Acta Herpetologica 13(2): 117-123, 2018
DOI: 10.13128/Acta_Herpetol-23189

New insights on the phylogenetic position and population genetic structure of the Critically Endangered Karpathos marsh frog *Pelophylax cerigensis* (Amphibia: Anura: Ranidae)

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Submitted on: 2018, 24th April; revised on: 2018, 9th August; accepted on: 2018, 26th August
Editor: Marcello Mezzasalma

Abstract. The genus *Pelophylax*, which currently comprises 26 species, is a well studied group due to its complex history and high diversification, although some phylogenies remain unresolved. Here we assess the phylogenetic position and the population genetic structure of the Critically Endangered Karpathos frog, *Pelophylax cerigensis*, endemic to Karpathos Island. A total of 42 cytb sequences were examined including specimens from Rhodes Island, and amplified fragment length polymorphisms (AFLPs) were generated to investigate the genetic structure and connectivity of the only two known populations on Karpathos. Molecular analyses reveal two major waterfrog lineages in the eastern Mediterranean: Clade A comprises *Pelophylax bedriagae* from the Middle East and the island of Cyprus, while clade B includes both *P. bedriagae* from the east Aegean Sea and *P. cerigensis*. Specimens from Karpathos and Rhodes constitute a single clade, contrasting previous studies, thus indicating the occurrence of Karpathos frog also to the neighboring Rhodes. The AFLP markers revealed low but statistically significant levels of genetic divergence between the two Karpathos' populations and similar levels of genetic diversity. Our results suggest that the current taxonomy of the species should be re-evaluated. We also strongly recommend the need of conservation actions to maintain the levels of diversity in the declining population of the Karpathos frog.

Keywords. *Pelophylax cerigensis*, insular populations, phylogeny, cytochrome b, genetic structure, AFLPs, conservation.

INTRODUCTION

The elucidation of phylogeographic history and the study of intra- and inter-population diversity are inextricably linked to the paleogeographic events that took place during the Pleistocene. The Balkan Peninsula, which is considered a Pleistocenic refugium, shows high levels of biodiversity and endemism (Sfenthourakis et al., 2001; Michaux et al., 2004; Sotiropoulos et al., 2007) and the long-term favorable environmental conditions allowed the preservation of genetically differentiated populations (Canestrelli et al., 2010) resulting in the complex history of many terrestrial species (Beerli et al., 1996; Douris et al., 1998; Poulakakis et al., 2003).

The Genus *Pelophylax* Fitzinger, 1843, which currently comprises 26 species, is one of the most well studied groups (Lymberakis et al., 2007; Plötner et al., 2012; Vervust et al., 2013; Litvinchuk et al., 2015) due to its

ISSN 1827-9635 (print)
ISSN 1827-9643 (online)

complex history and high diversification in the Balkan Peninsula and east Mediterranean. Isolation of land areas during the Pleistocene and the formation of islands in the Aegean region had a great impact in the genetic differentiation of the genus *Pelophylax*. Currently, researchers suggest long-separated lineages and diverse evolutionary histories of the western Palearctic water frogs (Lymberakis et al., 2007; Akın et al., 2010), although some phylogenies are still under question and further revision of their status is needed. Among the three major lineages in the eastern Mediterranean region, the Balkan-Anatolia (ridibunda/ bedriagae) lineage which comprises the species *P. cretensis*, *P. epeiroticus*, *P. bedriagae*, *P. cerigensis*, *P. kurtmuelleri* and *P. ridibundus*, is considered to have emerged from a widely-distributed common ancestor through vicariance events during the climatic changes in the Pliocene (Lymberakis et al., 2007).

Pelophylax cerigensis is a medium-sized, mainly insectivore water frog, endemic to Karpathos Island (Valakos et al., 2008; Pafilis et al., 2018). Karpathos is the second largest island of the Dodecanese archipelago (Aegean Sea, Greece), located approximately 47 kilometers southwest of Rhodes. The island is characterized by xeric habitats with dry pine and oak forests, Mediterranean maquis, and phrygana. According to Beerli et al. (1994), who first described the species, phylogenetic analyses based on electrophoretic data cluster the Karpathos and Rhodes populations together. In addition, Akın et al. (2010) found a shared haplotype in both Karpathos and Rhodes, a result further corroborated by Lymberakis et al. (2007). However, the taxonomy of the Rhodes populations remains unresolved and further evidence is needed for the clarification of the species status.

