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# Seven at one blow: the origin of major lineages of the viviparous Lycian salamanders (*Lyciasalamandra* Veith and Steinfartz, 2004) was triggered by a single paleo-historic event

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**Abstract.** The number of tectonic and climatic events that are used to explain speciation processes in the eastern Mediterranean region is low compared to the western Mediterranean. Among them, the emergence of the mid-Aegean trench and the Messinian Salinity Crisis (MSC) often concurred with speciation time estimates that were inferred from molecular data. We here present a dated molecular phylogeny of *Lyciasalamandra* from Turkey and Greece based on ca. 4500 bp of the mitochondrial genome (3000 bp of three nuclear genes appeared to be completely inconclusive due to their extremely low degree of variation among taxa). Seven major lineages emerged simultaneously from a basal hard polytomy. A scenario that dates this polytomy to 12.3 and 10.2 million years ago, around the final emergence of the mid-Aegean trench, appears to be most plausible. The MSC can be made responsible for first intraspecific divergence events within *L. luschani*, *L. fazilae* and *L. flavimembris*. Further diversification can be explained by Pliocene and Pleistocene glaciations. Based on levels of molecular differentiation we suggest the recently described species *L. arikani*, *L. irfani* and *L. yehudahi* to be treated as subspecies of *L. billae*.

*Keywords*: Anatolia, Bayesian molecular dating, hard polytomy, *Lyciasalamandra*, Messinian Salinity Crisis, mid-Aegean trench, MtDNA, ncDNA.

## Introduction

Our knowledge on the geological history of the Mediterranean region, and namely of its western part surrounding the Alboran and the Tyrrhenian Seas, is without comparison. Its reconstruction since the beginning of the Cenozoic, ca. 65 million years ago (mya) (among others reviewed by De Jong, 1998 and Rosenbaum et al., 2002) offers biogeographers a multitude of opportunities for inferring dated molecular phylogenies. In contrast, there exists less detailed information on the geological history of the Eastern Mediterranean.

During the Alpide orogeny in the late Mesozoic the Taurus Mountains formed mainly under the control of the Africa-Eurasia convergence and northward subduction of the African plate. The subduction and ensuing collision processes have led to the formation of mountain belts, from the Betic Cordillera and the Rif in the West, to the Hellenides and Taurides in the East (Jolivet et al., 2006). Additional geodynamic processes have been postulated to have contributed to the later formations of this orocline, including Miocene rotations of several mountain arches such as the Bey Dağları carbonate platform (Van Hinsbergen et al., 2010) or the emergence of the mid-Aegean trench, which was completed 10-9 mya in the late Miocene (Creutzburg, 1963).

In the Middle Miocene, numerous islands formed in the Aegean region which were repeatedly connected to, and isolated from, the mainland during their geological history, e.g., during the Messinian Salinity Crisis (5.96-5.33 mya) when the Mediterranean basin fell partly dry

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(Krijgsman et al., 1999a), or during the Pleistocene (2.58 mya-11.70 kya) due to eustatic sea-level changes (Perissoratis and Conispoliatis, 2003). Numerous evidence exists from a wide range of organisms, such as plants (Bittkau and Comes, 2009), invertebrates (e.g., Heller, 1976; Sfenthourakis, 1996; Welter-Schultes and Williams, 1999; Fattorini, 2002; Jesse et al., 2011) and vertebrates (e.g., Poulakakis et al., 2005; Busack and Lawson, 2008), that this may have triggered speciation in the Aegean region.

None of these younger tectonic events could be unambiguously linked to speciation events in Western Anatolia (but see Akin et al., 2010). Rather, severe climatic alterations were held responsible for species formation, such as those accompanying the Messinian Salinity Crisis (MSC; Hsü et al., 1977; Krijgsman et al., 1999a, 1999b; Lymberakis and Poulakakis, 2010) and the repeated pre- and post-Pleistocene climatic fluctuations (Perissoratis and Conispoliatis, 2003; Lymberakis and Poulakakis, 2010).

