

Cracking the nut: Geographical adjacency of sister taxa supports vicariance in a polytomic salamander clade in the absence of node support

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Received 16 May 2007; revised 8 November 2007; accepted 21 November 2007

Available online 5 December 2007

Abstract

The urodelan genus *Lyciasalamandra*, which inhabits a relatively small area along the southern Turkish coast and some Aegean islands, provides an outstanding example of a diverse but phylogenetically unresolved taxon. Molecular trees contain a single basal polytomy that could be either soft or hard. We here use the information of nuclear (allozymes) and mitochondrial (fractions of the 16S rRNA and ATPase genes) datasets in combination with area relationships of lineages to resolve the phylogenetic relationships among *Lyciasalamandra* species in the absence of sufficient node support. We can show that neither random processes nor introgressive hybridization can be invoked to explain that the majority of pairs of sister taxa form geographically adjacent units and interpret that this pattern has been shaped by vicariant events. Topology discordance between mitochondrial and nuclear trees mainly refers to an affiliation of *L. helverseni*, a taxon restricted to the Karpathos archipelago, to the western-most and geographically proximate mainland taxon in the nuclear tree, while in the organelle tree it turns out to be the sister lineage to the geographically most distant eastern clade. As this discordance cannot be explained by long-branch attraction in either dataset we suppose that oversea dispersal may have accounted for a second colonization of the Karpathos archipelago. It may have initiated introgression and selection driven manifestation of alien eastern mitochondrial genomes on a western nuclear background. Our approach of testing for area relationships of sister taxa against the null hypothesis of random distribution of these taxa seems to be especially helpful in phylogenetic studies where traditional measures of phylogenetic branch support fail to reject the null hypothesis of a hard polytomy.

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Keywords: Geographical adjacency of sister taxa; Hard polytomy; Soft polytomy; Vicariance; Node support; *Lyciasalamandra*; Salamanders; Amphibians

1. Introduction

The result of phylogenetic reconstruction largely depends on the resolving power of the characters used

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and relative lengths of internal lineages in the tree. If enough synapomorphies evolved between two successive speciation events, phylogenetic reconstruction produces dichotomous trees with sufficient support. This might be mirrored by high bootstrap support (Felsenstein, 1985), Bayesian posterior probabilities (Huelsenbeck et al., 2001) or similar estimates. Unfortunately, poorly supported dichotomies often leave the systematist with unre-

solved polytomies which can be soft or hard. Hard polytomies are the result of simultaneous divergence, whereas soft polytomies reflect relationships of organisms or genes due to the resolving power of the characters used (Page and Holmes, 1998). If the time between two speciation events is too short, the number of new character states that accumulated over time will be too low to affiliate the two descendant lineages to a common ancestor. The informative differences, once established along a branch, may become even smaller over time due to subsequent homoplastic evolution of characters that obscure phylogenetic signals (O’hUigin et al., 2002), thereby producing soft polytomies (Hoelzer and Melnick, 1994; McCracken and Sorenson, 2005). Therefore, distinguishing between hard and soft polytomies is often difficult, if not impossible.

Nine species and subspecies of *Lyciasalamandra* inhabit an area of ca. 350 km along the Turkish coast and some nearby islands (Fig. 1; Veith et al., 2001; Veith and Steinartz, 2004). The distinct species are narrowly distributed in patches following an east–west direction, most of them being located in a 30-km wide area away from the coast that receives more than 1000 mm annual precipitation and lies outside the January-0°-isotherm (Klewen, 1991). Local population size may add up to roughly 5000–10,000 individuals per hectare, thus exceeding known population densities of any other European amphibian species

(Polymeni, 1997; Veith et al., 2001). This is surprising as from spring to autumn the habitats occupied by *Lyciasalamandra* are exposed to extremely hot climatic conditions that are very unfavorable for terrestrial salamanders. Therefore, Lycian salamanders are almost exclusively restricted to boulder fields at the foot of carstic rock (Steinfartz and Mutz, 1999; Veith et al., 2001). These allow the salamanders to hide deep inside cold and humid crevices during the hot season. Salamanders are active outside these crevices only during the autumn and winter seasons when humidity is high and air temperature is below 20 °C.

In a recent paper, Weisrock et al. (2006) presented a mitochondrial DNA phylogeny of all currently known species of *Lyciasalamandra*. The major outcome of this study was that internal branches of the phylogeny were short while terminal branches were extraordinarily long. This resulted in poor support values, leaving the species phylogeny of *Lyciasalamandra* unresolved. The authors interpreted this weak resolution as an indicator of a rapid basal radiation, with time between splits too short to allow for the accumulation of sufficient synapomorphies to provide a robust phylogenetic signal between species pairs.

From a biogeographical point of view, one of the most interesting results of Weisrock et al. (2006) is the discordance between the present geographic distribution of *Lyciasalamandra helverseni* and its phylogenetic affiliation

