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Biochemical Systematics and Ecology 36 (2008) 297–311
biochemical systematics and ecology
www.elsevier.com/locate/biochemsyseco

Genetic structure of the alpine newt, *Mesotriton alpestris* (Salamandridae, Caudata), in the southern limit of its distribution: Implications for conservation

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Received 10 July 2007; accepted 5 October 2007

Abstract

Tissue samples of *Mesotriton alpestris veluchiensis* were collected from 11 localities in Greece, and the sequences of two mitochondrial genes (cytochrome *b* and 16S rRNA), as well as frequencies of 18 allozyme loci, were used in order to describe levels and patterns of genetic variation, identify possible evolutionary units, and reveal aspects of their conservation status. Two major lineages, displaying considerable amount of genetic differences, were supported by both analyses. These lineages, which have been geographically separated since the middle Pleistocene, constitute separate Evolutionary Significant Units (ESUs) and correspond to populations from the Greek mainland and Peloponnisos, respectively. The particularly high inter-population differentiation within each region implies long-term isolation in fragmented habitats, while severe bottlenecking is proposed to have resulted in the observed lack of heterozygotes in the majority of populations. Conservational implications are also discussed, particularly in relation to environmental factors and human activities, which seem to have contributed to the genetic impoverishment of the most marginal populations studied.

Keywords: *Mesotriton alpestris veluchiensis*; Allozymes; mtDNA; Genetic variation; Conservation units; Greece

1. Introduction

The use of molecular markers for estimating levels of intraspecific and intra-population genetic diversity, which seriously decreases the long-term survival probability of a population when lost, has been recognized by many authors (Avise, 1994, 1995; Newman and Pilson, 1997; Hedrick, 2000; Frankham et al., 2002; Reed and Frankham, 2003). Also, the tracking of intraspecific genetic differentiation – through diverse molecular characters such as nuclear and mitochondrial genes – has been proposed as a tool for the identification of population groups requiring

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0305-1978/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bse.2007.10.002

conservation (e.g. Avise, 1989; Goldstein et al., 2000). The delimitation and ranking of units that merit conservation below the species level (Evolutionary Significant Units and Management Units) are complicated on both theoretical and practical grounds (Ryder, 1986; Moritz, 1994a,b; Crandall et al., 2000; Fraser and Bernatchez, 2001). However, according to Moritz (1994a), subspecific entities that are reciprocally monophyletic for mtDNA haplotypes, and differ significantly in nuclear allele frequencies, fulfill the criteria for an ESU. Therefore, an ESU is a group of organisms that has been isolated from other conspecific groups for a period of time, capable of producing meaningful genetic divergence between those groups (Ryder, 1986; Paetkau, 1999). This concept is useful for the recognition and conservation of population clusters of appreciable genetic diversity, with an ultimate goal to maximally preserve biological diversity, as a definitive factor of species persistence over evolutionary time (Avise, 1994, 1995).

Considerable attention has been given to populations inhabiting the margins of species ranges. The risk of extinction in peripheral areas is highest and population densities are often lower than elsewhere (Hoffmann and Blows, 1994; Lesica and Allendorf, 1995), which predestines these populations to bottleneck events. This leads to the prediction that neutral genetic variations will be depleted in peripheral populations. However, marginal populations may contribute to a species' evolutionary potential (Gaston, 1998), since the suboptimal environmental conditions in peripheral populations will promote adaptation to local conditions, thus providing a source of adaptively significant variations (Garcia-Ramos and Kirkpatrick, 1997). The disagreement concerning the significance of these populations is still unresolved and requires further studies on the genetic variation in the periphery of species ranges.

Among amphibians, most urodeles express limited dispersal capabilities and show substantial, and often ancient, genetic structure at a relatively small spatial scale (Arano et al., 1991; Ward et al., 1992; Weisrock et al., 2001; Babik et al., 2005; Sotiropoulos et al., 2007).

The alpine newt (*Mesotriton alpestris*) inhabits central and eastern Europe and some parts of the Iberian, Italian and Balkan peninsulas (Fig. 1) (Zuiderwijk, 1997). Recent molecular studies identified numerous genetic lineages of *M. alpestris* in Europe, and revisited the relationships between existing phylogenetic entities (Sotiropoulos et al., 2007).

In Greece, which is the southernmost margin of the species range, the alpine newt forms three distinct mitochondrial lineages. Two of them are supposed to represent ssp. *veluchiensis* and are restricted to the central mainland and northern Peloponnisos. Thus, populations from the Greek mainland and Peloponnisos constitute the southern periphery of the species, and, particularly in the case of the later, exhibit a significant degree of isolation.

In this study, we sampled 11 local populations of *Mesotriton alpestris veluchiensis* (Table 1, Fig. 1). Also, the sequences of two mitochondrial gene fragments (cytochrome *b* and 16S rRNA) along with allelic frequencies of 18 allozyme loci were used in order to: (i) measure the extent of intraspecific genetic variation, (ii) detect divergent lineages or clades, and (iii) describe the intra- and inter-population genetic structure. The resulting patterns were connected to the geological history of the area, as well as to local environmental factors, in order to specify any Evolutionary Significant Units and to address the conservation necessities of the populations studied.

2. Materials and methods

2.1. DNA analysis

Muscle samples or tail tips, along with already published sequences (Sotiropoulos et al., 2007), were obtained from 33 individuals of *M. alpestris* from 11 localities in Greece (Table 1, Fig. 1). Tissue samples were homogenized in a digest buffer and total genomic DNA was extracted using Proteinase K dissolution. Samples were then purified by two extractions with phenol/chloroform/isoamyl alcohol (25:24:1), one with chloroform/isoamyl alcohol (24:1), and precipitated using isopropanol.

Two target genes were selected for molecular phylogenetic analysis: (1) a partial sequence (309 bp) of the mitochondrial protein-encoding cytochrome *b* gene (cyt *b*) and (2) a partial sequence of the mitochondrial gene encoding 16S rRNA (16S). The universal L14841 and H15149 primers (Kocher et al., 1989) were used to amplify the cyt *b* region of the mtDNA. The polymerase chain reaction (PCR) was performed as follows in the presence of 3 mM $MgCl_2$: 35 cycles of denaturation at 94 °C for 45 s, annealing at 47 °C for 45 s, and extension at 72 °C for 60 s. Primers 16Sar-L and 16Sbr-H (Palumbi et al., 1991) were used to amplify a segment of approximately 499 bp from the 16S rRNA region of the mtDNA, according to the following PCR profile and in the presence of 3 mM $MgCl_2$: 35 cycles of denaturation at 94 °C for 60 s, annealing at 47 °C for 60 s, and extension at 72 °C for 60 s. The light strands were sequenced using an ABI Prism 377 DNA fragments analyser by Macrogen Inc.

