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Conservation units in the insular endemic salamander *Lyciasalamandra helverseni* (Urodela, Salamandridae)

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Lyciasalamandra helverseni (Caudata, Salamandridae) is the only exclusively insular representative of the genus recently defined as *Lyciasalamandra*. The species is endemic to a three-island group in the southeast Aegean Sea: Kasos, Karpathos, and Saria (Greece). Tissue samples of *Lyciasalamandra helverseni* were collected from specimens at six localities on three islands, along with several outgroups. Sequences of two mitochondrial genes (cytochrome *b* and 16S rRNA), as well as frequencies of 18 allozyme loci, were used to describe levels and patterns of genetic variation, identify possible evolutionary units, and investigate issues of their conservation status. Two major clades of *L. helverseni*, displaying considerable amount of genetic differences, were found in both analyses. These clades, which have been separated since the late Pliocene, constitute separate evolutionary units and correspond to populations from the islands of Kasos and Karpathos, respectively. The particularly high interpopulation differentiation within relatively short geographical distances, especially in the island of Kasos, implies long-term isolation in fragmented habitats. Severe bottlenecking is proposed to have resulted in the observed low levels of polymorphism of local populations, while inbreeding and low population size might explain the observed lack of heterozygotes. Conservation implications are discussed, particularly in the case of Kasos, where extensive overgrazing of pasturelands has contributed to degradation of the habitat.

Introduction

Genetic analyses have greatly enhanced the ability to identify phylogenetically distinct entities, which were previously masked by taxonomic epithets (e.g. Goldstein *et al*. 2000). This, in turn, has accelerated the identification of population groups in need of conservation (Avise 1989). An interesting concept combining conservation needs with genetic characterization is the Evolutionary Significant Unit (ESU; Ryder 1986).

An ESU is a group of organisms that have been isolated from other conspecific groups for a period of time sufficient to display genetic divergence from those groups (Ryder 1986, Paetkau 1999, Fraser & Bernatchez 2001 and references therein). This concept is useful for the recognition and conservation of population clusters of

appreciable genetic diversity, with the ultimate goal being the preservation of biological diversity, as a definitive factor of species-persistence over evolutionary time (Avise 1994).

To be considered an Evolutionary Significant Unit (*sensu* Moritz 1994a) these entities should be reciprocally monophyletic for mtDNA haplotypes, and differ significantly in nuclear allele frequencies. The term ESU is somewhat similar to the "phylogenetic species" concept (Eldredge & Cracraft 1980, Cracraft 1983, Davis & Nixon 1992), but with special reference to cases that require separate protection strategies (Vogler & Desalle 1994).

Another conservation unit proposed by Moritz (Moritz 1994a), for the purpose of short term monitoring, is the Management Unit (MU). In short, the term corresponds to populations with statistically significant divergence in allele frequencies, whether nuclear, or mitochondrial, and has been suggested as synonymous with the term stock (Dizon *et al*. 1992). Currently, there is no non-arbitrary method to support the use of an "evolutionary significance" or "potential" concept for conservation purposes (Moritz 1994b, Goldstein *et al*. 2000). Much controversy over the subject has arisen since it was first introduced (Ryder 1986), and many authors have proposed various frameworks for identifying basic clusters for conservation (*see* Fraser & Bernatchez 2001 for a review of the proposed frameworks). The above authors suggest that a single and universal definition of an ESU may not be feasible across all species, and that different approaches should be applied to entities with dissimilar life histories and in diverse circumstances.

The use of molecular markers for estimating levels of intraspecific genetic diversity, which, if lost, seriously decreases the long-term survival probability of a population, has been recognized by many authors (Avise 1995, Newman & Pilson 1997, Reed & Frankham 2003).

Up until recently, the tailed amphibian genus *Mertensiella* comprised two allopatrically distributed species: *M. caucasica* and *M. luschani*. However, molecular studies have addressed the paraphyly of the genus (Titus & Larson 1995, Weisrock *et al*. 2001). Recently, Veith and Steinfarz (2004), made a taxonomic revision of the genus *Mertensiella*, and, based on mtDNA sequences, allozyme frequencies and external morphology placed *M. luschani* in a new genus: *Lyciasalamandra*. According to this revision, all previously recognized subspecies of *M. luschani* are raised to the species level. Among these, *Lyciasalamandra helverseni* is a strictly insular endemic species, inhabiting the southeastern Aegean islands of Kasos, Karpathos and Saria (Greece; Fig. 1). In contrast to *Lyciasalamandra* species from Turkey, which are mostly confined to forested localities with an average annual rainfall over 800 mm (Veith *et al*. 2001, Weisrock *et al*. 2001), *L. helverseni* occupies arid areas in one of the driest regions of Europe (average annual rainfall (source Hellenic National Meteorology Service): Karpathos 335 mm, Kasos 330 mm), and is restricted to favorable microhabitats (Polymeni 1988, 1994).