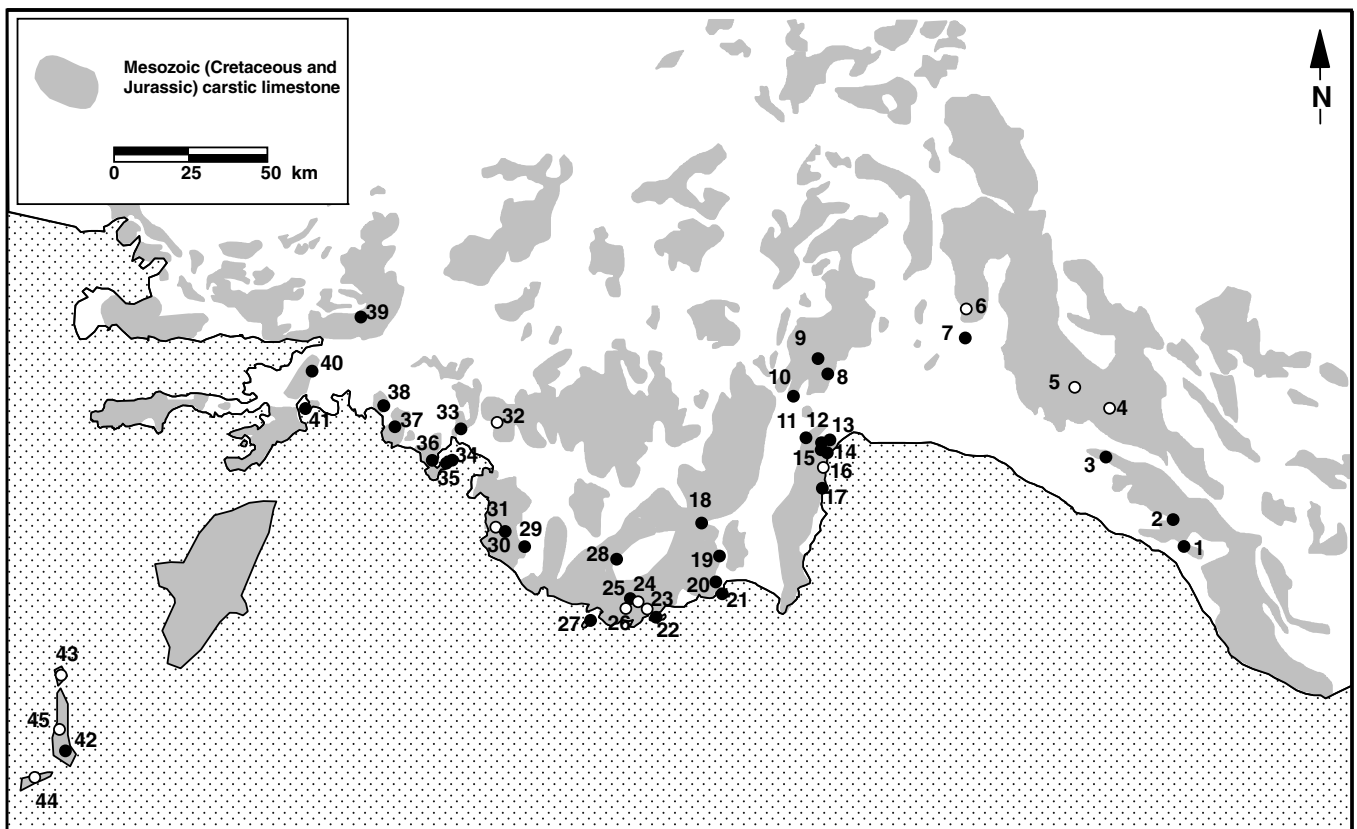


Fig. 1. Distribution of *Lyciasalamandra* along the Turkish coast and on some Aegean islands; population numbers refer to Veith et al. (2001): 1–7: *L. atifi*; 8–13: *L. antalyana*; 14–17: *L. billae*; 18–31: *L. luschani*; 32–38: *L. fazilae*; 39–41: *L. flavimembris*; 42–45: *L. helverseni*; salamanders were sampled from 33 of these populations (filled circles).

to the other species. *Lyciasalamandra helverseni* inhabits the Greek islands of Karpathos, Kasos and Saria opposite the westernmost mainland species *L. flavimembris*. Rhodes, the island between Karpathos and the Turkish mainland, was assumed to have served as a stepping stone for the colonization of Karpathos from the Anatolian mainland (Steinfartz and Mutz, 1999), although Lycian salamanders are nowadays absent from this island. The grouping of these populations based on geographic proximity is sharply contrasted by the mtDNA tree of Weisrock et al. (2006), in which *L. helverseni* is affiliated to a clade formed by *L. atifi*, *L. antalyana* and *L. billae* in the easternmost part of the species' range (Fig. 1). Although the mitochondrial tree presented by Weisrock et al. (2006) is poorly resolved, it might represent the real phylogeny, i.e., the species tree of *Lyciasalamandra*. However, at the moment we are unable to verify whether the mitochondrial phylogeny represents the true phylogeny of *Lyciasalamandra*, as such discordance could arise through long-branch attraction, ancient introgression or lineage sorting at the organelle level that produce gene trees that are different from species trees.

The goal of our study is to add mitochondrial and nuclear genetic data for 33 populations from all taxa of *Lyciasalamandra* to resolve its species phylogeny. Based on this new dataset we specifically test whether the null hypothesis of a hard polytomy can be rejected by area relationships of sister taxa formed by vicariant events and if introgressive hybridization can be invoked to explain geographical and phylogenetic adjacency.

2. Materials and methods

2.1. Sampling

We sampled 33 *Lyciasalamandra* populations, covering the entire distribution area of the genus (Fig. 1). Our sampling represents all known species and subspecies and includes all type localities of the current species and subspecies (Appendix A).

Since the genus *Salamandra* represents the sister group of *Lyciasalamandra* (Steinfartz et al., 2000, 2007; Weisrock et al., 2006) we used fire salamanders attributed to *S. salamandra* from Greece and Germany for outgroup rooting in the allozyme analyses. For the mitochondrial sequence analysis we used homologous sequences of *S. inframaculata* (Turkey), *Neurergus crocatus* (Turkey), *Pleurodeles waltl* (Spain) and *Mertensiella caucasica* (Turkey) for hierarchical outgroup comparisons.

Blood samples were taken from the caudal vein using insulin syringes filled with citric buffer (Joger and Lenk, 1997). Blood cells were separated from the plasma in a centrifuge at ca. 1000 rpm for 4 min. They were lysed in an equal amount of distilled water, and lysates were immediately used for electrophoresis. Plasma samples were stored at -80°C and later used for electrophoresis of plasma proteins. In addition, two toes were cut from each specimen,

one for electrophoresis of muscle proteins, and one for genomic DNA extraction. For allozyme electrophoresis, toes were homogenized in Pgm buffer (Hebert and Beaton, 1993) and centrifuged. The supernatant was immediately used for electrophoresis.

2.2. Allozyme electrophoresis

Allozyme electrophoresis was run for 483 specimens from 32 populations (Appendix A) on cellulose acetate (CA) plates of Helena Diagnostics, Texas. We used four different buffer systems for the separation of allozymes: phosphate buffer, pH 7.2 (PP 7.2); Tris–maleic buffer, pH 7.0 (TM 7.0); Tris–citric buffer, pH 7.2 (TC 7.2); Tris–glycine buffer, pH 8.5 (TG 8.5). Twenty enzyme systems and an unspecific protein staining provided data on 31 presumptive nuclear gene loci: aspartate aminotransferase (*aat*; EC 2.6.1.1), aconitase (*acon*; EC 4.2.1.3), adenylate kinase (*ak*; EC 2.7.4.3), creatine kinase (*ck*; EC 2.7.3.2), glucose-1-dehydrogenase (*gldh*; EC 1.1.1.47), glycerol-3-phosphate dehydrogenase (*gpdh*; EC 1.1.1.8), glucose-phosphate isomerase (*gpi*; EC 5.3.1.9), isocitrate dehydrogenase (*idh*; EC 1.1.1.42), lactate dehydrogenase (*ldh*; EC 1.1.1.27), NAD-dependent malate dehydrogenase (*mdh*; EC 1.1.1.37), NADP-dependent malate dehydrogenase (malic enzyme, *me*; EC 1.1.1.40), mannose-phosphate isomerase (*mpi*; EC 5.3.1.8), dipeptidase with phenylalanyl-proline as substrate (*pepD*; EC 3.4.11.-), tripeptidase with glycine-leucyl-leucine as substrate (*pepB*; EC 3.4.13.-), 6-phosphogluconate dehydrogenase (*6pgd*; EC 1.1.1.44), phosphoglucomutase (*pgm*; EC 5.4.2.2), pyruvate kinase (*pk*; EC 2.7.1.40), triosephosphate isomerase (*tpi*; EC 5.3.1.1), trehalase (*tre*; EC 3.2.1.28) and three plasma proteins: albumin (*alb*), prealbumin (*palb*) and transferrin (*tf*). Allozyme loci and alleles were numbered according to their electrophoretic mobility with the fastest being 1 and a, respectively.

2.3. DNA sequencing

We sequenced DNA from one to eight specimens per population (altogether 137 from 32 populations; all sequences newly generated in this study were deposited in GenBank; see Appendix A). DNA was extracted using the QiAmp tissue extraction kits (Qiagen). Double-stranded PCR was used to amplify mitochondrial DNA fragments of the 16S rRNA and ATPase genes. Primer pairs for 16S were 16SA-L and 16SB-H of Palumbi et al. (1991) and for ATPase were L-LYS-ML (5'-GGTGGCCCAACCAACCACCTTAATGAATG-3') and H-COIII-ML (5'-GTGGTAAGCGTGTGCTTGGTGTGCCAT-3'). Polymerase chain reaction cycling procedures were as follows: 16S rRNA gene: denaturation for 45 s at 94°C , primer annealing for 45 s at 55°C , extension for 60 s at 72°C ; final step at 72°C for 10 min after 35 cycles; ATPase gene: denaturation for 45 s at 94°C , primer annealing for 15 s at 55°C , ramp to 72°C with 0.5°C

per 10 s, extension for 15 s at 72 °C; final step at 72 °C for 10 min after 35 cycles. PCR products were purified using the Qiaquick purification kit (Qiagen). We sequenced single-stranded fragments on an ABI 377 automatic sequencer using standard protocols.