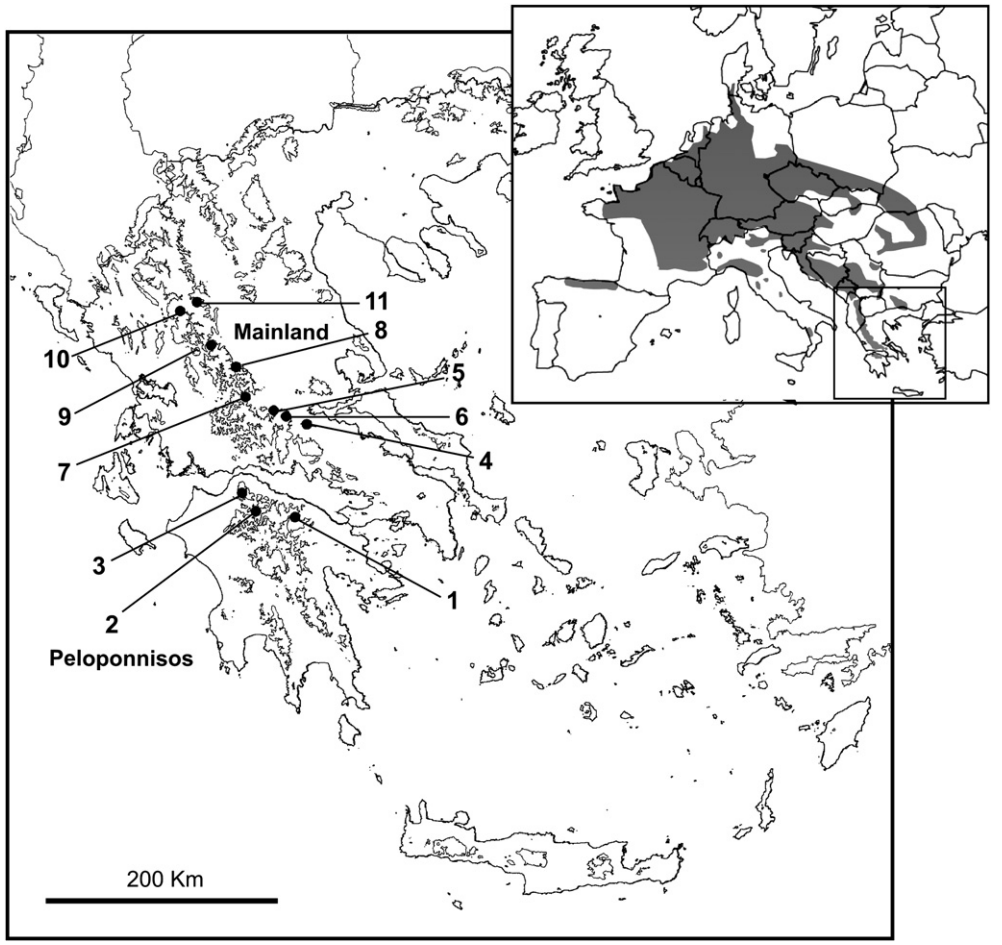

Fig. 1. Sampling locations of *Mesotriton alpestris veluchiensis* in Greece. The thin line represents the 1000 m contour (isophene). Population numbers correspond to those presented in Table 1. The European distribution of *M. alpestris* is marked by dark gray (from Sotiropoulos et al., 2007).

The alignment of the concatenated cyt *b* and 16S rRNA sequences was performed with Clustal X (Thompson et al., 1997) and corrected by eye. Additionally, the 16S was aligned based on its secondary structure, to facilitate proper alignments. Although some alignment gaps were inserted to resolve length differences between sequences, all positions could be unambiguously aligned and were therefore included in the analyses. Cytochrome *b* sequences were translated into amino acids prior to analysis and did not show any stop codons, suggesting that probably all were functional.

To examine whether the sequences from the two genes should be combined in a single analysis, a partition-homogeneity test was run in PAUP (v. 4.0b10, Swofford, 2002), and significance was estimated by 1000 repartitions. This test, which was described as the incongruence-length difference test by Farris et al. (1995), indicated no conflicting phylogenetic signals between the data sets ($p = 0.43$) and, given that the mtDNA genes are linked, data sets from both genes were analyzed together.

In order to visualize the relationships among the detected haplotypes, a median joining haplotype network (Bandelt et al., 1999) was constructed with the software Network 4.2.2 (available on the web at: http://www.fluxus-engineering.com).

Between-population uncorrected sequence divergences (*p*-distance) were estimated using the MEGA computer package (v. 3.1, Kumar et al., 2004). Additionally, haplotype and nucleotide diversity values within each recognized clade were calculated with ARLEQUIN v. 2001 (Schneider et al., 2000).

Table 1
Sampling localities and sample size (*N*) of *Mesotriton alpestris* specimens used in the study (see also Fig. 1)

ID	Locality	Altitude (m)	Humidity (%)	Pond seasonality	Coordinates	*N* (allozymes/DNA)	Haplotypes	Accession Nr. cyt *b*	Accession Nr. 16S rRNA
1	Kyllini Mt.	1600	65.0	Temporary	37°56′N 22°25′E	5/4	I (3) II (1)	DQ481513* EF650001	DQ481488* EF649992
2	Kalavryta	820	67.5	Temporary	38°03′N 22°02′E	19/4	IV (3) V (1)	EF650003 EF650004	EF649994 EF649995
3	Panachaiko Mt.	1150	67.5	Permanent	38°10′N 21°54′E	14/4	III (1) IV (3)	EF650002 DQ481514*	EF649993 DQ481489*
4	Kallidromo Mt.	980	66.3	Temporary	38°45′N 22°33′E	10/2	VI	EF650005	EF649996
5	Oiti Mt. 1	1805	67.5	Permanent	38°50′N 22°17′E	16/2	VII VIII	EF650006 EF650007	EF649997 EF649998
6	Oiti Mt. 2	1530	67.5	Temporary	38°46′N 22°19′E	25/3	IX	DQ481515*	DQ481490*
7	Velouchi Mt.	1890	67.5	Permanent	38°57′N 21°49′E	27/4	IX	DQ481516*	DQ481491*
8	Belokomitis	950	67.5	Temporary	39°16′N 21°44′E	16/2	X	EF650008	EF649999
9	Elati (Kerketio Mt.)	1050	70.0	Permanent	39°31′N 21°31′E	20/3	XI	DQ481517*	DQ481492*
10	Aoos' springs lake	1350	71.3	Permanent	39°50′N 21°12′E	22/2	XII	EF650009	EF650000
11	Zygos Mt.	1450	71.3	Permanent	39°53′N 21°17′E	23/3	XII	DQ481518*	DQ481493*

Asterisks indicate sequences that were available from a previous study (Sotiropoulos et al., 2007). Humidity refers to the mean annual value of the respective localities.