The Karpathos frog is regarded as the most threatened frog in Europe (Beerli et al., 2009; Temple and Cox, 2009) due to its limited distribution and current population decline. Habitat loss and degradation of aquatic terrains in this dry island are considered the major factors compromising the species survival whereas they could potentially affect the populations' genetic status. Therefore, studies on population genetic diversity and structure are necessary to design and implement proper management actions for the conservation of the species.

Due to their simplicity and cost-effectiveness as genetic markers, AFLPs have been widely used in population genetic studies in the past decades. Several studies have examined the genetic structure through AFLP markers using F-statistics (Wright, 1950; Meudt et al., 2007) and quantified the levels of differentiation and connectivity between populations of several species.

Here, we aim to evaluate the phylogenetic position of the Karpathos frog within the *bedriagae* lineage as well as to assess the genetic structure and connectivity of the population between the two known localities in the island of Karpathos.

MATERIAL AND METHODS

Sample collection and laboratory procedures

A total of 30 buccal swab samples of *P. cerigensis* were collected during 2016 from the two known breeding sites (Argoni and Nati rivers) on Karpathos (Table A1, Fig. 1). Additionally, three samples of water frogs (presumed *P. bedriagae*) from Rhodes Island (NHMC80.2.99.40, NHMC80.2.99.41, NHMC80.2.99.42) were used in the phylogenetic study. Total genomic DNA was extracted from buccal swabs using the NucleoSpin Tissue kit (Macherey-Nagel) following the manufacturer's protocol.

A partial sequence of approximately 340 bp from the mitochondrial cytb gene was successfully amplified for 26 samples (including the three from Rhodes, Table A1), with the universal primers L14841 and H15149 (Kocher et al., 1989). PCR amplifications were carried out in 12.5μl volume reactions containing 0.05 U Taq (Kapa Biosystems), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM of each primer, 1X Taq buffer (Kapa Biosystems) and 10-20 ng DNA template, under the following conditions: an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 60 sec, annealing at 47 °C for 60 sec, extension at 72 °C for 60 sec and a final extension for 7 min at 72 °C. PCR products were purified using the NucleoSpin ExtractII (Macherey-Nagel) cleanup kit and single strand sequencing was conducted by CEMIA (Cellular & Molecular Immunological Applications, Larisa, Greece).

AFLP analysis was performed according to a modified protocol from Vos et al. (1995), for 28 samples of high quality DNA, collected from the two breeding sites on Karpathos, Argoni (18 samples) and Nati (10 samples). Total genomic DNA was digested using two restriction enzymes TaqI and EcoRI (Takara Bio Inc.). Digestion was carried out in a final volume of 20 μl containing 1 mM TaqI buffer, 0.1 mM BSA, 0.5U of EcoRI and 200 ng of genomic DNA for 2 h at 37 °C. After the addition of 0.5U TaqI samples were incubated for two more hours at 65 °C. The ligation was carried out in a final volume of 30 μl containing 1X ligase buffer (Takara), 1.8 μM of each adaptor, 1U T4 DNA ligase and the digested DNA, and the reaction was incubated overnight at 16 °C. The digested-ligated DNA fragments were diluted 25-fold to be used as templates for the pre-amplification reaction. Each 50 μl pre-selective reaction contained 1X Taq buffer, 1.5 mM MgCl2, 0.2 mM of each dNTP, 0.3 μM of each primer (T01P2, T02P2), 0.3 μM of Presel ECO primer and 10 μl of the diluted ligation product. The PCR amplifications were carried out using the following profile: an initial denaturation step at 94 °C for 120 sec followed by 20 cycles of 30 sec at 94 °C, 60 sec at 56 °C, and 60 sec at 72 °C with a final extension step at 72 °C for 300 sec. The pre-amplification products were diluted 25-fold to be used as template for the selective amplification. The selective amplifications were performed in a total volume 25 μl containing 10 mM Taq buffer (Takara), 3 mM