Obtained sequences (lengths refer to the aligned sequences including gaps) comprised 540 bp in 16S and 465 bp in ATPase. Starting points are homologous to base pair positions 1937 (16S) and 7838 (ATPase) of the *Lyciasalamandra atifi* mitochondrial genome (formerly *Mertensiella luschani atifi*; GenBank Accession No. NC002756; Zardoya and Meyer, 2001; Zardoya et al., 2003). We almost consistently achieved ca. 1000 bp for all samples. Sequences were aligned automatically using Clustal W (Higgins and Sharp, 1993). The 16S alignment was slightly refined by eye in the loop regions.

2.4. Phylogenetic analysis of allozyme data

We used the Cavalli-Sforza and Edwards (1967) chord distance to calculate allele frequencies for the allozyme data and to build a Neighbor Joining tree (PHYLIP 3.6c Felsenstein, 2002). The tree was tested for statistical significance by 2000 bootstrap replicates that were run under the subroutine SEQBOOT as implemented in PHYLIP 3.6c.

2.5. Phylogenetic analysis of mtDNA data

A partition homogeneity test (ILD test; Farris et al., 1995; 100 replicates, heuristic search using the TBR branch swapping algorithm) indicated no difference among the 16S rRNA and ATPase gene fragments (analyzed with PAUP*; Swofford, 2001). Therefore we merged them into a single alignment, and also analyzed both gene fragments separately by determining the number, nature, and distribution of base substitutions for the combined alignment and for the single genes. Equal base composition among sequences was confirmed using a χ^2 -test with PAUP*. For the ingroup taxa we used the program Modeltest (Posada and Crandall, 1998) to search for the best-fitting substitution model for the single gene fragments and for the combined alignment. The significance level of the sequential Bonferroni correction was set to $\alpha = 0.01$ and the best-fitting models were selected according to the Akaike information criterion and used in neighbor joining and maximum likelihood analyses.

Sequence data were subjected to four different methods of phylogenetic reconstruction: (i) neighbor joining (NJ) according to Saitou and Nei (1987) using the selected substitution model; (ii) maximum parsimony (MP) with transitions and transversions given equal weight and insertions and deletions (so called gaps) treated as fifth character state (10 random additions of haplotypes; heuristic search with the TBR branch swapping algorithm); (iii) maximum likelihood (ML) analysis based on the selected substitution model by Modeltest (see above); (iv) Bayesian inference (Rannala and Yang, 1996; Huelsenbeck et al., 2001).

With the exception of the Bayesian approach (MrBayes; Huelsenbeck and Ronquist, 2001), all analyses were run in PAUP* (Swofford, 2001). Robustness of NJ and MP tree topologies was tested by bootstrap analysis, with 2000 replicates each. We used quartet puzzling (Strimmer and von Haeseler, 1996) with 100,000 permutations to infer reliability values for ML tree topologies. We applied the Bayesian method using the general time reversible model of nucleotide substitution (GTR; Rodríguez et al., 1990) with the proportion of invariable sites estimated from the data. We ran four simultaneous Metropolis-coupled Monte Carlo Markov chains for 500,000 generations. We repeated Bayesian analysis four times and plotted likelihood values against generation numbers to ensure that likelihood values converged at the same value as suggested by Leaché and Reeder (2002) and to determine the necessary burn-in. A tree was sampled every 100 generations and Bayesian posterior probabilities were calculated for 4000 trees by omitting the first 1000 trees as burn-in. For simplicity we subsequently use “support values” when referring to bootstrap values for NJ and MP, puzzle support values for ML and Bayesian posterior probabilities. Here we specify that only bootstrap values $\geq 70\%$ (Huelsenbeck and Hillis, 1993) and Bayesian posterior probabilities $\geq 95\%$ indicate sufficiently resolved topologies.

2.6. Testing for geographic adjacency

Given a vicariance event that separated a formerly widely distributed population into two neighboring lineages, A and B, each of them will accumulate autapomorphic base substitutions under isolation. If further vicariance events separate each of them into two sublineages 1 and 2, respectively, and if again time allows for the accumulation of autapomorphic substitutions in all sublineages, then a phylogenetic analysis will produce a clade structure of ((A1, A2), (B1, B2)). Even if the amount of apomorphic base substitutions is small, this clade structure will emerge, although with low node supports. In the absence of dispersal, this clade structure will also reflect the geographic affiliations of lineages.

We tested the null hypothesis that the clade structure inferred from the mitochondrial and nuclear marker systems reflects a random geographic association of lineages. We generated 1000 random species trees with MESQUITE (version 1.3; Maddison and Maddison, 2006) and compared the distribution of geographically adjacent taxa from random trees to that from the observed trees. A geographically adjacent partition is defined as a branch that splits into two geographically neighboring descendent lineages. With seven lineages, the possible number of geographically adjacent splits ranges from zero to six.

If the null-hypothesis has to be rejected, two processes may account for geographically adjacent sister lineages: a strict vicariance scenario or gene flow. To test if introgressive hybridization among neighboring lineages, for which evidence exists on both nuclear and mitochondrial levels

(see 3), may account for geographic adjacency of lineages, we tested for an isolation-by-distance pattern among taxa. We applied a Mantel test between average genetic distances and the number of loci diagnostic between a pair of taxa versus neighborhood hierarchy; the latter quantifies if a given taxon is the 1st-, 2nd-, 3rd-, ... nearest neighbor to any other lineages. We preferred neighborhood hierarchy over geographic distances since it is not prone to the large differences in distribution ranges among taxa or distribution range fluctuations over time.

2.7. Test for long-branch attraction

Long-branch attraction (LBA) may arise in “any situation in which similarity due to convergent or parallel changes produces an artifactual phylogenetic grouping of taxa due to an inherent bias in the estimation process” (Andersson and Swofford, 2004). LBA is not restricted to any particular phylogenetic inference method (Bergsten, 2005) and may even include cases with equal substitution rates along all lineages (Hendy and Penny, 1989).

To test for LBA we applied three approaches that might detect this phenomenon (Bergsten, 2005): (i) independent analysis of different gene partitions, (ii) long-branch extraction (for LBA to occur we need at least one other branch that attracted the potentially misplaced branch), and (iii) outgroup extraction. We applied all three approaches to the mitochondrial dataset and approaches (ii) and (iii) to the allozyme dataset. We used only species/subspecies level phylogenies for both datasets, restricting the mtDNA data to the respective longest branch (haplotype) within a lineage or pooling all conspecific/con-subspecific allozyme population data into a single sample. MtDNA test topologies were calculated with MEGA (version 3.1; Kumar et al., 2004) using the TrN+G substitution model and calculating neighbor joining trees with 2000 bootstrap replicates. Allozyme test phylogenies were constructed as UPGMA phenograms with Phylip (version 3.6c; Felsenstein, 2002) using Cavalli-Sforza and Edwards (1967) genetic distance and with 2000 bootstrap replicates each.

3. Results

3.1. Nuclear and mitochondrial molecular variability

Six out of 31 protein loci are monomorphic across all samples including outgroups (*idh2*, *me*, *pk*, *tre*, *acon2* and *gdh*; a table of allozyme frequencies is available from MV upon request). Another six loci are monomorphic within *Lyciasalamandra* (*ak1*, *apk*, *ldh2*, *ck1*, *ck2* and *gpdh*), leaving 19 loci within and among *Lyciasalamandra* species. Genetic variability within populations is extremely low on the nuclear level, and fixed intraspecific allelic differences are found only at locus albumin (*alb*) for the Cebireis and Selge populations of *L. atifi* and at locus *pepB-1* for the Nadarla and Akkuyu Mevkii populations of *L. l. basoglu*.

