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Genetic Diversity of *Pelophylax cerigensis*-like Population on the Anatolian Mainland in Türkiye (Amphibia: Anura: Ranidae)

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Abstract: Genetic diversity is increasingly used as a vital component in planning appropriate conservation strategies. Water frogs in the eastern Mediterranean consist of several endemic species. The Critically Endangered Karpathos water frog (*Pelophylax cerigensis*) is one such species, restricted to Karpathos Island, but recently *P. cerigensis* specific haplotypes were also found in Rhodes and southwestern Türkiye. Since, geologically Karpathos and Rhodes have been separated from the Anatolian mainland million years ago, the genetic diversity of *P. cerigensis*-like populations on the Anatolian mainland are not known. Here, we aim to evaluate the genetic diversity of this mainland population (N=52) in southwestern Anatolia by using five polymorphic microsatellite loci. According to results, a total of 38 alleles which five loci exhibited a moderate level of genetic diversity (observed heterozygosity, H_O=0.423). The population has not gone through a bottleneck anytime soon; however, signs of inbreeding were determined (F_{IS}=0.401). Due to restricted occurrence from Antalya to Aydın provinces in southwestern Türkiye and a moderate level of genetic diversity, they should be considered a third Management Unit (MU) of *P. cerigensis* populations in addition to previous Karpathos and Rhodes MUs. This approach is very crucial to formulate suitable management strategies for conservation of these threatened populations.

Türkiye'de Anadolu Anakarasında *Pelophylax cerigensis* benzeri (Amphibia: Anura: Ranidae) Popülasyonun Genetik Çeşitliliği

Anahtar Kelimeler
Genetik çeşitlilik, Su kurbağası *Pelophylax cerigensis*, Anadolu, Türkiye

Öz: Genetik çeşitlilik, uygun koruma stratejilerinin planlanmasında giderek daha hayati bir bileşen olarak kullanılmaktadır. Doğu Akdeniz'deki su kurbağaları çok sayıda endemik türden oluşmaktadır. Kritik Tehlike Altındaki Karpathos su kurbağası (*Pelophylax cerigensis*) bu türlerden biridir ve Karpathos Adası ile sınırlıdır, ancak yakın zamanda Rodos ve güneybatı Türkiye'de de *P. cerigensis*'e özgü haplotipler bulunmuştur. Jeolojik olarak Karpathos ve Rodos milyonlarca yıl önce Anadolu anakarasından ayrıldığından, Anadolu anakarasındaki *P. cerigensis* benzeri popülasyonların genetik çeşitliliği bilinmemektedir. Burada, beş polimorfik mikrosatellit lokusu kullanarak Güneybatı Anadolu'daki bu anakara popülasyonlarının (N=52) genetik çeşitliliğini değerlendirmesi amaçlanmıştır. Sonuçlara göre, beş lokusta bulunan toplam 38 alel orta düzeyde genetik çeşitlilik sergilemiştir (gözlenen heterozigotluk, H_O=0.423). Popülasyon yakın zamanda bir darboğaza girmemiştir; ancak yakın bireyler arasında üreme belirtileri tespit edilmiştir (F_{IS}=0.401). Türkiye'nin güneybatısında Antalya'dan Aydın'a kadar olan bölgede kısıtlı olarak görülmesi ve orta düzeyde genetik çeşitliliğe sahip olması nedeniyle, daha önceki Karpathos ve Rodos MU'larına ek olarak *P. cerigensis* popülasyonları üçüncü bir Yönetim Birimi (MU) olarak düşünülmelidir. Bu yaklaşım, tehdit altındaki bu popülasyonların korunması için uygun yönetim stratejilerinin formüle edilmesi açısından çok önemlidir.

1. INTRODUCTION

Genetic diversity is an indispensable component of populations to adapt to changing environmental conditions and to compensate them against unpredictable sudden events such as climate changes or outbreak of diseases [1]. Thus, genetic diversity is increasingly used as crucial component in the planning of suitable conservation and management strategies for many threatened populations, aiming to keep as much genetic diversity as possible [2, 3]. Anatolia, the Asian part of Türkiye, is one of the richest biodiversity areas since the Caucasus, Irano-Anatolian and Mediterranean Basin biodiversity hotspots all converge on the region. Besides its high biodiversity with many plant and animal species, it is also the one of the most threatened terrestrial regions of the world [4, 5].