2.2. Allozyme analysis

A total of 197 alpine newts from 11 localities in Greece (Fig. 1; Table 1) were examined in order to estimate levels of genetic variation and differentiation among local populations. Allozyme frequency data were obtained using Vertical Polyacrylamide Gel Electrophoresis (PAGE) and Cellulose Acetate Gel Electrophoresis (CAGE). Eighteen loci (Est-1, Est-2, Est-3, Est-4, G3pdh-1, G3pdh-2, G6pdh, Gdh, Gpi-1, Ldh-1, Ldh-2, Mdh-1, Pgm, Prot-1, Prot-2, Prot-3, Sdh, and Xdh), corresponding to 11 enzyme systems, were scored under standard histochemical procedures (Murphy et al., 1996). The mean number of alleles per locus (A), proportion of polymorphic loci (P), mean observed heterozygosity (Ho) and Nei's (1978) unbiased expected heterozygosity (He) were calculated by means of the program BIOSYS-1 (Swofford and Selander, 1981).

Departure from Hardy–Weinberg expectations was tested using the GENEPOP package, v. 3.1 (Raymond and Rousset, 1995). Estimation of the Hardy–Weinberg exact probability was performed for each locus in each population by the complete enumeration method (Louis and Dempster, 1987). A Fischer test was used to combine information over loci and over populations. The sequential Bonferroni correction (Rice, 1989) was employed over the multiple tests carried out.

Wright's F-statistics F, θ and f (estimators of F_{IT}, F_{ST} and F_{IS}, respectively) according to Weir and Cockerham (1984) were calculated with associated bootstrap estimates for determination of the 95% confidence interval (CI), employing FSTAT 2.9 (Goudet, 1995). Standard errors of the estimates were calculated by jackknifing over samples.

Genetic distances were calculated using Nei's unbiased method (Nei, 1978) using the package BIOSYS-1 (Swofford and Selander, 1981). Phylogenetic trees were constructed with the package PHYLIP 3.5 (Felsenstein, 1993). We used the neighbour-joining (Saitou and Nei, 1987) method based on Nei's (1978) genetic distances.

To complete a detailed investigation of the relationships among *M. alpestris* populations we also performed Principal Component Analysis (PCA) on allele frequencies by means of the package PCAGEN 1.2.1 (written by J. Goudet, http://www.unil.ch/izea/softwares/pcagen.html).

In order to investigate possible spatial patterns of genetic variation levels, we performed a multiple regression between genetic variation parameters and an array of seven potential explanatory variables (i.e. altitude, latitude, longitude, mean annual temperature, mean annual rainfall, humidity, and pond size) employing STATISTICA (Stat Soft Inc. 1997, Tulsa). Additionally, we tested the influence of pond seasonality (permanent vs. temporary ponds) and the presence of competitors (i.e. presence of other newt species) on the genetic variation levels. Mann–Whitney U-tests tested differences between means. All means are given with their standard deviations.

2.3. Demographic analysis

The demographic history of clades with sufficient numbers of sequences available was assessed by a mismatch distribution analysis, following Schneider and Excoffier (1999) with ARLEQUIN v. 2.001 (Schneider et al., 2000). Recent growth is expected to generate a unimodal distribution of the pairwise differences between sequences (Rogers and Harpending, 1992). The distribution is compared with that expected under a model of population expansion (Rogers, 1995). We report the values of Theta0, Theta1 and Tau, which are the estimators of theta before and after expansion, and the time since expansion (measured in mutational time units), respectively. These values were estimated using generalized nonlinear least squares. The goodness-of-fit to the sudden expansion model was tested using Monte Carlo simulations of 1000 random samples. The sum of squared deviations between observed and expected mismatch distributions was used as a test statistic and its p value represents the probability of obtaining a simulated sum of squared deviations larger than or equal to the observed number.

The history of effective population size was also assessed by means of other statistics including Tajima's D test (Tajima, 1989) and Fu's Fs test (Fu, 1997).

3. Results

3.1. DNA analysis

Of the 808 sites examined, there were 10 variable cyt *b* sites (eight of which were parsimony-informative), and 12 variable 16S rRNA sites (five of which were parsimony-informative). Twelve different haplotypes were identified

from among 33 *M. alpestris* sequences (Table 1). Three haplotypes were found in more than one population, while four populations showed more than one haplotype (Table 1).

For cyt *b*, between-population uncorrected sequence divergence (*p*-distance) ranged from 0.0 to 2.0%, while for 16S rRNA sequence divergence ranged between 0.0 and 1.4% (Table 2).

All *M. alpestris* haplotypes form two well-defined clades, which are in accordance with the geographical origin of the specimens: the ''mainland'' clade, containing populations from the central mainland and the ''Peloponnisos'' clade comprising Peloponnesian populations (Fig. 2A). The two clades express considerable genetic divergence (1.5 and 0.8% for cyt *b* and 16S, respectively). Within the mainland clade (eight populations with seven different haplotypes), mean genetic divergence was found to be 0.5 and 0.2% for cyt *b* and 16S, respectively. The three localities from Peloponnisos (clade ''Peloponnisos'') represent five different haplotypes that express a considerable amount of divergence in both gene fragments (0.3 and 0.1% for cyt *b* and 16S, respectively) (Table 2). Within the mainland clade (21 sequences constitute seven haplotypes), haplotype divergence (Hd) was 0.816 ± 0.058 SD and nucleotide divergence (Pi) was 0.00315 ± 0.00043. Within Peloponnisos (12 sequences constitute five haplotypes), Hd and Pi was 0.727 ± 0.113 and 0.00210 ± 0.00058, respectively.