We found 43 combined 16S/ATPase haplotypes within *Lyciasalamandra*, 36 of which (=86%) are private for a single population. More than half of the populations (56%) are polymorphic at the mtDNA level.

3.2. Phylogenetic relations of species

The Cavalli-Sforza and Edwards (1967) chord distance between species ranged from 0.377 to 0.676 (Table 1; pairwise distances between populations are given in Appendix B) with an average of 0.500 ± 0.100 .

None of the basal dichotomies in the allozyme NJ trees is sufficiently resolved on the level of 70% bootstrap support (Fig. 2). Basal branches are short, indicating a rapid and simultaneous radiation of major *Lyciasalamandra* lineages. In contrast, all conspecific populations consistently emerge as well supported clusters. Within *L. l. luschani*, only *L. l. luschani* appears to be monophyletic.

As for the nuclear phylogeny, the mtDNA data support the groupings of conspecific populations into highly supported species lineages (Fig. 3 and Table 2; mtDNA substitution model characteristics are given in Table 3). Again, branches of species are quite long when compared to branches between species. The inter-species relations are only poorly supported by bootstrap values and Bayesian posterior probabilities, with the exception of the (*L. billae*, *L. helverseni*) and the (*L. fazilae*, *L. flavimembris*) clades.

3.3. Test for LBA

Extraction of either *L. billae* (if LBA accounts for the position of *L. helverseni* in the tree, then *L. billae* is the attracting haplotype) or the outgroup from phylogenetic analysis does not significantly change tree topology. In both cases, *L. helverseni* still adheres to the eastern species group of *L. atifi*, *L. antalyana* and/or *L. billae*. Partitioning the ATPase gene into first, second or third codon positions removes *L. helverseni* from this group only when the more slowly evolving second codon position is analyzed. Also extraction of either *L. flavimembris* or outgroups from the nuclear tree does not alter the position of *L. helverseni*. In the first case *L. helverseni* remains the sister taxon of all other *Lyciasalamandra* species, while in the second case, *L. helverseni* remains with *L. flavimembris* accompanied by ca.

Table 1
Cavalli-Sforza and Edwards (1967) chord distance (below diagonal) and number of diagnostic allozyme loci (above diagonal) between species of *Lyciasalamandra*

Taxon	1	2	3	4	7	8	9
1 <i>L. atifi</i>	—	5	4	6	5	7	7
2 <i>L. antalyana</i>	0.339	—	4	6	7	8	10
3 <i>L. billae</i>	0.338	0.377	—	8	6	9	9
4 <i>L. luschani</i>	0.475	0.421	0.676	—	4	9	9
5 <i>L. fazilae</i>	0.379	0.476	0.532	0.492	—	8	6
6 <i>L. flavimembris</i>	0.515	0.478	0.657	0.590	0.531	—	7
7 <i>L. helverseni</i>	0.418	0.597	0.608	0.625	0.508	0.462	—

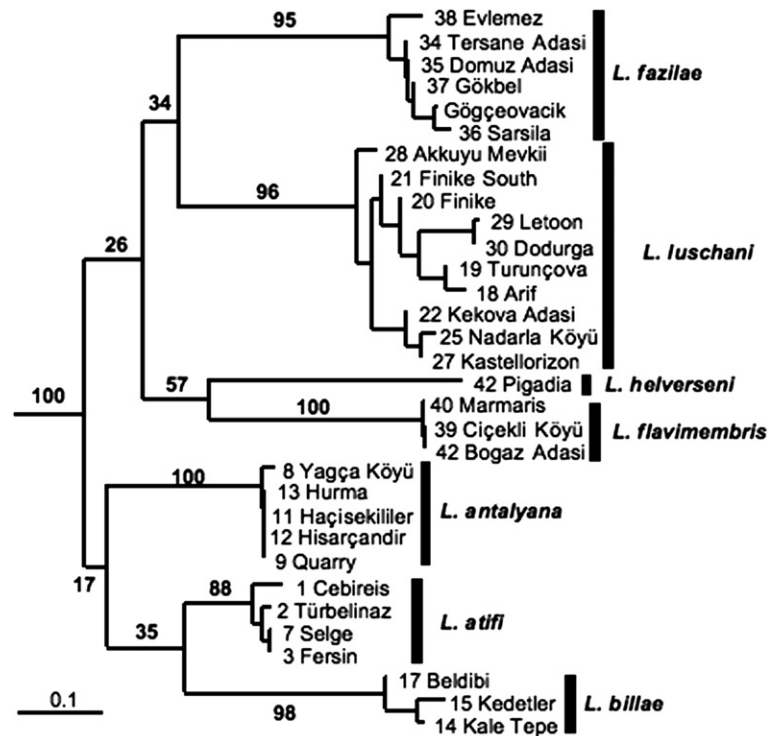


Fig. 2. NJ tree of *Lyciasalamandra* populations based on 31 protein loci; bootstrap p values for 2000 replicates are indicated; the tree is rooted via outgroup rooting using *S. s. salamandra* and *S. s. terrestris*.

50% bootstrap support. Hence, our tests give no indication that LBA is responsible for the well supported sister taxa relationship of *L. helverseni* and *L. billae* in the mitochondrial-based phylogenetic tree, and for the *L. helverseni* and *L. flavimembris* sister taxa relationship in the allozyme-based tree.

3.4. Test for geographic adjacency of sister taxa and introgression

If all conspecific or con-subspecific populations are pooled into one sample, the resulting nuclear tree almost perfectly explains the geographic distribution of taxa (Fig. 4). Four dichotomies in this tree separate geographically adjacent lineages *helverseni* versus *flavimembris*, *fazilae* versus *luschani* (*helverseni*, *flavimembris*) versus, (*luschani*, *fazilae*), and ((*helverseni*, *flavimembris*), (*fazilae*, *luschani*)) versus (*antalyana*, (*billae*, *atifi*)).

Only 19 out of 1000 random trees contain four or more splits between geographically adjacent lineages. Consequently, the observed number of geographically adjacent lineages in the allozyme tree ($n=4$) highly significantly lies outside the distribution of random trees (Fig. 5; $p < 0.01$). In contrast, the number of adjacent splits in the mtDNA tree ($n=2$) can easily be obtained by random tree topologies ($p > 0.05$; Fig. 5). Therefore, the grouping of lineages as reflected by the nuclear (organismal) tree is largely influenced by the species' geographic proximity, either due to gene flow or to a strict succession of vicariance events.

As the Mantel test between average genetic distances and the amount of loci diagnostic between a pair of taxa versus neighborhood hierarchy does not support a pattern of isolation-by-distance at the taxon level (Table 4), vicariance remains as the most plausible explanation for the geographic adjacency between the allozyme tree and the geographic distribution of *Lyciasalamandra*.

4. Discussion

Salamanders of the genus *Lyciasalamandra* are the only tailed amphibians that managed to persist now over several millions of years (Weisrock et al., 2001) in the xeric habitats of southwest Turkey and it is very likely that the survival in such an extreme habitat has been made possible by their adaptation to a partially underground-cavernicole life style (see Steinfartz and Mutz, 1998). At the same time, and compared to the rather small geographic area occupied, the genus evolved a species diversity unique among European and Near East amphibians. Based on our extensive phylogeographic analysis we will now discuss which mechanisms might have accounted for this situation and conclude that vicariance is the main driving mechanism that influenced the evolution of *Lyciasalamandra*.