Eastern Mediterranean water frogs (genus *Pelophylax*) are a genetically and phylogenetically diverse group which consist of both older and recently diverged lineages which are widely distributed or restricted to specific regions such as in southwestern Anatolia [6, 7]. The genus also comprises several cryptic lineages and endemic species such as *Pelophylax cretensis* on the island Crete, *P. cerigensis* on Karpathos [8], and *P. cypriensis* in Cyprus [9].

Because of its limited distribution and ongoing population decline, *P. cerigensis* is categorized as Critically Endangered (CR) according to the IUCN Red List of Threatened Species [10, 11]. It is recognized that anthropogenic factors, in particular the drying of wetlands for agriculture and tourism, most strongly affect the survival of the species, and are considered in management actions related to the restoration of their habitats [12].

The island of Karpathos is situated about 47 kilometres southwest of Rhodos. It is the second largest island of the Dodecanese archipelago on the South Aegean Sea. According to fossil data and research on tectonic movements in the Aegean, Rhodes and the Anatolian mainland cluster were isolated from Karpathos about 3 million years ago during Pliocene, while Rhodes was separated from the Anatolian mainland in early Pleistocene about 1.8 million years ago [13, 8].

Several studies were carried to understand phylogenetic relationships of *P. cerigensis* with other eastern Mediterranean water frog lineages, their population genetic structure and genetic diversity based on distinct marker systems. *P. cerigensis* was described as a new species characterized by a unique multi-locus combination of electrophoretic alleles, which also segregated within the Rhodes Island populations [8]. In previous phylogenetic studies, water frogs from Rhodes and Karpathos were grouped with *Pelophylax* cf. *bedriagae* from East Aegean and Türkiye, not with *P. bedriagae* from Cyprus and Syria [8, 14]. Various past studies [6, 7, 15, 16] indicated that both populations from Karpathos and Rhodes islands shared *P. cerigensis*-specific haplotypes. *Pelophylax cerigensis*-specific haplotypes were not only found on Karpathos and Rhodes, but also in the coastal parts of southwestern Anatolia [6, 7]. In southwestern Anatolia four distinct type of *P. cerigensis*-specific haplotypes were found on Avlan Lake (CER3), Dalaman (CER3), Fethiye (CER2, CER3, CER5), Kaş (CER1, CER3), Girdev Plataeu (CER3), Köyceğiz (CER1) where they were found to be coexist with *P.* cf. *bedriagae*-specific haplotypes or *P. cf. caralitanus*-specific haplotypes or both in some localities. CER3 were the most frequent *P. cerigensis*-specific haplotype [17]. Rhodes and Karpathos had one type of haplotype (CER4) and (CER1) respectively and CER1 was only identically shared haplotype with Anatolian populations and thus, the populations from Karpathos, Rhodes and southwestern Anatolia formed a single monophyletic group [6, 7, 17]. Akın Pekşen [17] found that serum albumin intron 1 (SAI1) of frogs from Karpathos and Rhodes was similar but not identical to populations from southwestern Anatolia which exhibited *cerigensis*-specific mtDNA. This indicates that the mainland populations can be differentiated from island populations based on nuclear genes, but not on mitochondrial haplotypes. The combination of low mitochondrial DNA diversity and different nuclear diversity can be explained by male-mediated gene flow [18]. Female natal philopatry and male-biased dispersal could be seen in species [19, 20]. Additionally, different regions of mitochondrial DNA show different polymorphisms, which may reflect different levels of mtDNA genetic diversity for populations of species.