3.2. Allozyme analysis

Four out of 18 gene loci were found to be monomorphic throughout the studied populations (Est-1, Gdh, Ldh-1, and Mdh-1). The remaining 14 loci showed more than one allele in at least one of the populations (Table 3). A total of 45 alleles were detected. All polymorphic loci showed banding patterns consistent with autosomal inheritance and with known quaternary structures (Richardson et al., 1986). A significant departure from Hardy–Weinberg equilibrium was detected over all populations and loci (Fisher test, $\chi^2_{156} = 867.9$, $p < 0.001$). Ten populations (Table 3) showed significant deviation from HW expectations against heterozygotes (Fisher test, $p < 0.01$). While, over all populations, the genotypic frequencies of 10 loci (Est-4, G3pdh-1, G6pdh, Gpi-1, Ldh-2, Prot-1, Prot-2, Prot-3, Sdh, and Xdh) deviated significantly from HW equilibrium (Fisher test, $p < 0.05$).

The mean number of alleles per locus (*A*) ranged from 1.2 to 2.0 (mean $= 1.6 \pm 0.2$ SD). The percentage of polymorphic loci (*P*) ranged from 22.2 to 72.2% (mean $= 56.1 \pm 14.2$% SD). Observed heterozygosity (Ho) ranged from 0.083 to 0.222 (mean $= 0.124 \pm 0.038$ SD) and expected heterozygosity (He) ranged from 0.106 to 0.312 (mean $= 0.210 \pm 0.049$ SD) (Table 3).

The *F*-statistics indicated apparent intra-population substructuring ($F_{IS} = 0.434 \pm 0.105$ SE, CI $= 0.231$–0.618, $F_{IT} = 0.628 \pm 0.072$ SE, CI $= 0.485$–0.757) and significant inter-population heterogeneity ($F_{ST} = 0.344 \pm 0.045$ SE, CI $= 0.274$–0.442) (Table 3). All values were significantly different from zero.

The unbiased genetic distances (Nei, 1978) between samples ranged from 0.009 to 0.416 (mean $= 0.195 \pm 0.122$ SD) (Table 4). The Neighbour-joining phenogram (Fig. 2B) revealed a remarkable division to two geographic groups of populations. The genetic distance between-groups averaged 0.318 ± 0.064 SD. The first group includes all mainland populations (mean $D_{NEI} = 0.100 \pm 0.051$ SD). Populations from Peloponnisos were clustered within the second

Table 2
p-Distances (%) among populations studied

Population ID	1	2	3	4	5	6	7	8	9	10	11
1	0.0/0.3	0.6	0.6	1.9	1.6	1.0	1.0	1.3	1.6	1.6	1.6
2	0.2	0.2/0.1	0.1	2.0	1.7	1.5	1.5	1.4	1.7	1.7	1.7
3	0.2	0.1	0.2/0.0	2.0	1.7	1.5	1.5	1.4	1.7	1.7	1.7
4	1.4	1.2	1.3	0.0/0.0	1.0	1.0	1.0	0.6	1.0	1.0	1.0
5	1.0	0.8	0.9	0.6	0.0/0.4	0.6	0.6	0.3	0.6	0.6	0.6
6	0.8	0.6	0.7	0.6	0.2	0.0/0.0	0.0	0.3	0.6	0.6	0.6
7	0.8	0.6	0.7	0.6	0.2	0.0	0.0/0.0	0.3	0.6	0.6	0.6
8	1.2	1.0	1.1	0.6	0.4	0.4	0.4	0.0/0.0	0.3	0.3	0.3
9	0.8	0.6	0.7	0.6	0.2	0.0	0.0	0.4	0.0/0.0	0.6	0.6
10	1.0	0.8	0.9	0.4	0.2	0.2	0.2	0.2	0.2	0.0/0.0	0.0
11	1.0	0.8	0.9	0.4	0.2	0.2	0.2	0.2	0.2	0.0	0.0/0.0

Population numbers correspond to those presented in Table 1 and Fig. 1. Above diagonal: cyt *b*; below diagonal: 16S; diagonal: within population *p*-distance (cyt *b*/16S).