4.1. Soft or hard? What accounts for the basal polytomy in *Lyciasalamandra*?

It is often difficult to distinguish between soft or hard polytomies. The true population history in soft polyto-

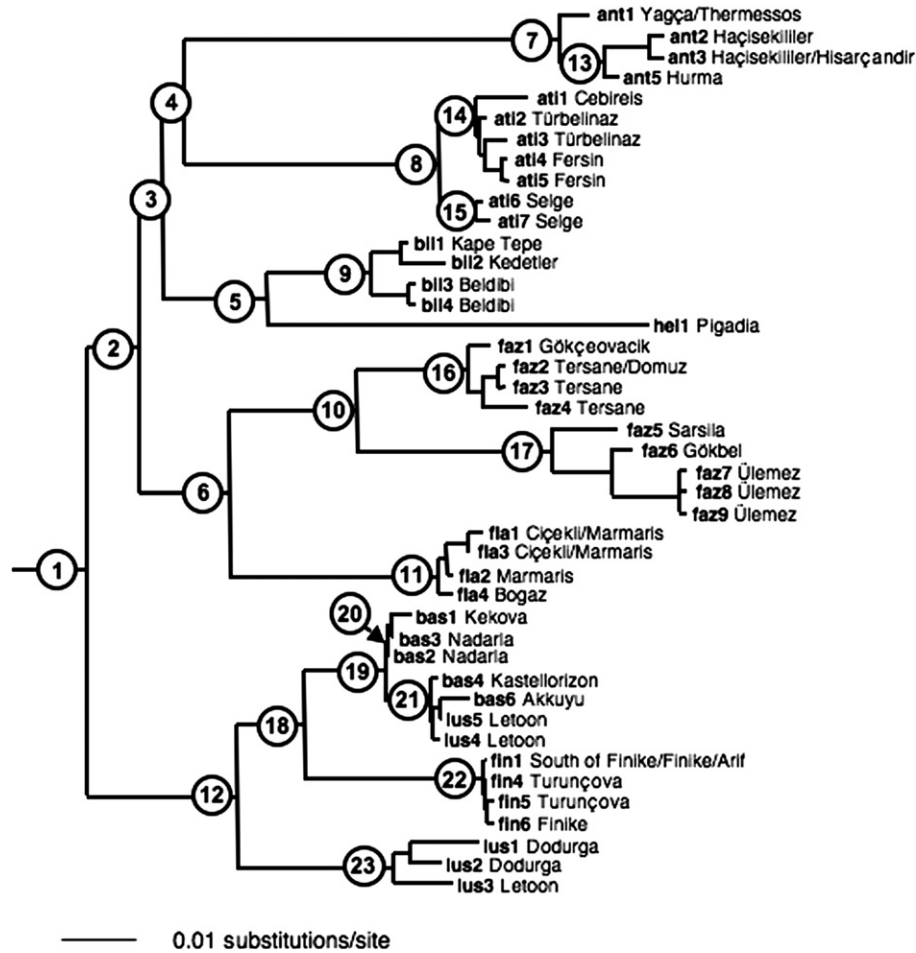


Fig. 3. Neighbor-Joining tree of 43 combined ATPase/16S *Lyciasalamandra* haplotypes applying the GTR+I+G substitution model. Major nodes are numbered. Behind haplotype names their populations of occurrence are indicated.

mies may be obscured by a reduced accumulation of synapomorphic base substitutions along internal branches, by homoplastic (convergent or back mutations) substitutions or by both. Depending on the age of a speciation event, the latter process may even totally erase any prior phylogenetic signal present on short internal branches. However, our approach testing for adjacency between geographical and phylogenetic taxon affiliation may serve as a model for distinguishing between soft or hard polytomies.

Let us assume that the basal polytomy in *Lyciasalamandra* in fact represents a hard polytomy. In evolutionary terms this is equivalent to a simultaneous allopatric fragmentation of a formerly widely distributed population into an array of lineages that subsequently evolved independently, and consequently synapomorphic characters found between single lineages would be homoplastic. However, identical alleles can be shared among lineages if the ancestral population was already polymorphic at the nuclear loci. The process of lineage sorting will lead to a stochastic sorting of these alleles, leaving some random pattern of population affiliation that does not reflect the true popula-

tion history. Under such a scenario we expect that the resulting phylogenetic tree will show random topologies of lineages, but would hardly favor a topology of lineages in which sister taxa are geographically adjacent. Our analysis of tree topologies (observed vs. random) clearly shows that a pattern of geographically adjacent taxa in the nuclear tree would hardly emerge merely by chance. Less than 1% of all randomly produced trees explained the geographic distribution of species equally well or even better than the observed nuclear tree. Hence, the basal polytomies in our organism trees of *Lyciasalamandra* are supposedly soft. Compared to the mtDNA tree, the allozyme tree is formally a population tree based on distances calculated from allele frequencies at many independent loci (Johannsen et al., 2006, showed that these protein loci are unlinked in *Lyciasalamandra*). It therefore more likely reflects the true organismal history than the mitochondrial gene tree. Since we have no indication that introgression shaped the observed phylogenetic affiliations of species, the pattern we observe in *Lyciasalamandra* evolution in fact must be the result of a successive speciation process following vicariant events.

Table 2
Clade support values (in %) for the mtDNA NJ-tree

Node	16S/ATPase				16S				ATPase				
	NJ	MP	ML	Bayes	NJ	MP	ML	Bayes	NJ	MP	ML	Bayes	
<i>Interspecific clades</i>													
1	<i>Lyciasalamandra</i>	98	100	96	100	87	100	98	100	91	98	<70	<95
2	(<i>ant</i> , <i>ati</i> , <i>bil</i> , <i>hel</i> , <i>faz</i> , <i>fla</i>)	<70	<70	<70	<95	<70	<70	<70	<95	<70	<70	<70	<95
3	(<i>ant</i> , <i>ati</i> , <i>bil</i> , <i>hel</i>)	<70	<70	<70	<95	<70	<70	<70	<95	<70	<70	<70	<95
4	(<i>ant</i> , <i>ati</i>)	<70	<70	<70	<95	<70	<70	<70	<95	<70	<70	<70	<95
5	(<i>bi</i> , <i>hel</i>)	84	92	<70	100	<70	91	71	100	<70	76	<70	<95
6	(<i>faz</i> , <i>fla</i>)	73	<70	88	100	93	87	96	100	<70	<70	<70	<95
<i>Intraspecific monophyly</i>													
7	<i>ant</i>	100	100	97	100	95	100	90	100	100	100	94	100
8	<i>ati</i>	89	100	71	100	98	99	<70	100	99	99	80	99
9	<i>bil</i>	97	100	<70	100	<70	99	83	<95	99	99	74	100
10	<i>faz</i>	99	100	82	100	91	97	<70	100	91	92	76	97
11	<i>fla</i>	100	100	96	100	100	100	94	100	85	100	<70	<95
12	<i>lus</i>	93	97	78	100	75	77	70	100	99	98	70	100
<i>Subclades</i>													
13	(<i>ant2</i> – <i>ant5</i>)	96	88	98	100	95	<70	<70	<95	94	87	95	99
14	(<i>ati1</i> – <i>ati5</i>)	89	75	<70	<95	79	<70	<70	<95	79	71	<70	<95
15	(<i>ati6</i> , <i>ati7</i>)	99	99	98	100	82	74	<70	99	98	96	96	100
16	(<i>faz1</i> – <i>faz4</i>)	100	100	96	100	96	100	83	100	100	100	90	100
17	(<i>faz5</i> – <i>faz9</i>)	100	100	90	100	99	93	88	100	100	100	84	100
18	mixed (<i>lus-bas-fin</i>)	82	91	<70	100	<70	72	<70	97	83	87	<70	99
19	mixed (<i>lus-bas</i>)	97	100	<70	100	<70	<70	<70	<95	98	100	<70	100
20	(<i>bas1</i> – <i>bas3</i>)	79	84	<70	<95	87	76	89	93	<70	<70	<70	<95
21	mixed (<i>bas4</i> , <i>bas6</i> , <i>lus4</i> , <i>lus5</i>)	86	84	<70	99	<70	<70	71	<95	78	86	<70	100
22	<i>l. fin</i>	100	100	91	100	82	84	<70	98	100	100	97	100
23	(<i>lus1</i> – <i>lus3</i>)	100	100	98	100	91	88	96	99	100	100	97	100