The study on the population genetic structure of *P. cerigensis* populations on Karpathos Island using AFLP markers revealed similar levels of genetic diversity and also a low but statistically significant genetic divergence between two breeding populations on Karpathos which indicates the importance of a conservation plan to maintain genetic diversity [15]. According to our mtDNA data, the *cerigensis*-lineage has a rather small distribution area on the Anatolian mainland [7, 17]. There is almost no information on the diversity of the nuclear genome of these mitochondrial lineages. Only we know from Beerli et al. [8] that Karpathos and Rhodes populations shared alleles at 30 of 31 loci, but the Rhodes population segregated for alternative alleles at 5 loci which were also seen in *P.* cf. *bedriagae* on the adjacent Anatolian mainland. They suggested that ancestral alleles could probably become extinct on the Anatolian mainland. Thus, we aim here to evaluate genetic structure and genetic diversity of this mainland population using five polymorphic microsatellite loci.

2. MATERIAL AND METHOD

2.1. Material and Laboratory Procedures

A total of 52 tissue samples were collected at 15 distinct localities in southwestern Anatolia, ranging from Antalya to Aydın provinces. The sample design was based build on the previous *P. cerigensis* specific haplotype records [17] (Table 1, Figure 1). Total genomic DNA was extracted as described by Akın et al. [7].

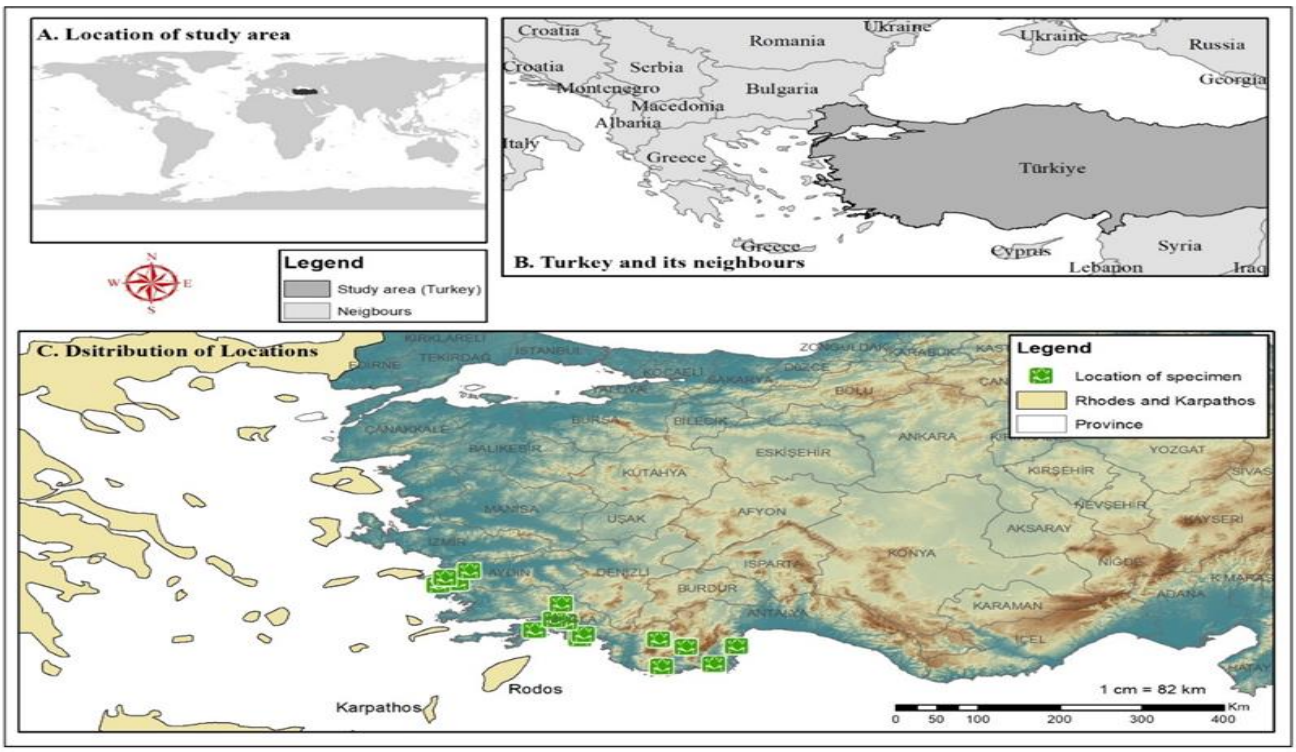

Figure 1. Distribution of the sampling sites.