Given that a considerable amount of genetic diversity has been detected between closely neighboring populations on the southwestern coast of Turkey (Weisrock *et al*. 2001), with the eventual formation of discrete phylogenetic species, and considering the limited dispersal capabilities of this particular amphibian, especially in arid environments, evaluation of the genetic diversity within *L. helverseni* is of great importance as a definitive factor for the assessment of the viability of the species (Avise 1994).

In this study, we sampled six local populations of the endemic *L. helverseni* from three islands (Table 1 and Fig. 1). 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 linked to the geological history of the area in order to identify any evolutionary significant units and to address the conservation needs of the populations studied.

Materials and methods

DNA analysis

Muscle samples or tail tips were obtained from 12 individuals of *Lyciasalamandra helverseni*

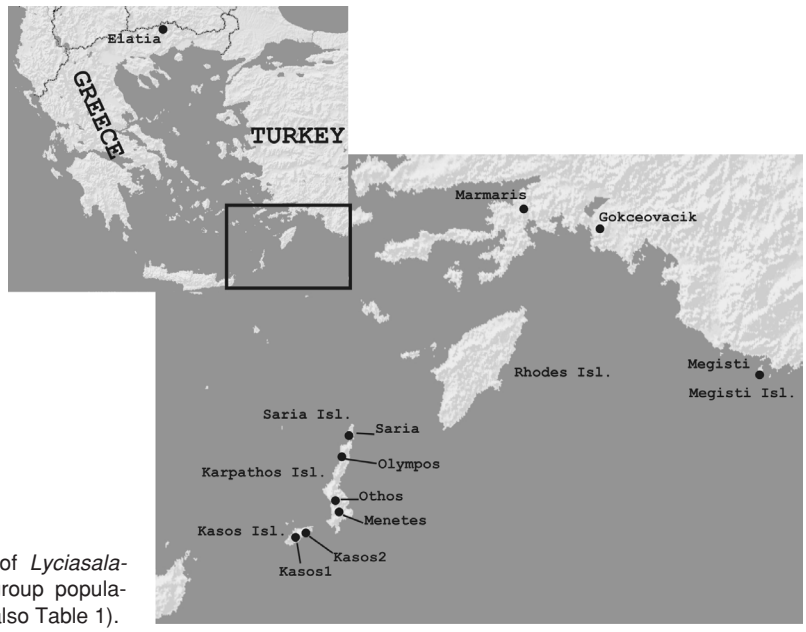

Fig. 1. Sampling locations of *Lyciasalamandra helverseni* and outgroup populations used in this study (*see* also Table 1).

from six localities in the southeast Aegean (Table 1 and Fig. 1). Tissue samples were homogenized in a digest buffer and total genomic DNA was extracted using Proteinase K dissolution; 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 (341 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 primers L14841 and H15149 (Kocher *et al.* 1989) were used to amplify the cyt-*b* region of the mtDNA. 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.

Table 1. Localities and sample sizes (*N*) of specimens used in the study (*see* also Fig. 1).

Locality	Island or location	Species	*N* (DNA/Allozymes)	Accession no. 16S rRNA	Accession no. cyt *b*
Othos	Karpathos	*L. helverseni*	2/20	DQ473589	DQ473598
Olympos	Karpathos	*L. helverseni*	2/10	DQ473590	DQ473599
Menetes	Karpathos	*L. helverseni*	2/14	DQ473591	DQ473600
Saria	Saria	*L. helverseni*	2/–	DQ473592	DQ473601
Kasos 1	Kasos	*L. helverseni*	2/–	DQ473593	DQ473602
Kasos 2	Kasos	*L. helverseni*	2/12	DQ473594	DQ473603
Megisti	Megisti	*L. l. basoglui*	–/25	–	–
Gökçeovacik	Turkey	*L. fazilae*	2/–	DQ473595	DQ473604
Marmaris	Turkey	*L. flavimembris*	2/–	DQ473596	DQ473605
Elatia	Rodope Mts.	*S. salamandra*	2/–	DQ473597	DQ473606

Complementary sequences were obtained for two specimens of *Lyciasalamandra flavimembris* (Accession numbers: DQ473605 and DQ473596 for cyt *b* and 16S respectively) and two specimens of *L. fazilae* (Accession numbers: DQ473604 and DQ473595 for cyt *b* and 16S respectively), from Turkey. In addition, two individuals of *Salamandra salamandra* from Rodope Mts. were included as outgroups (cyt *b*: DQ473606, 16S: DQ473597).

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 on the basis of its secondary structure to facilitate proper alignments. Alignment gaps were inserted to resolve length differences between sequences, and positions that could not be unambiguously aligned were excluded. Cytochrome-*b* sequences were translated into amino acids prior to analysis and did not show any stop codons, suggesting that they were all functional.

For several recognized clades and for the outgroup, sequence divergences were estimated in MEGA computer package (ver. 3.1, Kumar *et al*. 2004) using the Tamura-Nei model of evolution (Tamura & Nei 1993) to adjust for differences in nucleotide frequencies and substitution-rate heterogeneity.