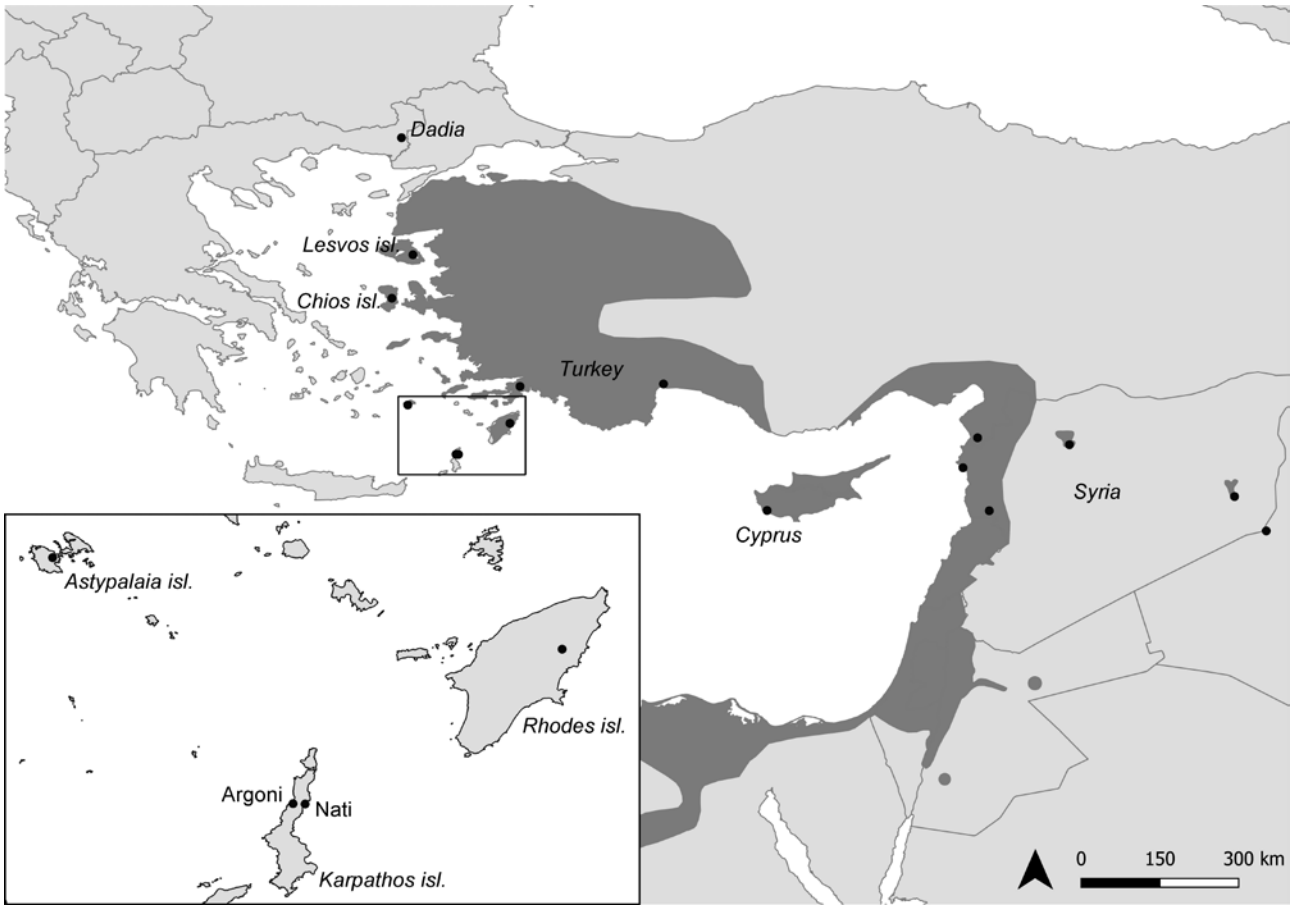

Fig. 1. Sampling localities of *P. cerigensis* used in the study. Shaded areas correspond to the current distribution of *P. bedriagae*.