Five nuclear polymorphic microsatellite loci were used to assess genetic diversity: Res5, Res16 [21], RlCA1b5, RlCA1b20 and RlCA5 [22]. Locus-specific fluorescently labelled (HEX or FAM) forward primers were used at the 5′-end in a multiplex reaction (Table 2). Because RICA5 exhibited inconclusive results, it was excluded from multiplex PCR and amplified separately.

All PCR were carried out in a final volume of 25 µl containing 5x HOT FIREPol Blend Master Mix RTL (HOT FIREPol DNA polymerase, 5x blend master mix, 12.5 mM MgCl2, 1 mM dNTP, BSA, blue and yellow dyes) (Solis BioDyne), 0.3 µM of each primer pair, and 1 µl of genomic DNA. Using the touchdown method, PCR was performed under the following conditions: 15 min of 95 °C heat activation followed by 34 cycles of 20 s at 95 °C, 1 min at 57 °C, 1 min at 72 °C, and a final extension step of 10 min at 72 °C. PCR products were checked on 2% agarose gels. PCR products were analysed on an ABI-PRISM 3100 sequencer (Applied Biosystems).

Allele sizes obtained from raw data were evaluated with PEAK SCANNER 2.0 (Applied Biosystems) by means of the GS400HD size standard. In the case of low peak signal, genotyping was repeated three times per sample.

2.2. Data Analyses of Microsatellites

Frequencies of null alleles were calculated with FREENA [23] with 10 000 replicates. Using MICROCHECKER 2.2.3 [24], genotyping errors, scoring of stutter peaks, and allelic dropout were evaluated statistically.

Genotypic linkage disequilibrium between each pair of microsatellite loci and basic statistics, e.g., number of alleles and allelic richness, were calculated using FSTAT 2.9.3 [25]. Deviations from Hardy-Weinberg equilibrium (HWE) was tested with CERVUS 3.0.7 [26, 27] and GenAlEx 6.5 [28]. Inbreeding coefficients [FIS] for each locus and population were predicted with GENEPOP 3.4 [29]. In addition, polymorphic information content (PIC), indicating the possible utility of markers in identifying individuals, was estimated with CERVUS 3.0.7 [26, 27]; where PIC > 0.5 is highly informative, 0.5 > PIC > 0.25 is highly informative, and PIC < 0.25 is somewhat informative [30].

To reveal any recent bottlenecks, the software BOTTLENECK 1.2.02 [31, 32] was used. Under the two-phase mutation model (TPM), the statistical significance of heterozygosity excess was measured by means of Wilcoxon's sign rank test; the mode-shift graphical method was applied to estimate allele frequency shift after a bottleneck event [33]. It is expected that allele carrying intermediate frequencies (about 0.1–0.2) is more widespread than alleles carrying low frequencies (< 0.1) [34]

Table 1. Locality information, samples IDs and sex of the investigated individuals (F: female; M: male)