Analyses of phylogenetic inference were conducted using Bayesian inference (BI). Nucleotides were used as discrete, unordered characters. To examine whether the sequences from the two genes should be combined in a single analysis, a partition-homogeneity test was run in PAUP (ver. 4.0b10, Swofford 2002), and significance was estimated with 1000 repartitions. This test, described as the incongruence-length difference test by Farris *et al*. (1995), indicated no conflicting phylogenetic signals between the datasets ($p = 0.59$) and, given that the mtDNA genes are linked, datasets from both genes were analysed together.

We performed Bayesian analysis of the combined dataset with the program MrBayes (ver. 3.1, Huelsenbeck & Ronquist 2001). The best-fit model of DNA substitution and the parameter estimates used for tree construction were chosen by performing hierarchical likelihood-ratio tests (Huelsenbeck & Crandall 1997) in MrModeltest (ver. 2.2, Nylander 2004). Likelihood-ratio tests and Akaike Information Criterion (AIC; Akaike 1974) indicated that the General Time Reversible (GTR) model + G had the highest likelihood score ($-\ln L = 1918.9617$), and showed a significantly better fit than the other less complicated models (model parameters: GTR + G, G = 0.1873; base frequencies A = 0.3274, C = 0.2055, G = 0.1847, T = 0.2823; rate matrix A–C = 4.7446, A–G = 18.9796, A–T = 4.8539, C–G = 1.1065, C–T = 30.3054, G–T = 1.00).

Salamandra salamandra was used as the designated outgroup. Analysis was run with four chains for 10^6 generations and the current tree was saved to file every 100 generations. This generated an output of 10^4 trees in each run. The $-\ln L$ stabilized after approximately 10^4 generations, and the first 10^3 trees (10% "burnin" in Bayesian terms, chain had not become stationary) were discarded as a conservative measure to avoid the possibility of including random, sub-optimal trees. We used PAUP (ver. 4.0b10, Swofford 2002) to obtain a 50% majority-rule consensus tree. The percentage of samples recovering any particular clade in a Bayesian analysis represents that clade's posterior probability (Huelsenbeck & Ronquist 2001).

We used one of the methods of Leaché and Reeder (2002) to ensure that our analyses were not trapped on local optima. In particular, the posterior probabilities for individual clades obtained from separate analyses (4 runs) were compared for congruence (Huelsenbeck & Imennov 2002), given the possibility that two analyses could appear to converge on the same ln-likelihood value while actually supporting incongruent phylogenetic trees.

Allozyme analysis

A total of 81 salamanders from 5 localities (3 of *L. helverseni* from Karpathos, 1 of *L. helverseni* from Kasos, and 1 of *L. luschani basoglui* from Megisti, islands in Greece) (Fig. 1 and 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-2*, *Est-4*, *Est-5*, *G3pdh2*, *G6pdh*, *Gpi-1*, *Idh*, *Ldh*, *Mdh-1*, *Mdh-2*, *Pgm*, *Prot-1*, *Prot-2*, *Prot-3*, *Sdh*, *Sod-1*, *Xdh*, *6pgdh*), corresponding to thirteen enzyme systems, were scored under standard histochemical procedures (Murphy *et al*. 1996). 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 & Selander 1981).

Departure from Hardy-Weinberg expectations was tested using the GENEPOP ver. 3.1 (Raymond & Rousset 1995). Estimation of the Hardy-Weinberg exact probability was performed by the complete enumeration method (Louis & Dempster 1987) for each locus in each population. 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 with jackknifing over samples.

Genetic distances were calculated using Nei's unbiased method (Nei 1978) by means of the package BIOSYS-1 (Swofford & Selander 1981). Phylogenetic trees were constructed with the package PHYLIP 3.5 (Felsenstein 1993). We used the neighbour-joining (Saitou & Nei 1987) method based on Nei's (1978) genetic distances. *Lyciasalamandra luschani basoglui* from the island of Megisti was used as the designated outgroup.

To investigate the relationships among *Lyciasalamandra helverseni* populations in detail, we also performed Principal Component Analysis (PCA) on allele frequencies by means of the package PCAGEN 1.2.1 (written by J. Goudet, http://www2.unil.ch/popgen/softwares/pcagen.htm).

Divergence time and molecular-clock testing

A likelihood ratio test (LRT) (Muse & Weir 1992), as well as a non-parametric clock test (Tajima 1993), which has no need for a specific model of nucleotide substitution, were employed to uncover heterogeneity among haplotypes. Since the results indicated a significant rate of heterogeneity among clades, we used a semi-parametric method implemented in the software r8s (Sanderson 2002, 2003) to scale branch lengths over the tree topology. This method allows evolutionary rates to vary between branches within certain limits using a penalized-likelihood function (PL) that includes a roughness penalty and a smoothing parameter.