Node numbers refer to Fig. 3. ns, not supported by bootstrap analysis. We regard support values <70% in NJ, MP and ML and <95% in Bayes as insufficient.

Table 3

Selected substitution models (Akaike information criterion, AIC) and model parameters; $-\ln = \log$ likelihood, $\pi_x =$ nucleotide frequencies, $\text{rate}_{[X-Y]}$ = transformation rates, $I =$ proportion of invariable sites, $\alpha =$ gamma shape parameter

Model	Data partition		
	16S TVM+I	ATPase TrN+G	16S+ATPase GTR+I+G
$-\ln$	1386.76	2179.88	3688.77
AIC	2789.52	4371.77	7397.54
π_A	0.3452	0.3596	0.3527
π_G	0.1830	0.0882	0.1395
π_C	0.1970	0.2789	0.2279
π_T	0.2748	0.2732	0.2799
$\text{rate}_{[A-G]}$	19.7797	18.9272	33.6336
$\text{rate}_{[C-T]}$	19.7797	6.6461	21.5482
$\text{rate}_{[A-C]}$	2.9447	1	4.0936
$\text{rate}_{[A-T]}$	5.8105	1	3.2302
$\text{rate}_{[C-G]}$	1.0685	1	2.2020
$\text{rate}_{[G-T]}$	1	1	1
I	0.8362	0	0.5309
α	Equal rates	0.4237	0.7682

4.2. Can introgressive hybridization account for topology incongruence between nuclear and mitochondrial genes?

The non-monophyly of the *L. l. fnikensis* populations at the nuclear gene level may be interpreted as the result of

comparatively recent gene flow among subspecies. Introgression may also be seen in the *L. fazilae* population from Evlemez (=Uelemez). This population forms the sister group to all other *L. fazilae* populations in the allozyme tree, while mtDNA haplotypes from Evlemez are deeply

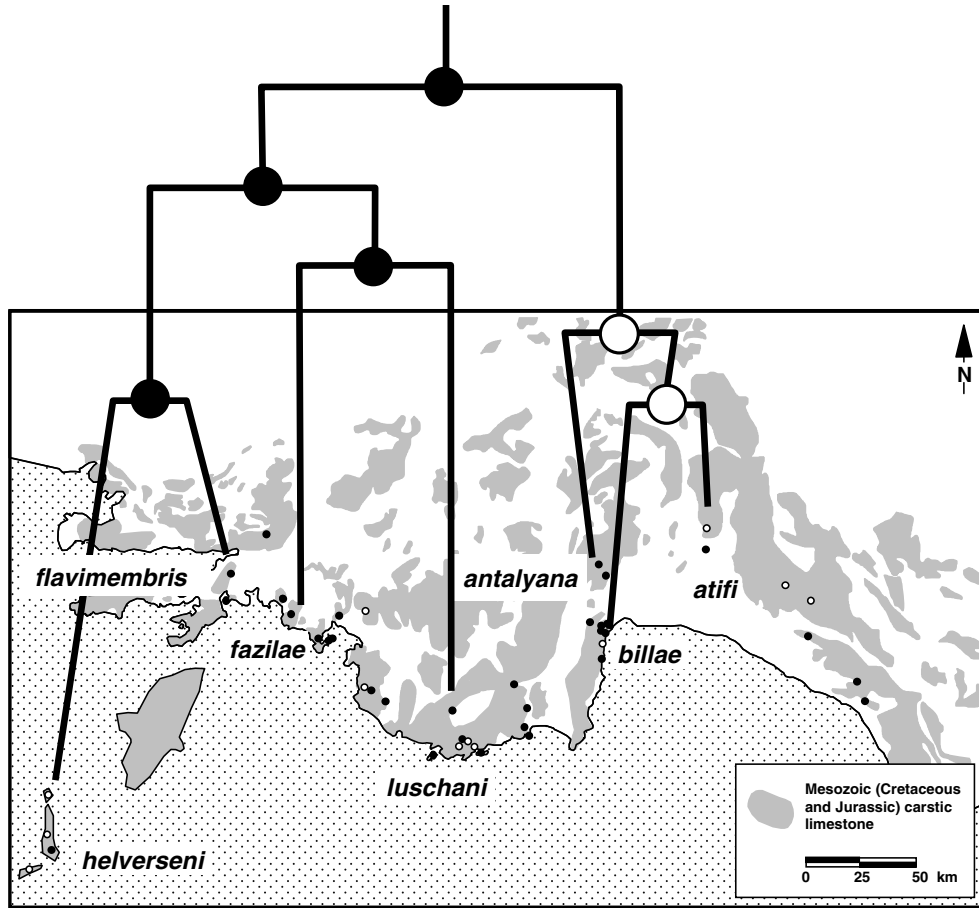


Fig. 4. Geographic projection of the *Lyciasalamandra* species topology of the NJ tree; 2000 bootstrap replicates; the tree is based on Cavalli-Sforza and Edwards (1967) chord distance; branch lengths do not reflect degrees of differentiation; filled or open circles indicate if splits are geographically adjacent or not, respectively.

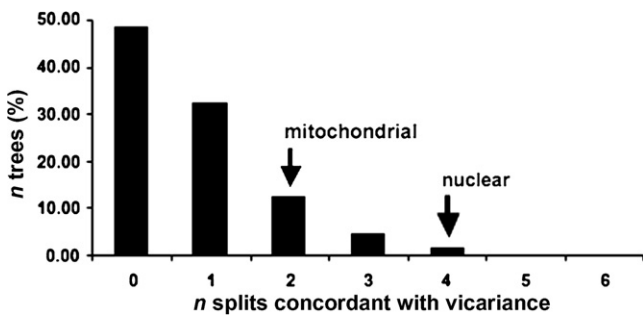


Fig. 5. Number of splits of geographically adjacent lineages in 1000 random trees for seven *Lyciasalamandra* species. Observed numbers of geographically adjacent splits in the allozyme and mtDNA trees are indicated; note that five splits cannot be achieved since one discordant partition automatically affects a second partition, reducing the maximum number of six geographically adjacent partitions directly to four.

nested within the *L. fazilae* clade. Most striking is the presence of *L. l. basoglui* haplotypes in the geographically closest *L. l. luschani* population, which shows that introgressive hybridization occasionally occurred in the past.