SAMPLE ID	Sex	Locality	Latitude	Longitude
HS07165	unknown	Antalya Avlan Lake	36.5825	29.9482
CA07218	unknown	Antalya Kaş	36.2769	29.6839
CA07219	unknown			
CA1865	M	Antalya Kemer	36.5954	30.5045
CA1866	F			
CA1868	unknown	Antalya Kumluca	36.3168	30.2512
CA1801	unknown	Aydın Azap Lake	37.5844	27.447
CA1802	unknown			
CA1803	unknown			
CA1785	F	Aydın Bıyıkali pond	37.7716	27.5741
CA1787	F			
CA1788	F			
CA1789	F			
CA1796	M	Aydın Menderes River	37.5476	27.2371
CA1798	M			
CA1790	unknown	Aydın Söke water channel	37.6607	27.3087
CA1792	F			
ISCA307	unknown	Muğla Dalaman	36.7138	28.7856
ISCA308	unknown			
ISCA309	unknown			
ISCA310	unknown			
ISCA311	unknown			
ISCA312	unknown			
CA1845	F	Muğla Dalaman Tersakan	36.7794	28.8252
CA1846	M			
CA1847	M			
CA1848	M			
CA1849	M			
CA1850	unknown			
CA1851	F	Muğla Girdev Plataeu	36.7007	29.6509
CA1853	F			
CA1854	F			
CA1855	F			
CA1856	unknown			
CA1857	unknown			
CA1858	unknown			
CA1859	unknown			
CA1860	unknown			
CA1861	unknown			
CA1840	F	Muğla Köyceğiz Zaferler Village	36.9718	28.6299
CA1841	F			
CA1842	F			
CA1843	F			
CA1844	F			
AKCA48212	unknown	Muğla Marmaris	36.8467	28.2879
CA1837	unknown	Muğla Ula Nannan River	37.0129	28.5105
CA1839	unknown			
CA1830	F	Muğla Yemişendere	37.2516	28.5810
CA1832	unknown			
CA1833	M			
CA1834	M			
CA1835	unknown			

Table 2. Locus name and characterization of five polymorphic microsatellite primers used to analyse P. cerigensis population.

Locus	Primer Sequence (5' – 3')	Repeat motif	Tag	TA (0C)	Expected allele size	Observed Allele size
RES16	F:GATCCTGATTTCCTGCT R:GTTTATTTACTCTGTTCGTCTT	$(CA)_{10}$	HEX	57	102-114	114-126
RES5	F:ATACTGCCAATAAGCTGGCAATGTTTAGC R:GGCCGACTTCAAAGGGGTGCTC	$(GT)_{15}$	FAM	57	129-151	141-157
RICA1b5	F:CCCAGTGACAGTGAGTACCG R:CCCAACTGGAGGACCAAAAG	$(CA)_{17}$	HEX	57	145	122-140
RICA5	F:CTTCCACTTTGCCCATCAAG R:ATGTGTCGGCAGCTATGTTC	$(CA)_{17}$	FAM	57	250	238-264
RICA1b20	F:GGGCAGGTATTGTACTCAATATCAC R:CAACACAAGGACTCCACTGC	$(CA)_8(C)_{13}$	FAM	57	87	78-86

3. RESULTS

3.1. Reliability of Data

DNA isolation from tissue samples of 52 individuals was successfully performed (Supplementary Table S1). All microsatellite loci were amplified successfully in all individuals (Table S1). All loci proved to be polymorphic in the *P. cerigensis* population studied. No loci were detected to contain null alleles (r≤0.2) when tested with FREENA.

Looking at possible comparisons between pairs of loci in the population, no significant linkage disequilibrium was detected after Bonferroni correction (p=0.005 for 5% nominal level), and each locus was evaluated independently.

Analyses using FREE showed no evidence for the presence of null alleles among the five loci in the population, but MICROCHECKER detected null alleles which may be present due to the general excess of homozygotes for most allele size classes in loci RICA1b5 and RICA1b20. However, because this finding was not consistent with other software, we kept the locus for further analysis. There was also no evidence of genotyping errors due to stuttering or large allele dropout at any of the five loci in the study population based on MICROCHECKER.

3.2. The Genetic Diversity, Inbreeding and Bottleneck

Five nuclear loci were successfully genotyped for all samples (n=52). A total of 35 alleles were detected in all loci throughout the population. These five loci exhibited moderate genetic diversity based on number of alleles (Na), observed heterozygosity (HO) and gene diversity (Table 3). The number of alleles was the highest in loci RICA1b5 and RICA5 (Table 3). The five loci used were found to be highly informative loci (PIC > 0.5) in the *P. cerigensis* population (Table 3). The loci RICA1b5 and RICA1b20 were not in HWE. No private population-specific allele was also detected in any of the population. All the estimates of inbreeding coefficient (FIS) differed from zero, so the high signs of inbreeding were determined which means non-random mating (Table 3).