These control the trade-offs between the smoothing of rate change across adjacent branches and the goodness-of-fit in the model. A cross-validation procedure (Sanderson 2002) was used to find the optimal smoothing parameter value. The analyses with r8s were performed using the PL method and the truncated-Newton algorithm. As a calibration point we used a reliably-dated geological event: the separation of the island of Karpathos from the Anatolian mainland in the late Pliocene, some 3.5 Mya (Dermitzakis & Papanikolaou 1981, Meulenkamp 1985), and assigned between the *fazilae-flavimembris* node and the *helverseni* node on the BI tree. Empirical 95% confidence intervals to the temporal estimates were obtained from estimating branch lengths in 100 bootstrapped data sets, keeping topology and the model of evolution constant, and running r8s analysis for each branch-length set. Analyses were performed with the r8s bootstrap kit (Eriksson 2003).

Results

DNA analysis

Of the 840 sites examined, there were 42 variable cyt-*b* sites, 20 of which were parsimony-informative (67 and 26 respectively, when the outgroup was included in the analysis), and 35 variable 16S rRNA sites, 17 of which were parsimony-

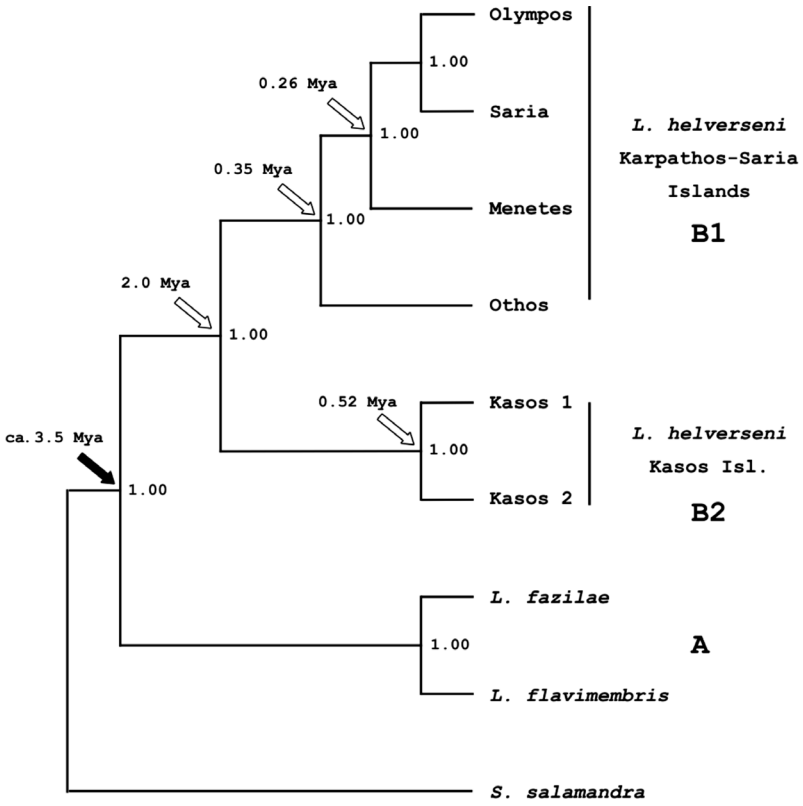

Fig. 2. 50% majority rule consensus tree produced by Bayesian analysis. Branch numbers present posterior probabilities of BI. Plain arrows indicate the estimated date of separation of the respective branches. The black arrow indicates the calibration point used for dating of clades and subclades. Population locations are presented in Table 1 and Fig. 1.

informative (56 and 19 respectively, including outgroup). Eight different haplotypes were identified among 16 *Lyciasalamandra* sequences. For cyt *b*, ingroup sequence divergence (Tamura and Nei, 1993) ranged from 0.3% to 8.2%, while for 16S rRNA sequence divergence ranged between 0% and 4.6% (Table 2).

The Bayesian inference method under the GTR + G model, produced identical topologies for each of the 4 runs with the full dataset, although posterior probabilities for some of the nodes differed slightly between each of the Bayesian runs (Fig. 2). Figures on the resolved branches are posterior probabilities. The mean –ln likelihood of these trees was –1928.58 (G-shape parameter with four discrete rate categories = 0.2117; nucleotide frequencies: A = 0.326, C = 0.208, G = 0.184, T = 0.282). In total, all seven

Table 2. Tamura-Nei distances (%) among the salamander populations studied. Population localities correspond to those presented in Table 1 and Fig. 1. Above diagonal: cyt *b*; below diagonal: 16S.