Table 4
Mantel test statistics for neighborhood hierarchy (nh) versus number of diagnostic loci (dl) and Cavalli-Sforza and Edwards (1967) chord distance (D); 500 permutations

Matrix comparison	Mantel <i>t</i>	<i>r</i> (matrix corr.) (normalized Mantel Z)	<i>p</i> ($Z_{\text{rand}} < Z_{\text{obs}}$)
nh versus dl	1.235	0.287	0.892
nh versus <i>D</i>	0.418	0.097	0.662

Discordant nuclear and mitochondrial molecular patterns that are likely to stem from introgression have frequently been shown. They are usually caused by mitochondrial introgression that is not paralleled by nuclear introgression (reviewed by Ballard and Whitlock, 2004). The presence of *L. l. basoglui* mitochondrial haplotypes in an *L. l. luschani* population follows this hypothesis. Morphology and allozymes clearly affiliate the Letoon population to *L. l. luschani*. However, introgression of ‘alien’ mitochondrial haplotypes must have been accompanied by introgression of nuclear alleles. Hence, it is likely

that the introgression of *L. l. basoglui* alleles into an *L. l. luschani* background originated from the occasional immigration of only a few females into the Letoon population with a selective advantage conferred by the *L. l. basoglui* mitochondrial genome.

Differential selection of mtDNA haplogroups was experimentally shown to foster the replacement of one haplotype by a better adapted one (Mishmar et al., 2003; Ballard and Whitlock, 2004). In an extreme case the original haplotypes may be replaced, making the host population virtually equivalent to the alien population at the mtDNA level. Babik et al. (2005) demonstrated such a case for *Triturus montandoni*, a newt species well distinguishable from its congener *Triturus vulgaris* on morphological, behavioral and nuclear genetic grounds. However, its mitochondria have been entirely replaced by *T. vulgaris* mitochondria. A similar case is known from the two European bat species *Eptesicus serotinus* and *E. nilssonii*, where the *E. serotinus* mitochondria entirely replaced the *E. nilssonii* mitochondria, although for morphological and nuclear markers both are clearly distinguishable at the species level (Meyer and van Helversen, 2001).

4.3. Rafting salamanders?

The relative position of *L. helverseni* within the phylogeny of *Lyciasalamandra* is the most striking discord between the nuclear and the mitochondrial markers. Its affiliation to *L. flavimembris* in the allozyme tree seems plausible given that the only land bridge between Karpathos and the Turkish mainland close to Marmaris, the type locality of *L. flavimembris*, was possible via Rhodes. However, as in the mitochondrial analysis of Weisrock et al. (2006), *L. helverseni* links in the mtDNA tree to the *L. billae/L. antalyana* group that lives geographically far away from Karpathos.

However, a second explanation remains. Assuming that the allozyme tree would reflect the true genus history of Lycian salamanders (as suggested by geographical analysis of the allozyme tree topology), the *L. billae/L. helverseni* haplolineage reflects introgression from a *L. billae* ancestor into the gene pool of an ancestral *L. helverseni* ‘host’ population. Branch lengths indicate that this would have happened a long time ago. Unfortunately, confidently identifying introgression becomes more difficult the further in the past such gene flow had occurred (Funk and Omland, 2003). The *L. l. luschani* example from Letoon and the *L. billae/L. antalyana* example from near Antalya show us that introgressive hybridization between *Lyciasa-*

lamandra lineages occurred at different times in the group’s history, perhaps even as long ago as shortly after the major lineages had diverged.

How could salamanders from the Beydaglari Mountains where *L. billae* lives have reached Karpathos without leaving any traces on the mainland in between? The Aegean islands had reached their current positions by 10–9 million years ago (Creuzburg, 1963; Dermitzakis and Papanikolaou, 1981). Tectonic events therefore cannot be invoked to explain a close *L. billae*–*L. helverseni* relationship. The only plausible explanation is rafting. A single pregnant female of these viviparous salamanders would suffice to colonize an island and to found a new population or to immigrate into an already existing one. Today, Mediterranean currents, namely the Rhodes Gyre (Pinardi and Masetti, 2000), may easily transport a log with salamanders from the Bay of Antalya towards Karpathos. Such currents are pronounced, especially in winter (Robinson et al., 1991). This alone may not suffice to explain salamander rafting several million years ago. However, in the light of Mediterranean paleoclimatic maps for the Pliocene (Haywood et al., 2000) and the recurrent climatic fluctuations since it seems plausible that surface water circulation patterns similar to those of today may have repeatedly occurred in the history of the Eastern Mediterranean. At least for the western Mediterranean we know that since the mid Pleistocene regional wind patterns and hence also water currents were comparable to those of today (Haywood and Cook, 2005). Rafting therefore may provide a plausible mechanism to explain how *L. billae* may have reached Karpathos. During the last years increasing evidence for overseas rafting accumulated for a variety of organisms (reviewed by de Queiroz, 2005), including amphibians, (e.g., Vences et al., 2003; Veith et al., 2003; Measey et al., 2007).

Acknowledgments

Thomas Mutz and Burkhard Thiesmeier provided samples. Mehmet Rizvan-Tunc, Olaf Godmann and Thomas Mutz assisted during field work in Anatolia and Dagmar Klebsch in the laboratory. Jes Johannesen and an anonymous referee gave valuable comments on an earlier draft of this paper. Renata Platenberg improved this manuscript linguistically. Several general and many specific comments made by Allan Larson helped to increase the quality of this paper significantly. Thanks to all of them.

Appendix A

Localities of *Lyssalamandra* analysed in the present study; populations numbers refer to Veith et al. (2001); sizes of allozyme and mtDNA samples as well as abundances of combined 16S/ATPase haplotypes are indicated (in brackets); GenBank accession number refer to haplotypes printed in bold (*tt* = type locality)

Pop. No.	Locality	Taxon	Coordinates		Sample size			GenBank accession numbers	
			N	E	Allozymes	mtDNA	Haplotypes	16S	ATPase
1	Cebireis	<i>L. atifi</i>	36°32.57	32°06.16	15	4	ati1 (4)	EU430959	EU430917
2	Türbelinaz (<i>tt</i>)	<i>L. atifi</i>	36°35.53	32°01.52	8	3	ati2 (2) ati3 (1)	EU430960	EU430918 EU430919
3	Fersin	<i>L. atifi</i>	36°48.39	31°45.85	24	6	ati4 (3) ati5 (3)	EU430962	EU430920 EU430921
7	Selge	<i>L. atifi</i>	37°12.27	31°09.97	5	3	ati6 (1) ati7 (2)	EU430963	EU430922 EU430923
8	Yagça Köyü	<i>L. antalyana</i>	36°03.28	30°33.39	16	5	ant1(5)		
9	Quarry	<i>L. antalyana</i>	37°06.48	30°33.80	5	—			
10	Thermessos	<i>L. antalyana</i>	37°01.01	30°29.31	—	1	ant1 (1)	EU430956	EU430913
11	Haçisekililer	<i>L. antalyana</i>	36°50.59	30°30.11	10	5	ant2 (1) ant3(4)	EU430957	EU430914
12	Hisarçandır	<i>L. antalyana</i>	36°49.62	30°32.56	5	4	ant3 (4)	EU430957	EU430915
13	Hurma Köyü (<i>tt</i>)	<i>L. antalyana</i>	36°51.10	30°35.31	17	6	ant5 (6)	EU430958	EU430916
14	Kale Tepe (<i>tt</i>)	<i>L. billae</i>	36°47.09	30°34.03	25	5	bil1 (5)	EU430967	EU430929
15	Kedetler	<i>L. billae</i>	36°48.16	30°32.00	15	4	bil2 (4)	EU430968	EU430930
17	Beldibi	<i>L. billae</i>	36°41.08	30°34.34	15	4	bil3 (3) bil4 (1)	EU430969	EU430931 EU430932
18	Arif/Catalar	<i>L. l. finnikensis</i>	36°30.34	30°03.73	5	3	fin1(3)		
19	Turunçova	<i>L. l. finnikensis</i>	36°23.95	30°08.48	14	2	fin4 (1) fin5 (1)	EU430984	EU430942 EU430943
20	Finike (<i>tt</i>)	<i>L. l. finnikensis</i>	36°18.39	30°08.28	13	6	fin1(5) fin6 (1)	EU430978	EU430942
21	South of Finike	<i>L. l. finnikensis</i>	36°16.58	30°08.92	9	3	fin1 (3)	EU430977	EU430942
22	Kekova Adası	<i>L. l. basoglui</i>	36°11.12	29°52.63	22	5	bas1 (5)	EU430964	EU430924
25	Nadarla Köyü (<i>tt</i>)	<i>L. l. basoglui</i>	36°15.50	29°48.10	11	4	bas2 (2) bas3 (2)	EU430964	EU430925 EU430926
27	Kastellorizon	<i>L. l. basoglui</i>	36°08.79	29°35.85	20	5	bas4 (5)	EU430965	EU430927
28	Akkuyu Mevkii	<i>L. l. basoglui</i>	36°23.22	29°42.45	28	5	bas6 (5)	EU430966	EU430928