According to the Wilcoxon test, the observed proportion of heterozygotes showed no deviation from expectation under mutation-drift equilibrium using a TPM (one-tailed for H excess: P = 0.5) in the pooled sample. This indicates that the mainland *P. cerigensis* population was not going through a bottleneck. The proportion of alleles in different allele frequency classes (0-0.1 low; 0.9-1 high allele frequency class) showed the normal L-shaped distribution rather than the mode-shifting distribution that would be expected for bottleneck populations (Figure 2). This also supports the idea that the population is in mutation-drift equilibrium and is not going through a bottleneck anytime soon.

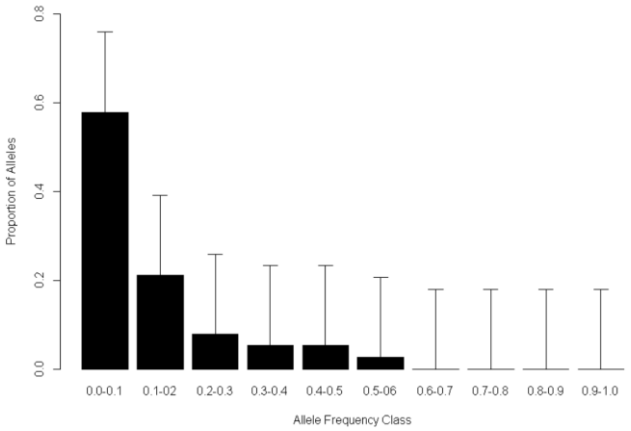

Figure 2. Mode shift of the allele frequencies indicating the occurrence of a recent genetic bottleneck.

4. DISCUSSION AND CONCLUSION

The level of heterozygosity observed was consistent with estimates from other water frog populations [35, 36]. In the study of Leuenberger et al. [36] 16 individuals yielded no amplicons for the marker RlCA5, but all water frog samples in our study were amplified in all loci. In the analysis of the *Pelophylax esculentus* complex (*Pelophylax lessonae*, *Pelophylax esculentus*, and *Pelophylax ridibundus*) and Anatolian *Pelophylax* cf. *bedriagae* revealed that four loci (RICA1b5, Res16, RICA1b20, and RICA5) from eight microsatellites are the most discriminative loci between species [35] which were all used in this study. As found in *P. lessonae* and *P. ridibundus* populations in Belgium, genetic diversity results indicated that observed heterozygosity values (Ho) for all loci (RES 16, RES 5, RICA1b5, RICA5, RICA1b20) in mainland *P. cerigensis* populations are less than expected heterozygosity values like our results. Although the limited number of individuals (about 52 individuals) were analysed in the *P. cerigensis* population in comparison with *P. lessonae* and *P. ridibundus* (both 150 individuals each), the observed heterozygosity value (0.423) is moderate and higher than those found in *P. lessonae* and *P. ridibundus* (0.319 and 0.389 respectively) [35]. Additionally, a total of 35 alleles was found in the Anatolian population. The average number of alleles per locus ranges from 5 to 13. Although Holsbeek et al., [35] studied the same five loci, they found only 21 alleles in *P. lessonae* populations and 32 alleles in *P. ridibundus* populations. Regarding the genetic diversity of *P. cerigensis* populations on Karpathos Island, there is only one study based on AFLP markers, where samples collected from two known breeding sites also revealed moderate levels of genetic diversity and differentiation [15].

The inbreeding coefficient (FIS) varied between 0.257 to 0.562, while the Hardy-Weinberg ratios were not met in the two loci which showed an excess of homozygotes for most allele size classes. This may be due to the fact that small number of individuals involved in reproduction or in the last few decades mating has been occurred among closely related animals. The heterozygote deficiency could also be explained as a Wahlund effect if population subdivision is

Table 3. Genetic diversity at five loci studied. Mean allele number (Na), observed heterozygosity (HO), expected heterozygosity (HE), polymorphic information content (PIC), Hardy-Weinberg Equilibrium (HWE) and inbreeding coefficient (Fıs) values.