	L. helverseni								
	Othos	Olympos	Menetes	Saria	Kasos 1	Kasos 2	*L. fazilae*	*L. flavimembris*	*S. salamandra*
Othos	–	0.6	0.9	1.5	4.2	3.6	5.9	7.9	13.9
Olympos	0.0	–	0.3	0.9	4.2	4.3	5.2	7.2	14.3
Menetes	0.0	0.0	–	1.2	4.5	4.6	5.5	7.5	14.3
Saria	0.0	0.0	0.0	–	4.8	4.9	6.2	7.8	14.3
Kasos 1	2.3	2.3	2.3	2.3	–	1.8	7.5	8.2	14.6
Kasos 2	2.3	2.3	2.3	2.3	0.4	–	7.9	8.2	14.6
L. fazilae	4.6	4.6	4.6	4.6	4.2	4.2	–	6.6	14.8
L. flavimembris	4.2	4.2	4.2	4.2	4.2	4.0	3.3	–	14.4
S. salamandra	7.5	7.5	7.5	7.5	7.7	7.7	8.0	7.1	–

ingroup nodes were significantly supported by their posterior probabilities (Fig. 2). All *Lyciasalamandra* haplotypes form two well-supported clades: clade A containing *L. flavimembris* and *L. fazilae* from Turkey and clade B that constitutes *L. helverseni* (Fig. 2). Mean genetic divergence between the two major clades was 7.1% and 4.3% for cyt *b* and 16S, respectively.

Within *L. helverseni*, two subclades, which are in accordance with the geographical origin of the specimens, were revealed; subclade B1 includes populations from the islands of Karpathos and Saria, while subclade B2 includes populations from the island of Kasos (Fig. 2). The two subclades express considerable genetic divergence (4.4% and 2.3% for cyt *b* and 16S, respectively). Within subclade B1, the mean genetic divergence was found to be 0.9% and 0% for cyt *b* and 16S, respectively. The two localities from Kasos (subclade B2), represent different haplotypes expressing a considerable amount of divergence in both gene fragments (1.8% and 0.4% for cyt *b* and 16S, respectively) (Table 2).

Allozyme analysis

Nine out of 18 gene loci were found to be monomorphic throughout the studied populations (*Est-2*, *G3pdh-2*, *G6pdh*, *Idh*, *Mdh-1*, *Prot-1*, *Prot-3*, *Sdh*, *Sod-1*). The remaining nine loci showed more than one allele in at least one of the populations (Table 3). A total of 32 alleles were detected. All polymorphic loci showed banding patterns consistent with autosomal inheritance and with known quaternary structures (Richardson *et al*. 1986).

The unbiased genetic distances (Nei 1978) between samples ranged from 0.005 to 0.408 (mean ± SD = 0.194 ± 0.161) (Table 4). The Neighbour-joining phenogram clearly separated *L. helverseni* from *L. l. basoglui* (Fig. 3). The two taxa exhibited a mean unbiased genetic distance (Nei 1978) of 0.367 ± 0.029 (mean ± SD). A significant subdivision was detected within *L. helverseni*, comprising a northern and a southern group of populations. The genetic distance between *helverseni* groups averaged at 0.147 ± 0.016 (mean ± SD). The northern group includes the three populations of Karpathos (mean D_{NEI} = 0.008 ± 0.003 SD), while the southern group includes the population from Kasos (Fig. 3). Principal Component Analysis showed an identi-

Table 3. Allele frequency, average number of alleles per locus (*A*), average number of polymorphic loci (*P* %), average observed (Ho) and average expected heterozygosities (He). Mean F_{IT} and F_{ST} values (Weir & Cockerham 1984) with ± SE and 95% CI in parentheses. Significant departures from HW expectations are given in bold.

Locus allele	Population				
	Othos	Olympos	Menetes	Kasos 2	Megisti
Est-4					
A	0.214	0.550	0.727	0.000	0.980
B	0.429	0.250	0.182	1.000	0.000
C	0.357	0.200	0.091	0.000	0.020
Est-5					
A	0.050	0.000	0.000	0.000	0.000
B	0.000	0.000	0.000	1.000	0.000
C	0.000	0.000	0.000	0.000	1.000
D	0.850	1.000	1.000	0.000	0.000
E	0.100	0.000	0.000	0.000	0.000
Ldh-2					
A	0.000	0.000	0.000	0.000	1.000
B	1.000	1.000	1.000	1.000	0.000
Mdh-2					
A	0.000	0.000	0.000	0.000	0.040
B	1.000	1.000	1.000	1.000	0.960
Gpi-1					
A	1.000	1.000	1.000	1.000	0.760
B	0.000	0.000	0.000	0.000	0.240
Pgm-1					
A	1.000	1.000	1.000	1.000	0.000
B	0.000	0.000	0.000	0.000	1.000
Prot-2					
A	0.000	0.000	0.000	0.000	1.000
B	0.000	0.000	0.000	1.000	0.000
C	1.000	1.000	1.000	0.000	0.000
Xdh					
A	0.950	1.000	1.000	1.000	1.000
B	0.050	0.000	0.000	0.000	0.000
6pgdh					
A	0.000	0.000	0.000	0.000	1.000
B	0.925	1.000	0.964	0.833	0.000
C	0.075	0.000	0.036	0.167	0.000
A	1.33	1.11	1.17	1.06	1.17
P (%)	22.2	5.6	11.1	5.6	16.7
Ho	**0.030**	0.028	0.024	**0.000**	**0.007**
He	**0.067**	0.035	0.029	**0.016**	**0.027**