MgCl2, 0.3 mM of each dNTP, 0.2 μM of EcoRI primers, 0.5 μM of 5 selective primers (T101P2, T105P2, T106P2, T204P2 & T205P2) and 5 μl of diluted pre-amplified DNA. Selective amplification was carried out using a touchdown protocol with an initial denaturation step at 94 °C for 120 sec, 30 sec at 94 °C, 60 sec at 65 °C followed by 11 cycles where the annealing temperature was gradually reduced 0.7°C per cycle followed by 23 cycles of 30 sec at 94 °C, 30 sec at 56°C, and 60 sec at 72 °C. The primers used in each pre-selective and selective PCRs are presented in Table A2. Selective products were separated in Fragment Analyzer (Advanced Analytical Technologies Inc.) using the dsDNA 910 Reagent Kit. AFLP patterns were visualized and processed using the PROsize 2.0 Software (Advanced Analytical Technologies) simultaneously by two persons to reduce scoring bias. Scoring bias was quantified by calculating Cohen's kappa coefficient for inter-rater agreement. AFLP profiles were scored according to presence/absence of peaks.

Genetic analyses

Mitochondrial sequences were edited by eye in MEGA v.7 (Kumar et al., 2016) and aligned with CLUSTAL W (Thompson et al., 1994). Additionally, 15 sequences of *P. bedriagae*, which were retrieved from GenBank, and one of *P. cretensis* that was used as outgroup, were added in the analyses. To visualize the relationships among the detected haplotypes, a median joining (MJ) network (Bandelt et al., 1999) was constructed with the software PopArt (Leigh et al., 2015), excluding the outgroup sequence and setting the parameter ε equal to zero. Additionally, a neighbor-joining (NJ) tree (Saitou and Nei, 1987) was constructed according to the Kimura-2 parameter substitution model, implemented in MEGA. The reliability of the nodes was assessed by 50000 bootstrap replications. Between-population uncorrected sequence divergences (p-distance) were estimated using MEGA. Additionally, haplotype and nucleotide diversity values within each recognized clade were calculated with dnaSP v.5 software (Librado and Rojas, 2009).

AFLP data were used to estimate Nei's (1973) gene diversity and levels of genetic differentiation between the two populations (Argoni and Nati), using a Bayesian approach with non-uniform prior distribution through 1000 bootstrap replicates implemented in the software AFLP-SURV (Vekemans, 2002) which is based on the methods described by Lynch and Milligan (1994). To evaluate the presence of genetic substructure in the population, a Principal Coordinates Analysis (PCoA) was

performed in R 3.4.1 (R core team, 2017) to visualize the clustering of individuals based on their AFLP band patterns. Individuals with missing data were excluded from the analysis.

RESULTS

The final dataset included 42 sequences (Table A1) and the analysis of 286 bp of Cytb revealed 12 haplotypes with high levels of haplotypic diversity (Hd= 0.62). We detected 49 variable sites (excluding the outgroup) of which 27 were parsimony-informative. Both the haplotype network (Fig. 2) and the NJ phylogenetic tree (Fig. A2) revealed two well-defined lineages, which are in accordance with the geographical origin of the specimens. Clade A (5 haplotypes, Hd = 0.89, Pi = 0.014) corresponds to *bedriagae* specimens of Syria and Cyprus, while Clade B (7 haplotypes, Hd =0.42, Pi = 0.019) consists of the *bedriagae* specimens from Turkey, the Aegean islands and *P. cerigensis*. Between clade p-distance was 6%, while p-distances between the various geographical locations ranged from 0.1 to 7.2% (Table 1). Specimens from the islands of Karpathos and Rhodes share one haplotype (B-1) while one additional haplotype (differing by one substitution) was found in Rhodes (B-2).