	Locus	N	Na	Ho	H_E	Gene diversity	PIC	HWE	F_{is}
cerigensis	**RES16**	52	6	0.442	0.588	0.595	0.533	NS	0.257
	RES5	52	6	0.404	0.647	0.656	0.578	NS	0.384
	RICA1b5	52	8	0.462	0.723	0.733	0.678	***	0.370
	RICA5	52	13	0.519	0.876	0.888	0.863	NS	0.415
	RICA1b20	52	5	0.288	0.649	0.659	0.592	***	0.562
Mean		**52**	**7.6**	**0.423**	**0.697**	**0.706**	**0.648**		**0.401**

NS: non-significant; ***: significant (with Bonferroni correction, P value<0.001)

occurring, linkage with loci under selection (genetic hitchhiking), population heterogeneity, null alleles (non-amplifying alleles) or inbreeding. Positive FIS value suggested inbreeding to be one of the main causes for shortage of heterozygotes in this population [37]. Therefore, a significant inbreeding event (Fıs>0.25) can be true for *P. cerigensis* population since they were found range within the restricted geographic region from Antalya province to Aydın province (i.e. only the southwestern Anatolia), a fact that was previously derived from mitochondrial ND2 and ND3 and nuclear SAI-1+*Rana*CR1 data [17], but also now supported by specific allele sizes of RES16 and RICA1b5 microsatellite loci. In the study of Coltman and Slate [38], to detect inbreeding depression on a life history trait using microsatellites have recruited more than 600 individuals to detect an average effect size (r = 0.10) with reasonable statistical power (0.80) shows that sampling is required. However, as known, very few published studies have used sample sizes approaching this value. On the other hand, Hale et al. [39] showed that there appears to be little benefit in sampling more than 25 to 30 individuals per population for population genetic studies based on microsatellite allele frequencies. The mainland *P. cerigensis* population is bounded in the north, west and east by two other Anatolian water frog lineages *Pelophylax* cf. *caralitanus* and *P.* cf. *bedriagae*. Therefore *P. cerigensis* population was unable to expand further and got entrapped within this region (possibly also due to mountain barriers). This would lead to increased inbreeding and excess homozygosity, and hence, moderate levels of heterozygosity within populations. However, a full understanding of the consequences of inbreeding in wild populations would require not only detection of inbreeding but also long-term measurements of reproductive success and survival [40, 41].

We detected no signs of a genetic bottleneck. This finding is not surprising since southwestern Anatolia, where *P. cerigensis* population is located, is considered an important refuge for many animal and plant species during the Quaternary ice ages. Populations that could sustain themselves during glacial periods often spread throughout Europe and the Caucasus during the following interglacial intervals [42]. Due to the mountainous topography up to 3000 m, densely forested areas could have acted as a biodiversity center for certain Anatolian water frogs (*Pelophylax*) lineages. Several other reptile and amphibian species, for example *Lyciasalamandra flavimembris* [43], *Lyciasalamandra fazilae* [44], *Ophiomorus kardesi* [45], and *Anatololacerta pelasgiana* (Mertens, 1959) are known to be largely restricted in the same geographic area [46].

Phylogenetic studies based on one or two mitochondrial genes [6, 7, 15, 17) and complete mitogenomes [16] revealed that *P. cerigensis* occurs on Karpathos Island but is also present in Rhodes Island. Interestingly, Akın et al. [7] and Akın Pekşen [17] indicated that all *P. cerigensis* specific haplotypes from Karpathos, Rhodes and southwestern Anatolia formed a single monophyletic group. However, analyses of nuclear SAI-1+*Rana*CR1 gene Akın Pekşen [17] revealed that southwestern Anatolian population (*P. cerigensis*) on the other hand, and Karpathos and Rhodes Island populations on the others have distinct albumin alleles, and they formed two distinct but closely related clades.