F_{IT}all	0.929 ± 0.047 (0.830–0.991)
F_{IT}helver	0.777 ± 0.134 (0.616–1.000)
F_{ST}all	0.856 ± 0.074 (0.676–0.958)
F_{ST}helver	0.631 ± 0.197 (–0.003–0.926)

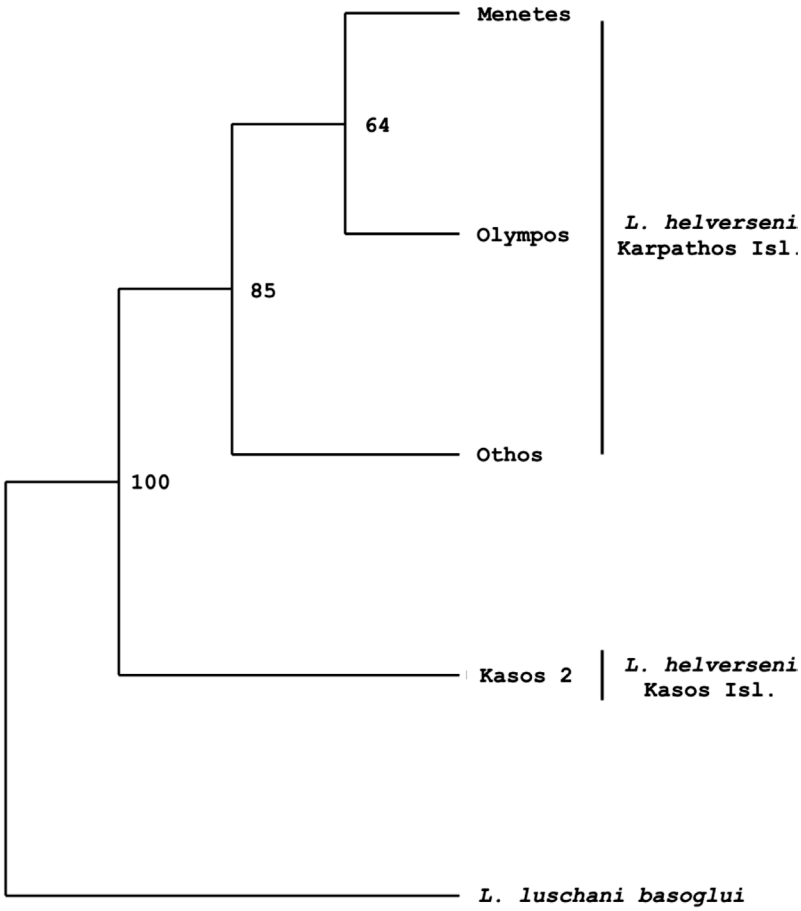

Fig. 3. Neighbor joining tree based on Nei's (1978) unbiased genetic distances of the five populations of *Lyciasalamandra helverseni* and *L. luschani basoglui* from southeast Aegean area. The two genetic lineages of *helverseni* cluster at D = 0.147. Population locations are presented in Table 1 and Fig. 1.

cal discrimination of these two genetic groups (Fig. 4). The PCA on 26 *helverseni* allele frequencies produced one significant vector ($p < 0.05$), which summarized 92.4% of the detected genetic variation, separating Karpathos (– loadings) from Kasos (+ loadings) populations (Fig. 4). The separation was due to the major contribution of the following alleles: Est-4A, Est-4B, Est-5B, Est-5D, Prot-2B, Prot-2C. The latter four alleles are diagnostic of the islands of Karpathos and Kasos, respectively (Table 3).

Levels of genetic variation varied between *helverseni* populations (Table 3). The population from Othos (Karpathos) was found to be the most polymorphic while the Kasos population displayed the lowest levels of genetic variation

Table 4. Nei's (1978) unbiased genetic distances between the populations studied. Population locations are presented in Table 1 and Fig. 1.

	L. helverseni				*L. l. basoglui*
	Othos	Olympos	Menetes	Kasos 2	Megisti
Othos	–				
Olympos	0.0040	–			
Menetes	0.0101	0.0004	–		
Kasos 2	0.1289	0.1510	0.1601	–	
Megisti	0.3665	0.3523	0.3414	0.4071	–

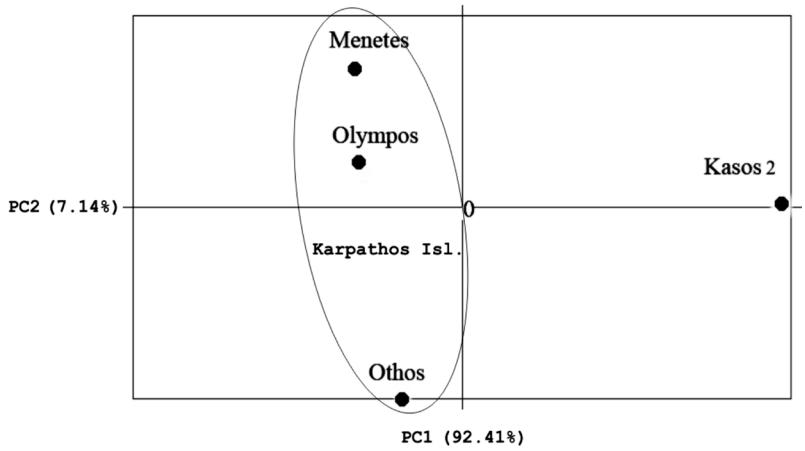

Fig. 4. Discrimination of Kasos (+ loadings) and Karpathos (– loadings) populations, in the first Principal Component of the PCA on 26 allele frequencies. Population locations are presented in Table 1 and Fig. 1.

