ND2 unique haplotypes of Palearctic water frogs available from GenBank nucleotide database (National Center for Biotechnology Information, U.S. National Library of Medicine, USA). A maximum-likelihood tree among the haplotypes retrieved was inferred with PhyML 3.10 (Guindon et al. 2010), using the neighbour nearest interchange (NNI) for tree improvement and the substitution model selected by the Smart Model Selection (SMS) procedure in PhyML (Lefort et al. 2017), under the Bayesian Information Criterion (BIC). The robustness of the inferred tree topology was assessed using the non-parametric bootstrap method with 1000 pseudo-replicates. Phylogenetic relationships among haplotypes were further characterized the statistical parsimony method implemented in TCS 1.2.1 (Clement et al. 2000). Following a first run with the whole dataset under the 95% criterion for a parsimonious connection among haplotypes, we re-ran the analysis after removing all the haplotypes which did not clustered with our samples. The putative geographical origin of our alien water frogs was inferred looking at the provenience of the phylogenetically more closely related haplotypes from GenBank.

## Results and discussion

The Aspromonte mountain massif, at the southern tip of the Italian peninsula, is emerging as an outstanding hotspot of endemic Palearctic biodiversity, at



**Figure 2.** Images and call structure of two individuals belonging to the native species *Pelophylax lessonae* (A and C), and alien species *Pelophylax kurtmuelleri* (B and D) found within the study area. Photo by G. Martino.

both the species and the intraspecific levels (e.g. Canestrelli et al. 2006, 2008, 2010). Guarding in mind this unique biodiversity, regional conservation priorities should be to manage threats such as those posed by alien species.

Mating call patterns of frogs from site 1 clearly suggested the occurrence of individuals of non-indigenous marsh frogs belonging to *P. ridibundus* species complex (see Figure 2).

Subsequent bioacoustic surveys in the area, during spring 2016–2018, allowed us to identify eight sites of occurrence of putative alien marsh frogs, distant 10 km at most from the first site surveyed (see Table 1). At three of these sites (sites 1, 7, 8), marsh frogs were the only water frogs present, whereas at the remaining five sites (sites 2, 3, 4, 5, 6), marsh frogs co-occurred with the native species *P. lessonae*.

Interviews with local people revealed a single event of foreign people settlement in the area, active for a few years since the early '80s (exact dates unavailable): a small settlement of Greeks, who attempted a hotel business

just at the site where we first identified alien frogs (site 1 in Table 1 and Table S1). This occurrence would indicate an introduction of marsh frogs in the early '80s, possibly connected with their use as food (frog leg consumption), a common practice in several areas of Greece.

We collected tissue samples from 32 putative marsh frogs, 4 from each site of occurrence, and we successfully sequenced a 517 bp fragment of the ND2 gene from all the samples. All individuals analysed shared a single common haplotype (GenBank accession number: MK116532), suggesting a single introduction event or a single area of origin. After aligning our sequences with those retrieved from GenBank, we generated a final alignment made up of 91 unique haplotypes. The best substitution model selected by the SMS under the BIC was TN93+I+G. The tree inferred with PhyML placed our samples within the *P. ridibundus* species groups (tree log-likelihood score: -3983.82; data not shown). Moreover, the phylogenetic network (Figure 1A) showed that the haplotype from our samples was closely related to *P. kurtmuelleri* haplotypes from coastal areas of southwest Balkans (Figure 1B; GenBank accessions: AM749704 and AM900662).

During the last century, marsh frogs from the Balkan region have been extensively introduced and bred for the food market in several areas of Europe, including northern Italy, and they are now considered the most problematic invasive amphibians in Europe (i.e. frog leg consumption; Holsbeek et al. 2008). However, they were not yet reported in southern Italy.

The limited number of sites of occurrence identified to date indicates that the introduction of marsh frogs in the Aspromonte National Park has not yet triggered a massive biological invasion. However, to the state of knowledge, we cannot know whether this limited distribution is due to a stable co-existence between alien and native frogs (Leuenberger et al. 2014), to environmental effects on the dispersal ability of marsh frogs within the Aspromonte massif, or if too little time has passed for marsh frogs to enter the invasive phase (Hui and Richardson 2017). However, regardless of their current demographic phase, and considering their invasive potential as clearly documented in other areas, the protected status of the study area (as a National park), as well as the presence of a unique evolutionary lineage of the native pool frog *P. lessonae* in this area (Canestrelli and Nascetti 2008 and references therein), we urge for timely actions aimed at monitoring population dynamics, limiting their further spread and, ultimately, to eradicate them from the area.

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### Supplementary material

The following supplementary material is available for this article:

**Table S1.** Geographic location and altitude of the eight sites within the Aspromonte National Park (southern Italy) where *Pelophylax kurtmuelleri* individuals have been documented.

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