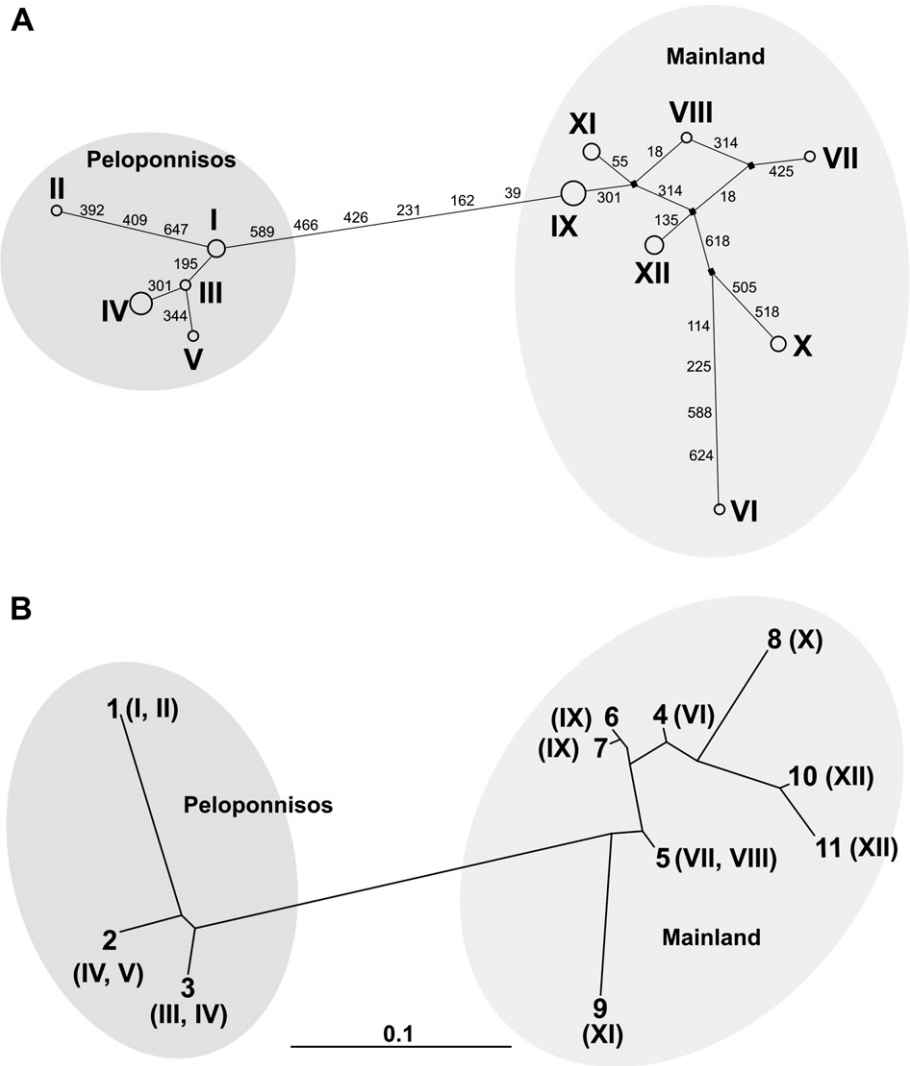

Fig. 2. (A): median joining network connecting haplotypes (Roman numerals) found in the populations studied. Positions defining haplotypes are mapped onto the network. The size of the ellipses representing haplotypes is proportional to their observed frequency. (B): neighbour-joining tree based on Nei's (1978) unbiased genetic distances of the 11 analyzed populations of *Mesotriton alpestris veluchiensis* from Greece. The two genetic lineages cluster at $D = 0.318$. Roman numerals represent mtDNA haplotypes found in each population. Population locations are presented in Table 1 and Fig. 1.

group (mean $D_{NEI} = 0.100 \pm 0.036$ SD). Principal Component Analysis showed an identical discrimination of these two genetic groups (Fig. 3). The PCA on 45 allele frequencies produced one highly significant vector ($p < 0.001$), which summarized 46.4% of the detected genetic variation, separating Peloponnesian (+ loadings) from mainland (− loadings) populations (Fig. 3). The separation was due to the major contribution of the following alleles: Prot-2C, Est-2C, Est-3A, Est-3B, Est-4A, G3pdh-2A, and G3pdh-2B.

Among mainland populations, F_{ST} was high and significantly different from zero: $F_{ST} = 0.243 \pm 0.046$ SE (CI = 0.162−0.341) (Table 3). Similarly, F_{ST} was found to be significantly different from zero among Peloponnesian populations: $F_{ST} = 0.231 \pm 0.082$ SE (CI = 0.068−0.373).

A significant positive correlation of the mean number of alleles per locus (A) was observed in relation to the mean annual humidity of the corresponding localities. Populations from the less humid areas (in the east-southeast) expressed lower levels of variation (model $R^2 = 0.967$; Beta in = 3.091; Partial correlation, $r = 0.95$; $p = 0.012$).

Table 3
Allele frequency, average number of alleles per locus (*A*), average number of polymorphic loci (*P*), average observed (Ho) and average expected heterozygosity (He)

Locus		Allele	Populations										
			1	2	3	4	5	6	7	8	9	10	11
Est-2	*N*		5	19	14	10	16	25	27	16	20	22	23
		A	–	0.184	0.286	1.000	0.563	0.860	0.741	0.625	0.800	0.955	0.565
		B	–	–	–	–	0.313	0.100	0.185	0.250	0.050	0.045	0.435
		C	1.000	0.816	0.714	–	–	–	–	0.125	0.150	–	–
		D	–	–	–	–	–	0.040	0.074	–	–	–	–
		E	–	–	–	–	0.125	–	–	–	–	–	–
Est-3	*N*		5	19	14	19	16	25	27	16	20	22	23
		A	1.000	1.000	1.000	0.211	0.563	0.420	0.481	0.250	0.200	0.273	0.543
		B	–	–	–	0.789	0.438	0.580	0.519	0.750	0.800	0.727	0.457
Est-4	*N*		5	19	14	14	14	24	23	16	20	22	23
		A	–	–	–	0.714	0.500	0.667	0.500	0.875	0.700	0.864	0.739
		B	0.500	0.421	0.714	0.143	0.143	0.104	0.500	–	–	–	–
		C	–	–	–	–	0.071	–	–	0.125	0.200	0.136	0.261
		D	–	–	–	0.143	0.286	0.229	–	–	0.100	–	–
		E	0.500	0.579	0.286	–	–	–	–	–	–	–	–
G3pdh-1	*N*		3	9	12	9	12	21	12	16	16	22	23
		A	1.000	1.000	0.667	0.889	0.917	0.952	0.958	0.125	0.375	0.773	1.000
		B	–	–	0.333	0.111	0.083	0.048	0.042	0.875	0.625	0.227	–
G3pdh-2	*N*		3	9	14	12	12	25	23	16	20	22	23
		A	–	–	–	0.875	0.667	0.960	1.000	1.000	0.050	1.000	1.000
		B	1.000	1.000	1.000	0.125	0.333	0.040	–	–	0.950	–	–
G6pdh	*N*		5	17	14	19	16	23	27	16	16	22	23
		A	–	0.647	1.000	0.526	0.563	0.609	0.630	0.875	0.125	0.432	0.304
		B	–	–	–	0.474	0.438	0.304	0.370	0.125	0.875	0.250	0.130
		C	1.000	0.353	–	–	–	0.087	–	–	–	–	–
		D	–	–	–	–	–	–	–	–	–	0.318	0.565
Gpi-1	*N*		5	19	14	19	16	25	25	16	20	22	23
		A	0.600	0.632	0.286	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
		B	0.400	0.368	0.714	–	–	–	–	–	–	–	–
Ldh-2	*N*		5	19	14	19	16	25	27	16	20	22	23
		A	1.000	1.000	1.000	1.000	1.000	0.860	1.000	1.000	0.700	0.636	0.500
		B	–	–	–	–	–	0.140	–	–	0.300	0.364	0.500
Pgm	*N*		5	19	14	19	16	25	27	16	20	22	23
		A	1.000	0.947	0.929	0.947	0.688	0.900	0.815	0.938	0.700	0.955	0.804
		B	–	–	–	0.053	0.313	0.100	0.185	0.063	0.300	0.045	0.196
		C	–	0.053	0.071	–	–	–	–	–	–	–	–