(Table 3). No departure from the Hardy-Weinberg equilibrium was detected over all populations and loci (Fisher test: $\chi^2_{14} = 23.20$, $p > 0.05$). However, the Othos population from Karpathos (Table 3) showed significant deviation from HW expectations against heterozygotes in one (Xdh) out of four polymorphic loci ($p < 0.05$). Additionally, the unique polymorphic locus of the Kasos population (*6Gpdh*) deviated significantly from HW equilibrium ($p < 0.01$).

The *F*-statistics indicated apparent intra-population substructuring (F_{IS} ± SE = 0.352 ± 0.182, 95% CI = 0.280–1.000, F_{IT} ± SE = 0.777 ± 0.134, 95% CI = 0.616–1.000), but marginally significant inter-population heterogeneity (F_{ST} ± SE = 0.631 ± 0.197, 95% CI = –0.003–0.926) (Table 3).

Time of clade separation

Separation time between the Kasos and Karpathos clades was dated at 2.03 Mya (95% CI: 1.81–2.41). A divergence time of 0.52 Mya (95% CI: 0.43–0.58) was calculated between the two units from Kasos. The Saria lineage diverged from the Karpathos lineages at 0.35 Mya (95% CI = 0.31–0.40) while within Karpathos, the separation of lineages was estimated at 0.26 Mya (95% CI = 0.23–0.31) (Fig. 2).

Discussion

The identification of clusters to be assign as separate ESUs is of major importance as *L. helverseni* represents an exclusively insular endemic salamander species. Two major units were established, each displaying significant differences in nuclear gene frequencies, a number of diagnostic alleles, and considerably diverse mitochondrial haplotypes. These units consistently formed separate, highly supported clades (Figs. 2 and 3).

The first clade (B1) contains populations from the islands of Karpathos and Saria, while the second clade (B2) contains the two populations of Kasos. The two clades express an average genetic distance of 4.4% for cyt *b* and 2.3% for 16S rRNA, which is well above the intraspecific level of divergence of numerous amphibian taxa (Vogler & Desalle 1994, Johns & Avise 1998). Accordingly, the reported Nei's (1978) mean genetic distance among all the *Lyciasalamandra* taxa (Veith & Steinfartz 2004), proves to be lower than that calculated between *L. helverseni* populations from the islands of Karpathos and Kasos ($D_{NEI} = 0.147$ although based on different enzyme systems). This amount of divergence has, according to our data, accumulated over the last ca. 2 My, since clade separation (late Pliocene). This date merely coincides with the onset of Pleistocene glaciations (Dynessius & Jansson 2000).

The limited dispersal abilities of this amphibian (Polymeni 1988, 1994) might have become reduced further when the climate started to shift from near-tropical to a much dryer climate, resembling the Mediterranean type, after the first glacial period (Axelrod 1973). This probably

forced the entrapment of populations in favorable environments such as limestone outcrops, which are suitable for a cryptic lifestyle.

It is clear that in the subsequent extensive reconnections of the two islands throughout the repeated glacial stages, when the sea level dropped beneath the maximum depth between islands, there must have been no contact between the respective salamander populations. This scenario, which was initially suggested for *Lyciasalamandra* species in Turkey (Veith *et al*. 2001), is supported in the case of *L. helverseni* because of the drier climate and the relative absence of dense vegetation in southern Karpathos and over most of Kasos. (Turkish *Lyciasalamandra* species are mostly limited to pine forests with higher humidity levels).

Salamander populations inhabiting the two major island groups meet the "discrete and significant" criteria of management units (Moritz 1994a, 1994b), meaning that they are physically separated and genetically distinct, and therefore warrant separate conservation status. This genetic substructuring may promote local adaptation in marginal populations, which is important in maintaining the adaptive potential of a species (Garcia-Ramos & Kirkpatrick 1997).

The Saria island population, however, is well within the Karpathos clade. The 13-m-deep water barrier between the two islands might have facilitated an almost continuous connection during the Pleistocene. The Tamura-Nei genetic distance between Saria and the closest locality of Karpathos (Olympos), was found to be 0.9% for the cyt-*b* fragment, while all four populations of the clade (B1) harbor a single 16S haplotype. This great similarity within the Karpathos clade implies a long-term absence of barriers to gene-flow in an otherwise continuously distributed population.