Geological studies indicate that Karpathos separated from Rhodes and Asia Minor during the Pliocene [47, 8], and it is believed that Rhodes served as a springboard for the colonisation and spread of species from Asia Minor (mainland Anatolia) [48]. However, the results show that the mainland and island populations could be differentiated in terms of nuclear markers, but still have the shared mitochondrial haplotypes from most probably human mediated dispersal like that seen in Cyprus water frog populations [49]. These results clearly show the need for a taxonomic reassessment of *P. cerigensis* using more samples from island populations of Karpathos and Rhodes by applying and nuclear markers as a very limited sample were originated from those islands.

In conclusion, both mtDNA and nuclear marker [17] showed that Karpathos and Rhodes Islands and mainland populations of *P. cerigensis* are closely related. Because of their restricted range and moderate genetic diversity of *P. cerigensis* population in southwestern Anatolia, they should be a considered as a third Management Unit (MUs) in addition to those as previously suggested for Karpathos and Rhodes populations [16]. It is crucial to develop and implement appropriate conservation strategies to assure future survival and conservation of these threatened *P. cerigensis* populations.

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Appendices

Appendix A. Table S1

Table S1. Sample ID and microsatellite profiles for 52 individuals.

ID	RES 16		RES 5		RICA1b5		RICA5		RICA1b20	
1842	118	118	143	143	124	124	242	254	82	82
1843	114	118	143	143	122	122	254	254	80	80
1847	114	118	141	145	122	130	240	262	82	82
1848	114	118	141	145	122	122	240	240	82	82
1854	118	118	141	141	124	130	242	258	84	84
1855	114	118	141	143	122	130	258	258	82	82
1856	118	118	143	143	124	130	250	264	82	82
1865	118	118	143	153	126	128	250	254	80	80
1868	118	118	153	157	124	128	242	252	82	82
1840	118	118	143	143	122	122	254	254	80	84
1841	118	118	141	143	122	122	240	254	80	80
1845	118	118	143	143	122	122	262	262	80	86
1846	114	118	143	143	122	132	262	262	82	82
1851	118	122	141	143	128	128	250	250	82	84
1853	114	124	143	143	128	128	248	250	86	86
309	116	118	143	143	122	122	240	254	80	86
310	118	118	141	143	122	122	242	254	82	82
311	118	118	143	143	124	124	238	244	80	84
312	114	118	141	143	122	122	238	242	82	82
218	114	118	141	143	122	130	240	240	80	84
219	114	118	141	143	130	130	240	240	82	82
212	118	118	141	143	130	130	242	242	82	82
165	118	118	141	141	128	140	250	262	80	86
308	114	118	143	143	122	122	254	254	82	86
307	114	118	143	143	122	122	254	254	82	86
1830	114	118	141	141	130	130	240	240	80	80
1832	118	118	141	145	130	132	242	250	82	82
1833	118	118	141	145	130	130	242	242	82	86
1834	118	118	141	145	130	130	242	254	82	82
1835	118	118	141	141	130	130	248	248	80	80
1837	118	118	145	145	122	122	250	250	82	86
1839	114	118	143	143	122	130	260	260	82	86
1844	116	118	143	143	122	122	240	240	82	82
1849	118	118	143	145	122	122	240	242	82	82
1850	114	118	145	145	122	122	240	242	82	82
1857	114	118	141	143	122	130	250	250	80	86
1858	114	116	141	143	128	130	244	250	86	86
1859	118	118	141	141	124	130	242	258	82	82
1860	118	118	143	143	124	124	240	248	82	82
1861	118	124	143	143	128	130	250	250	82	82
1866	118	126	151	153	128	132	254	264	80	80
1796	116	116	141	141	122	130	240	240	80	80
1798	114	116	141	141	122	130	240	244	80	80
1801	116	116	141	141	124	124	246	264	82	82
1802	114	114	141	141	124	130	246	246	82	82
1803	114	114	141	141	124	130	242	248	82	82
1785	116	116	145	145	122	122	244	250	80	80
1787	114	114	141	141	130	130	242	248	80	80
1788	116	116	141	141	124	130	242	242	80	86
1789	116	116	141	145	124	134	244	248	80	82
1790	114	114	141	145	122	130	248	248	80	80
1792	116	118	141	141	122	130	244	244	78	78