Prot-1	*N*		5	19	14	16	16	25	27	16	20	22	23	
		A	1.000	0.263	0.857	1.000	0.500	0.960	1.000	0.938	0.800	0.750	0.913	
		B	–	0.737	0.143	–	0.500	0.040	–	0.063	0.200	0.250	0.087	
Prot-2	*N*		5	11	14	14	12	22	21	16	20	22	23	
		A	0.700	0.545	0.643	0.357	0.500	0.750	0.786	0.375	0.500	0.045	0.174	
		B	0.300	0.455	0.357	–	–	–	–	–	–	–	–	
		C	–	–	–	0.643	0.500	0.250	0.214	0.625	0.500	0.955	0.826	
Prot-3	*N*		5	19	14	16	16	25	27	16	18	22	23	
		A	0.800	0.526	0.357	0.625	–	0.260	0.407	0.375	–	0.841	0.957	
		B	0.200	0.474	0.643	0.375	0.563	0.620	0.593	0.625	0.778	0.159	0.043	
		C	–	–	–	–	0.438	0.120	–	–	0.222	–	–	
Sdh	*N*		5	13	14	12	12	25	27	16	20	22	23	
		A	1.000	1.000	1.000	0.542	0.750	0.840	0.722	1.000	0.950	0.727	1.000	
		B	–	–	–	0.458	0.083	0.140	0.278	–	–	0.273	–	
		C	–	–	–	–	0.167	0.020	–	–	0.050	–	–	
Xdh	*N*		5	19	14	19	16	25	27	16	20	22	23	
		A	–	0.737	0.500	0.842	0.813	0.980	1.000	0.375	0.700	0.545	0.565	
		B	1.000	–	0.357	0.158	0.188	0.020	–	0.625	0.300	0.455	0.435	
		C	–	0.263	0.143	–	–	–	–	–	–	–	–	
														Mean ± SD
A			1.2	1.5	1.6	1.6	2.0	1.9	1.6	1.6	1.8	1.7	1.6	1.6 ± 0.2
P			0.222	0.500	0.500	0.556	0.722	0.667	0.500	0.556	0.722	0.667	0.556	0.561 ± 0.142
Ho			0.111	**0.083**	**0.095**	**0.087**	**0.129**	**0.222**	**0.111**	**0.139**	**0.136**	**0.114**	**0.138**	**0.124 ± 0.038**
He			0.106	**0.205**	**0.206**	**0.201**	**0.207**	**0.312**	**0.197**	**0.184**	**0.252**	**0.221**	**0.215**	**0.210 ± 0.049**
F_{IS}			−0.053	0.602[a]	0.547[a]	0.554[a]	0.297[a]	0.382[a]	0.443[a]	0.250	0.467[a]	0.492[a]	0.364[a]	

F_{IT} all	0.628 ± 0.072	(0.485−0.757)
F_{IT} main	0.558 ± 0.092	(0.366−0.715)
F_{IT} pel	0.653 ± 0.129	(0.366−0.855)
F_{ST} all	0.344 ± 0.045	(0.274−0.442)
F_{ST} main	0.243 ± 0.046	(0.162−0.341)
F_{ST} pel	0.231 ± 0.082	(0.068−0.373)

Mean F_{IT} and F_{ST} values (Weir and Cockerham, 1984) with ±SE and 95% CI in parenthesis. Significant departure from HW expectations is given in bold. Population numbers correspond to those presented in Table 1 and Fig. 1.

all: Values for all populations; main: values for mainland populations; pel: values for Peloponnesian populations.

[a] Associate *p* values significant after Bonferroni corrections (5000 randomizations, indicative adjusted nominal level (5%) for one table is 0.00025).

Table 4
Nei's (1978) unbiased genetic distance between-populations studied, based on the allele frequencies of 18 allozyme loci

Population ID	1	2	3	4	5	6	7	8	9	10	11
1	–										
2	0.1214	–									
3	0.1200	0.0590	–								
4	0.3824	0.3162	0.3025	–							
5	0.3374	0.1897	0.2188	0.0730	–						
6	0.3587	0.2559	0.2471	0.0293	0.0395	–					
7	0.3429	0.2460	0.2243	0.0337	0.0528	0.0092	–				
8	0.4018	0.3772	0.2828	0.0914	0.1224	0.0980	0.1163	–			
9	0.3637	0.2949	0.2647	0.1381	0.1006	0.1340	0.1694	0.1418	–		
10	0.4162	0.3759	0.3833	0.0355	0.1184	0.0878	0.1087	0.0840	0.1771	–	
11	0.3440	0.3446	0.3596	0.0886	0.1324	0.1050	0.1191	0.1339	0.2288	0.0305	–

For population numbers and localities see Table 1 and Fig. 1.

Moreover, a significant reduction of heterozygosity was found in populations that inhabited temporary ponds (permanent ponds: He = 0.243 ± 0.045 SD, temporary ponds: He = 0.172 ± 0.050 SD; *U*-test, $p = 0.018$).

3.3. Demographic analysis

The results of the mismatch analysis for the two clades (mainland, Peloponnisos) are presented in Table 5 and Fig. 4. The sudden expansion model was not rejected at $\alpha = 0.05$ in both clades. However, in the mainland clade, the shape of the mismatch distribution is multimodal, with the higher modes centered around zero, two, and three pairwise differences, suggesting older episodes of growth (Tau = 3.770 mutational units). Similarly, in the Peloponnesian clade, the shape of the mismatch distribution is multimodal, with the higher modes centered on zero and two pairwise differences, suggesting older episodes of growth (Tau = 2.131 mutational units). Moreover, other tests of changes in population size, Tajima's *D* and Fu's Fs, were not significant for both clades (Table 5).

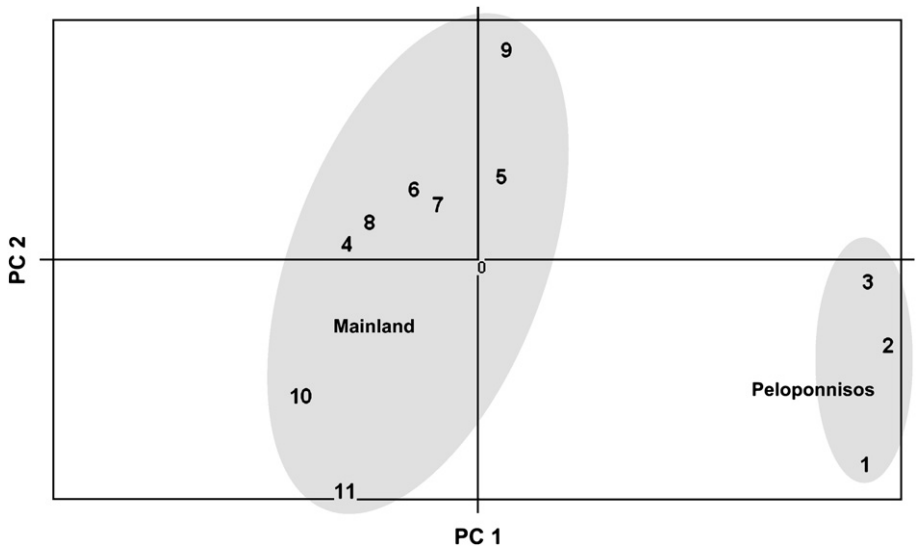

Fig. 3. Discrimination of Peloponnesian (+ loadings) and mainland (− loadings) populations, in the first Principal Component of the PCA on 45 allele frequencies. Population locations are presented in Table 1 and Fig. 1.