According to our data, the low divergence within Karpathos might have accumulated over quite a short period, since the late Pleistocene. Additionally, allozyme data suggest an "isolation by distance" model of differentiation within the island of Karpathos (Eleftherakos *et al*. 2004), which is further supported by the fact that populations of *L. helverseni* are still evenly distributed over the island (authors' pers. obs.), following an unfragmented habitat (extensive pine forest and limestone outcrops).

However, the relatively recent — as dating estimates suggest — restriction of populations to favorable microhabitats might have caused bottlenecks resulting in reduced average heterozygosity and allelic diversity (Table 3). Average heterozygosity is primarily affected by the rate of population growth after a given founder event or bottleneck, as it declines in each generation at a rate inversely dependent on population size (Hedrick 2000). *Lyciasalamandra* species are viviparous and, according to observations from *L. antalyana*, give birth to only two fully metamorphosed offspring per female per year (Özeti 1979). Population growth rate is therefore likely to be reduced, relative to other amphibians, leading to difficulty in restoring any lost variation.

On the island of Karpathos, only the population from the area of Mount Othos departs from HW equilibrium (Table 3). This area is an isolated limestone outcrop, a habitat type usually preferred by populations of this genus (Weisrock *et al*. 2001). As such, the relative isolation of the Othos population from neighboring populations may have promoted extensive inbreeding (Frankham 1995, Frankham & Ralls 1998, Keller & Waller 2002). However, individuals from this population were collected over different years, so the heterozygote deficiency observed might be the result of random allelic frequency fluctuations between different age classes, rather than inbreeding (Hranitz & Diehl 2000).

In contrast, extensive genetic divergence was revealed within the Kasos clade (B2). The genetic distances found between the two localities, a mere 4 km apart, were considerably higher (1.8% for cyt *b* and 0.4% for 16s), corresponding to a long-term separation episode of Pleistocene origin (ca. 0.5 Mya). The "Kasos 2" population, when analyzed for allelic variation, was found to be monomorphic in all but one (*6gpdh*) loci examined (Table 3). Moreover, this unique polymorphic locus departed significantly from HW equilibrium against heterozygotes. The sampling locality "Kasos 2" is a south-facing cliff with high levels of humidity blown in by almost constant north winds which carry moisture to that side without striking it directly. Data from the Hellenic National Meteorology Service (Direction of Climatology, Statistical Climatology Section, Karpathos Station), record NW

and NE winds present for a total of 55.6% of the year. This population is probably restricted to that favorable microclimate, and has no contact with other populations. The low levels of genetic variability and the lack of heterozygotes can be attributed to severe bottlenecking along with inbreeding, with the undermining effects on reproductive fitness unavoidable in small closed populations (Frankham 1995, Frankham & Ralls 1998, Keller & Waller 2002).

The locality "Kasos 1" was sampled for DNA analysis only, since insufficient specimens for population analysis could not be found. In general, apart from the first locality mentioned above, sampling proved to be a difficult task all over the island. Kasos is probably the warmest (20 °C mean annual temperature), and the second driest (330 mm mean annual rainfall [source Hellenic National Meteorology Service]) place in Europe (Sarlis 1998). Its main vegetation consists of small bushes (*Sarcopoterium spinosum*, *Corydothymus capitatus*, *Juniperus phoenicea*, *Asphodelus* sp., etc.), while taller vegetation has dwindled, cut down for firewood, and browsed by sheep and goats.

Sheep and goats are considered an important undermining factor for vegetation and biodiversity in general, when introduced and left relatively uncontrolled (Koopowitz & Kaye 1990). Data from a study for the management of pasturelands on Kasos indicate severe over-exploitation of the island's livestock-carrying capacity: the island is inhabited by 5–6 times more livestock than its carrying capacity, grazing twice to three times more than advisable for a specific location (Sarlis 1998). Consequently, local salamander populations of Kasos seem to have been isolated in spatially-restricted favorable locations, with limited or even totally absent gene-flow among them. These conditions, along with a possibly reduced population size, raise the concern that Kasos populations will eventually face the danger of genetic impoverishment and lowered reproductive fitness, thus unable to adapt to altering environmental changes (Frankham & Ralls 1998, Keller & Waller 2002).

Although some authors have disputed the role of genetic factors in the risk of extinction, arguing that ecological and/or demographic factors would drive a species to extinction before genetic impoverishment would take effect (e.g. Lande 1988, Young 1991, Elgar & Clode 2001), a recent study that compared the heterozygosity of 170 threatened species with the heterozygosity of the nearest related nonthreatened species, show a clear trend of threatened species having lower heterozygosity than nonthreatened (Spielman *et al*. 2004). In 77% of the paired comparisons, overall heterozygosity was reduced by an average of 35%. However, the relatively small sample size examined in the allozyme analysis could possibly hide the presence of shared alleles, thus affecting the definition of conservation units by allowing misinterpretation of diagnostic characters (Walsh 2000).

We therefore 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) to improve our knowledge of actual population characteristics, to understand the true measure of gene pool fragmentation, and to demonstrate the importance of discrete populations as units requiring protection.

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