In total, 87 AFLP fragments (Fig. A1) were produced from all primer combinations and the mean number of segregating peaks per individual were 59. Cohen's kappa coefficient value was 0.83, suggesting that no scoring bias was present in our dataset. The results are summarized in Table 2. The two breeding populations showed low but statistically significant levels of genetic differentiation, while levels of gene diversity are similar between them (Table 2). In the PCoA plot, individuals from each breeding population formed two separated groups that widely overlapped (Fig. 3). The first and the second axis accounted for 24% and 15.9% of the total variance, respectively.

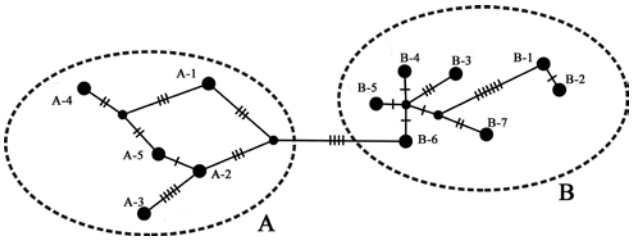

Fig. 2. Median joining network constructed using the 12 detected haplotypes presenting the two major waterfrog lineages (A, B). Vertical lines correspond to the mutational steps observed between the different haplotypes.

Table 1. % p-distances (below diagonal) with the corresponding standard error (above diagonal) between the geographical locations of *P. bedriagae* and *P. cerigensis*.

Karpathos isl.		0.1	1.4	1.3	1.9	1.2	1.2	1.7	1.3
Rhodes isl.	0.1		1.4	1.3	1.9	1.3	1.2	1.8	1.3
Astypalaia isl.	4.6	4.7		0.9	1.7	0.9	0.9	1.5	1
Chios isl.	3.6	3.8	1.8		1.5	0.6	0.6	1.3	0.8
Cyprus	6.5	6.6	4.6	3.7		1.4	1.5	1	1.4
Dadia	3.6	3.8	1.8	0.9	2.7		0.6	1.2	0.8
Lesvos isl.	3.6	3.8	1.8	0.9	3.7	0.9		1.3	0.8
Syria	7.1	7.2	5.2	4.2	2.8	3.3	4.1		1.3
Turkey	4.1	4.2	2.3	1.3	3.2	1.3	1.3	3.9	

Table 2. Gene diversity within the two populations of *P. cerigensis*. Hj: Nei's gene diversity, S.E.: standard error, P %: level of polymorphism and fixation index (Fst value).

Population	Sample size	Hj	S.E.	P % Fst (95% CI)
Argoni	18	0.35	0.013	71.2
Nati	10	0.38	0.011	67.8
				0.04 (-0.03-0.01)

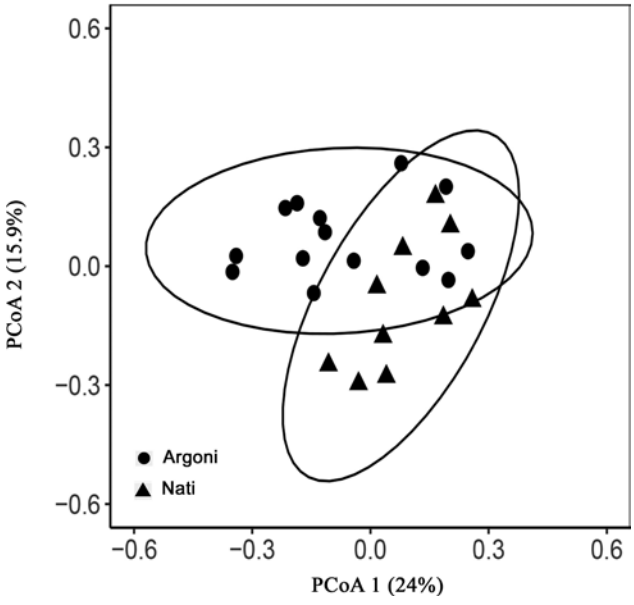

Fig. 3. A two-dimensional PCoA representation of the dissimilarities among individuals with 95% confidence ellipses. Points represent sampled individuals from the two breeding sites (Argoni, Nati).