29	Letoon	<i>L. l. luschani</i>	36°22.62	29°18.54	21	8	lus3 (3) lus4 (2) lus5 (3)	EU430982 EU430983 EU430983	EU430950 EU430951 EU430927
30	Dodurga (<i>tt</i>)	<i>L. l. luschani</i>	36°24.30	29°13.00	17	4	lus1 (2) lus2 (2)	EU430980 EU430981	EU430948 EU430949
33	Gögçeovacik (<i>tt</i>)	<i>L. fazilae</i>	36°47.33	28°58.69	24	5	faz1 (5)	EU430971	EU430934
34	Tersane Adasi	<i>L. fazilae</i>	36°49.50	28°54.70	19	4	faz2 (1) faz3 (2) faz4 (1)	EU430972 EU430973 EU430972	EU430935 EU430935 EU430936
35	Domuz Adasi	<i>L. fazilae</i>	36°49.90	28°54.03	17	5	faz2 (5)		
36	Sarsila Isk.	<i>L. fazilae</i>	36°50.45	28°50.40	12	4	faz5 (4)	EU430974	EU430937
37	Gökbel	<i>L. fazilae</i>	36°46.91	28.39.90	18	4	faz6 (4)	EU430975	EU430938
38	Ülemez	<i>L. fazilae</i>	36°50.45	28°37.60	8	3	faz7 (1) faz8 (1) faz9 (1)	EU430976 EU430976 EU430976	EU430939 EU430940 EU430941
39	Çiçekli Köyü	<i>L. flavimembris</i>	37°05.70	28°29.31	11	6	fla1 (1) fla3 (5)	EU430979 EU430979	EU430944 EU430946
40	Marmaris (<i>tt</i>)	<i>L. flavimembris</i>	36°55.53	28°16.78	30	3	fla1 (1) fla2 (1) fla3 (1)	EU430979	EU430945
41	Bogaz Adasi	<i>L. flavimembris</i>	36°48.69	28°17.47	11	2	fla4 (2)	EU430979	EU430947
42	Pigadia, GR (<i>tt</i>)	<i>L. helverseni</i>	36°30.35	27°13.26	13	6	hell (6)	EU430970	EU430933
	Greece, Pelion Mts.	<i>Salamandra s. salamandra</i>			5				
	Germany, Hunsrück	<i>S. s. terrestris</i>			5				
	Turkey	<i>S. s. infraimmaculata</i>				1		EU430954	EU430911
	Turkey	<i>Mertensiella caucasica</i>				1		EU430955	EU430912
	Turkey	<i>Neurergus crocatus</i>				1		EU430953	EU430910
	Algeria	<i>Pleurodeles poireti</i>				1		EU430952	EU430909

Pop.	17	18	19	20	21	22	25	27	28	29	30
18	0.7099										
19	0.6795	0.0121									
20	0.6371	0.0423	0.0197								
21	0.6177	0.0743	0.044	0.0077							
22	0.6351	0.1289	0.1007	0.0407	0.0271						
25	0.6702	0.1645	0.141	0.0768	0.0655	0.0124					
27	0.6369	0.1462	0.1191	0.0572	0.0444	0.0069	0.0087				
28	0.6105	0.1308	0.101	0.0596	0.0399	0.0578	0.0964	0.0662			
29	0.6093	0.1062	0.0758	0.0845	0.0989	0.1564	0.1982	0.175	0.1533		
30	0.6093	0.1005	0.0702	0.0789	0.0932	0.1507	0.1926	0.1694	0.1505	0.0056	
33	0.4984	0.5793	0.549	0.5074	0.4876	0.5102	0.552	0.5279	0.48	0.4788	0.4788
34	0.4629	0.5438	0.5135	0.4719	0.4521	0.4747	0.5165	0.4924	0.4445	0.4433	0.4433
35	0.464	0.5449	0.5145	0.4729	0.4532	0.4757	0.5175	0.4935	0.4456	0.4444	0.4444
36	0.5046	0.5941	0.5672	0.5264	0.5069	0.529	0.5698	0.5418	0.4755	0.4981	0.4981
37	0.4405	0.5513	0.5209	0.4793	0.4596	0.4821	0.5239	0.4999	0.452	0.4508	0.4508
38	0.5368	0.5203	0.4899	0.4483	0.4285	0.4511	0.4929	0.4688	0.4209	0.4197	0.4197
39	0.6166	0.597	0.597	0.5634	0.5461	0.5643	0.597	0.5801	0.5373	0.6567	0.6567
40	0.6175	0.597	0.597	0.5634	0.5461	0.5643	0.597	0.5801	0.5373	0.6567	0.6567
41	0.6166	0.597	0.597	0.5634	0.5461	0.5643	0.597	0.5801	0.5373	0.6567	0.6567
42	0.5616	0.6567	0.6567	0.6231	0.6058	0.624	0.6567	0.6398	0.597	0.597	0.597
<i>S. s. sal.</i>	1.2537	1.3731	1.3731	1.3731	1.3731	1.3731	1.3731	1.3731	1.3731	1.3731	1.3731
<i>S. s. ter.</i>	1.3134	1.3731	1.3731	1.3731	1.3731	1.3731	1.3731	1.3731	1.3731	1.3731	1.3731
Pop.	33	34	35	36	37	38	39	40	41	42	Ssal
34	0.012										
35	0.0113	0									
36	0.0208	0.038	0.0373								
37	0.0159	0.0054	0.0053	0.0369							
38	0.0463	0.0559	0.0552	0.0723	0.0598						
39	0.5385	0.503	0.5041	0.55	0.5052	0.5824					
40	0.5385	0.503	0.5041	0.55	0.5052	0.5824	0.0009				
41	0.5385	0.503	0.5041	0.55	0.5052	0.5824	0	0.0009			
42	0.5254	0.503	0.5041	0.491	0.4855	0.5373	0.4618	0.4627	0.4618		
<i>S. s. sal.</i>	1.3731	1.3388	1.3399	1.3458	1.341	1.3731	1.3731	1.3731	1.3731	1.3134	
<i>S. s. ter.</i>	1.4328	1.3985	1.3996	1.4055	1.4007	1.4328	1.4328	1.4328	1.4328	1.3731	0.2985

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