Table 5
Mismatch analysis and neutrality tests for the two Greek alpine newt lineages

	Mainland $n = 21$	Peloponnisos $n = 12$
Tau	3.770	2.131
Theta0	0.000	0.000
Theta1	11.152	4.554
Goodness-of-fit test		
SSD	0.0471	0.0667
p	0.0870	0.1350
Tajima's *D* test	−0.6388	−0.5564
p	0.2680 (NS)	0.3210 (NS)
Fu's Fs test	0.0096	−0.6090
p	0.5270 (NS)	0.2920 (NS)

n: Number of sequences; NS: nonsignificant.

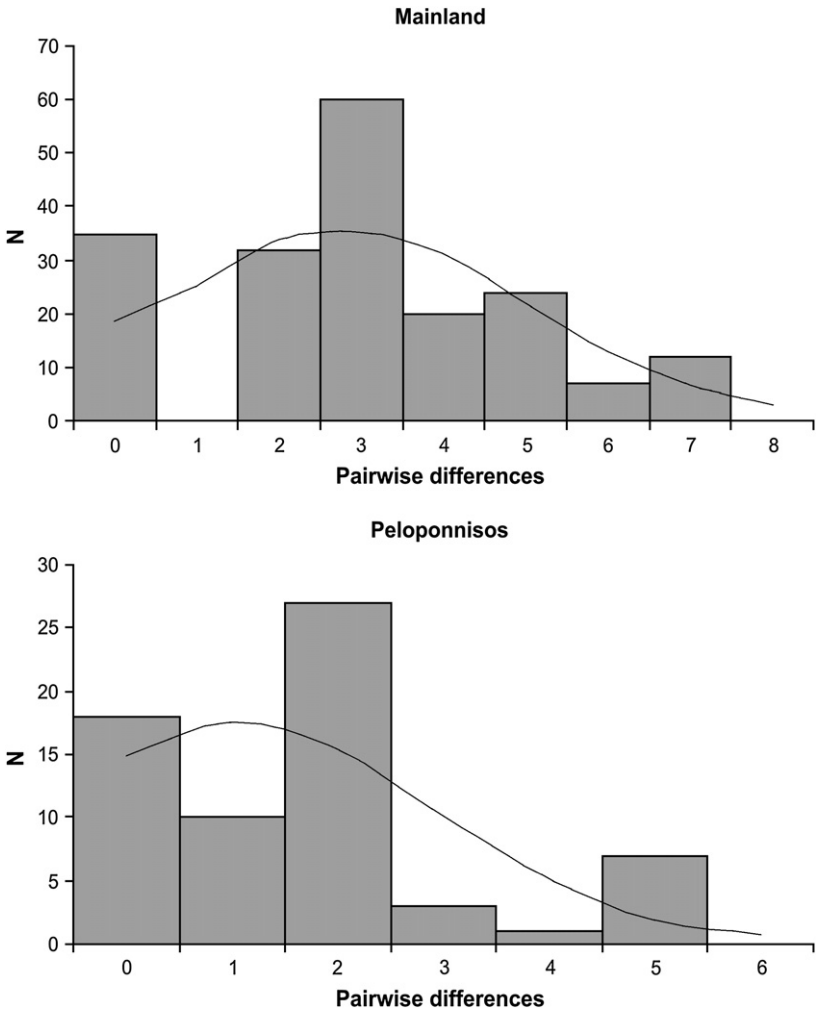

Fig. 4. Mismatch distributions in the two alpine newt clades examined. The multimodal distribution in both clades suggests long-term demographic stability. Black curves show shapes of theoretical distributions under the sudden expansion model.

4. Discussion

Our data, on the allozyme and mtDNA variation of *M. alpestris veluchiensis* from Greece, support the recently detected genetic subdivision between two disjunct groups of populations (Sotiropoulos et al., 2007). Populations from the Greek mainland and Peloponnisos exhibit significant differences in nuclear gene frequencies, coupled with considerably diverse mitochondrial haplotypes, and consistently form separate, highly supported clades (Figs. 2 and 3). The two clades express an average genetic distance of 1.5% for cyt *b* and 0.8% for 16S rRNA, which is well within the intraspecific level of divergence of numerous amphibian taxa (Vogler and Desalle, 1994; Johns and Avise, 1998). Accordingly, the degree of allozyme divergence between the two population groups ($D_{NEI} = 0.318$) was found to be within the reported range of amphibian interspecific differentiation (Thorpe, 1982; Avise, 1994; Highton, 2000). It was found to be higher than between other alpine newt species (Kalezić and Hedgecock, 1980) and subspecies: *Mesotriton alpestris alpestris* − *Mesotriton alpestris apuanus*: $D = 0.11$; *M. a. apuanus* − *Mesotriton alpestris cyreni*: $D = 0.20$; *M. a. alpestris* − *M. a. cyreni*: $D = 0.26$ (Macgregor et al., 1990).

This amount of divergence accumulated during the last *c*. 0.4–0.5 My (middle Pleistocene), since clade separation (Sotiropoulos et al., 2007). The contemporary separation of the two clades by the Gulf of Corinth, coupled by the well-documented palaeohistory of the Greek region (Dermitzakis, 1990; Perissoratis et al., 2000; Perissoratis and Conispoliatis, 2003), favors an allopatric–vicarianistic scenario. According to this, a widespread ancestral lineage might have suffered significant range fragmentation during Pleistocene, since extensive salt barriers isolated Peloponnesian populations from mainland ones. Although Peloponnisos was repeatedly connected to continental Greece over the Gulf of Corinth until the last glacial period (Würm, ∼18 Kya) (Dermitzakis, 1990; Perissoratis et al., 2000; Perissoratis and Conispoliatis, 2003), the observed amount of divergence favors such an earlier separation of the respective alpine newt populations. Additionally, the results of the mismatch distributions and other tests of neutrality indicate long-term demographic stability of both clades. Therefore, a recent expansion model can be ruled out.