DISCUSSION

Taxonomic implications

Our results reveal two major water frog lineages in the eastern Mediterranean: clade A comprises *P. bedriagae* from the Middle East, while clade B includes both *P. bedriagae* specimens of east Aegean Sea and *P. cerigensis* specimens. The results suggest homogeneity in respect to mitochondrial DNA for the specimens from Karpathos and Rhodes and reveal one shared haplotype between the Karpathos and Rhodes specimens and a second one in Rhodes Island. The two island populations comprise a single clade, in contrast to previous studies, where *Pelophylax* specimens of Rhodes clustered with specimens of *P. bedriagae* from Asia Minor (Lymberakis et al., 2007). This does not seem to be the case in the present study, thus raising an argument concerning the taxonomic validity of the Karpathos frog. However, the results of the above study were underpinned by different genetic markers, validating the need of utilizing both mitochondrial and nuclear markers in each study.

The shared haplotype present in both islands in addition to the low levels of genetic divergence, support the presence of *P. cerigensis* in the neighboring Rhodes, as proposed by Akin et al. (2010). However, according to geological data Karpathos and Rhodes were already completely isolated during the Pleistocene (Beerli et al., 1996). Although it is not clear when the two islands were separated from Asia Minor, geological records propose that Karpathos was isolated earlier while Rhodes was still connected to Anatolia until late Pliocene or early Pleistocene (Boger and Dermitzakis, 1985; Beerli et al., 1994). This disagreement with the paleogeographic history should be investigated using nuclear data from several species along with geological information, which could unravel the complex biogeographic history of the Aegean archipelago. The high pairwise p-distances within clade B (3.6-4.7%) corroborate the divergent and complicated history of the genus *Pelophylax*, which is linked with the events during the Pleistocene.

Our results confirm the necessity of revision of the current taxonomy of the species by utilizing both mitochondrial and nuclear markers. In addition, further analysis of bioacoustic data could possibly shed light on the subject as they are proven to play an important role in anuran taxonomy (Schneider and Sinsch, 1999; Padial et al., 2008). Additionally, the use of more specimens covering the species' distribution is highly recommended to verify its taxonomic validity.

Population structure and implications for conservation

According to the population genetic analysis, both Karpathos populations show moderate levels of genetic variation and differentiation. The AFLP markers showed low but statistically significant levels of genetic divergence between the two population groups and similar levels of gene diversity. According to the PCoA analysis, individuals from Argoni and Nati form two largely overlapping clusters. The observed low inter-group differentiation reflects ongoing gene flow hence dispersal movements between the two localities may occur. In fact, these two localities are small brooks located in the opposite sides of the central mountain ridge and are separated by the main island road. The smallest distance between them is few hundred meters (approx. 500 m), a distance that lies within the dispersal capability of the species, thus dispersal movements along an elevational gradient could take place leading in a higher connectivity between them. Further analysis with other genetic markers such as microsatellites could offer an insight using more polymorphic loci, and assess recent events of gene flow (Manel et al., 2005).

A primary objective of conservation actions is to maintain levels of diversity and heterozygosity, which are strongly recommended due to the observed population decline on Karpathos. Currently, the largest threat to populations of *P. cerigensis* is the loss of suitable breeding sites due to fragmentation and climatic change (Beerli et al., 2009). Small wetlands are unique ecosystems preserving amphibian populations and providing patches of suitable habitats and the overall pressure of climatic change is also apparent in other species inhabiting such fragile ecosystems. Hence, there should be increased efforts in the conservation of these areas that provide suitable habitats for the Karpathos marsh frog along with future studies in the biology and behavior of the species.

ACKNOWLEDGEMENTS

Sampling took place according to the Hellenic National Law (Presidential Decree 67/81) and under a special permit (code: 6ΨΤ04653Π8-ΥΟ3) issued by the Ministry of the Environment. The study was fully funded by the Mohamed bin Zayed Species Conservation Fund.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <http://www.unipv.it/webshi/appendix> manuscript number 23189.

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