However, during the glacial reconnections, the presence of ice caps on mountaintops of the Greek mainland and Peloponnisos (Brunn, 1956; Denton and Hughes, 1981; Hughes et al., 2003) possibly caused movements of previously isolated mountainous populations to lower altitude valleys, where secondary intergradation might have taken place in zones of contact (e.g. Hewitt, 1996). The detection of common alleles unevenly distributed in both regions (Table 3) could indicate such contacts and introgression as well as differential fixation due to drift. However, the lack of common mtDNA haplotypes between the two regions indicates male-biased dispersal, which is frequently observed in the alpine newt (Joly and Grolet, 1996). Nevertheless, the low dispersal ability and homing behavior of the alpine newt (Joly and Miaud, 1989) could have prevented the extensive admixture of the two gene pools, thus maintaining genetic divergence.

However, the between-groups observed differences in allozymes are higher than in mtDNA. A plausible explanation, commonly invoked in cases of small isolated populations, involves random fluctuations of allele frequencies due to drift which consequently increases levels of divergence among isolated populations within short periods of time (e.g. Hedrick, 2000; Frankham et al., 2002).

In any case, populations inhabiting these two major groups meet the ‘‘discrete and significant’’ criteria of Evolutionary Significant Units (*sensu* Moritz, 1994a,b). This means that they are physically separated and genetically distinct and, therefore, warrant separate conservation status.

Extensive genetic isolation was observed among populations within each ESU. The degree of subdivision among mainland ($F_{ST} = 0.243$) and Peloponnesian ($F_{ST} = 0.231$) populations was high and significantly different from zero (Table 3). The detection of a private allele (Est-2E) from population Oiti-1 (ID = 5), the rare Est-2D and PgmC from populations Oiti-2 (ID = 6), Velouchi (ID = 7) and Kalavryta (ID = 2), Panachaiko (ID = 3), respectively, as well as the high frequency of G6pdhD from the two northernmost populations from Aoos (ID = 10) and Zygos (ID = 11) (Table 3), supports the hypothesis of the independent history of local populations accompanied by limited, or even absent, gene flow for great periods of time (e.g. Slatkin, 1987; Avise, 1994). This is supported further by the existence of private and well-differentiated haplotypes in many populations: e.g. haplotype VI in Kallidromo (ID = 4), X in Belokomitis (ID = 8), I–II in Kyllini (ID = 1) (Table 1).

Significant differences in allele frequencies between three alpine newt population groups from the northeastern limit of its distribution (Pabijan et al., 2005; Pabijan and Babik, 2006) led to the description of separate management units (MUs). Similarly, the extent of divergence observed between local alpine newt populations within each previously described ESU, due to their alternative allelic and haplotype composition, could indicate the existence of several

management units in the southern limit of the species distribution. In most cases these populations are situated in different mountain massifs (Fig. 1). The separation of these massifs by low–mid altitude ridges, along with the breeding-site fidelity and low vagility that alpine newts display (Joly and Miaud, 1989), might explain the high levels of genetic divergence observed and low gene flow rates among local populations.

Additionally, the majority of local populations show increased genetic variation levels (He), further supporting their old history but also their increased potential to adapt in a variable environment (Avise, 1994; Willi et al., 2006). However, the strong deviation from the HW expectations (low or zero Ho), along with the respective and strongly positive inbreeding coefficients (F_{IS}) (Table 3), might indicate strong inbreeding, but could also be the result of recent and severe bottlenecks (Hedrick, 2000; Frankham et al., 2002; Keller and Waller, 2002). This situation has often been connected to increased extinction risk mostly due to environmental alterations and/or human activities (Rowe and Beebee, 2003; Saccheri et al., 1998).

Annual fluctuations of effective size of amphibian populations are related to a series of factors that determine the degree of harshness of the terrestrial habitat (Hairston and Wiley, 1993; Griffiths, 1996). These fluctuations may be dramatic from year to year mainly due to increased animal mortality, as a result of prolonged aridity and intense human activities (Verrell, 1987; Accordi et al., 1990). The observed reduction of genetic variability in low-humidity areas and temporary ponds is mainly attributable to the contribution of populations from Mt. Kallidromo (ID = 4) and Mt. Kyllini (ID = 1). Both populations, which constitute the most marginal populations of the species (see Fig. 1), inhabit temporary ponds situated in the less humid parts of the species' range in Greece. These two populations each bear a unique haplotype. The Mt. Kallidromo population also has extensive anthropogenic activity (e.g. motor-sports in annual basis; personal observations), which probably causes the reduction of variation as well. These results coincide with the hypothesis of reduced variability of peripheral populations (Highton and Webster, 1976; Larson et al., 1984; Hoffmann and Blows, 1994; Lesica and Allendorf, 1995). Population densities are often expected to be lower (Brown, 1984; Caughley et al., 1988; Lawton, 1995) and more variable (Curnutt et al., 1996) near the periphery of the range. As a result, peripheral populations might be less resilient to stochastic threats than core populations are (Curnutt et al., 1996). This increases the risk of extinction (e.g. Goodman, 1987).

As a whole, all examined populations seem to have been affected by the degree of isolation, and possibly their small size. Apart from the existence of two evolutionary independent and long lasting population groups or lineages, populations within each require management and conservation. Management should focus on 'controlling' the factors that cause habitat fragmentation and degradation, as well as human activities that could eventually compromise local populations' survival (Araújo and Williams, 2001).

However, the relatively small sample size could possibly hide the presence of shared alleles and/or haplotypes, thus affecting the definition of conservation units by misinterpreting diagnostic characters (Walsh, 2000). As a result, we suggest a more thorough population study, with increased sample sizes and sampling localities, as well as the use of more neutral markers (e.g. microsatellites), in order to unveil the true measure of gene pool fragmentation, and evaluate further the importance of discrete populations as units requiring protection.

Acknowledgments

The present study was partially funded through the Operational Program for Education and Initial Vocational Training (O.P. ''Education'') in the framework of the project ''Pythagoras II – Support of University Research Groups''. The Editor and anonymous reviewers provided valuable comments and suggestions on the manuscript.

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