Amphibians are especially suitable for vicariance biogeography, since transmarine dispersal is rare in these physiologically highly sensible organisms (but see e.g. Vences et al., 2003; Veith et al., 2004). Hence, calibrated molecular clocks in the Mediterranean area mainly rely on dated vicariance events caused by marine isolation of populations. However, there are some examples of specious amphibian genera that live in comparatively small areas which cannot easily be attributed to such vicariance events. Five species of the plethodontid salamander genus *Hydromantes* live on Sardinia (Carranza et al., 2008; Van der Meijden et al., 2009; Chiari et al., 2012); climatic events most probably accounted for intra-Sardinian diversification (Carranza et al., 2008). Ten species of the intriguing genus *Lyciasalamandra* have been described from mountainous areas of south-western Anatolia, within a strip approximately 20-30 km broad and 550 km long, and from some Greek and Turkish islands (Veith et al., 2001; Göçmen et al., 2011, 2012). With only one exception (*L. helverseni*), *Lyciasalamandra* populations are restricted to boulder fields at the foot of karstic limestone formations where the salamanders live partially underground (e.g. Steinfartz and Mutz, 1998; Veith et al., 2001; Veith and Steinfartz, 2004).

Veith et al. (2008) analysed nuclear (31 allozyme loci) and mitochondrial (ca. 1000 bp of fractions of the 16SrRNA and ATPase genes) data to reconstruct the phylogenetic relationships of the seven described species at that time. However, due to tree topology discrepancies between both data sets, they did not apply a molecular clock. Estimates of the split between *Lyciasalamandra* and its sister genus *Salamandra* (Titus and Larson, 1995; Veith et al., 1998) dated this split at 43.4-27.7 mya (Zhang et al., 2008), 15-14 mya (Veith et al., 1998) and 11 mya (Weisrock et al., 2001). Consequently, diversification within *Lyciasalamandra* must have started much later. Based on the mitochondrial DNA sequence data of six of the seven at that time known species, Weisrock et al. (2001) suggested the intrageneric divergence to have started around 7.9-5.9 mya, which they attributed to Late Miocene vicariance caused by the rise of Anatolia due to acceleration of northward movement of the Gondwana fragment of Arabia (Quennell, 1984, cited in Weisrock et al., 2001). All published intrageneric phylogenies consistently showed that lineages within *Lyciasalamandra* diverged relatively rapidly (Veith et al., 1998; Weisrock et al., 2001; Veith et al., 2008), making it likely that a single event or period may have been responsible for the seemingly simultaneous rise of so many species within a relatively small area. However, the description of three new *Lyciasalamandra* species within the last years (Göçmen et al., 2011, 2012) raises the question if the seemingly simultaneous speciation within the genus has to be reassessed, since the new species may have evolved earlier or later than the others. Therefore, we analysed ca. 4500 bp

of mitochondrial and ca. 3000 bp of nuclear genes for all species of *Lyciasalamandra* and present a first dated phylogeny of all described species. We test the hypothesis of a hard polytomy being a likely scenario for the evolution of all *Lyciasalamandra* species. Based on our phylogenetic trees and levels of molecular divergence we also re-assess the validity of all described species.

## Material and methods

*Samples*

We analyzed specimens of all major *Lyciasalamandra* lineages as identified by Veith et al. (2008), as well as newly described species and subspecies (Göçmen et al., 2011, 2012; Akman and Godmann, 2014; Üzüm, 2015) (see table S1 in the online supplementary material). For hierarchical outgroup rooting, as well as for molecular clock calibration, we added homologous gene fragments from complete mitochondrial genomes of further Salamandridae species from Zhang et al. (2008): *Salamandra salamandra* (EU880331), *Chioglossa lusitanica* (EU880308), *Mertensiella caucasica* (EU880319), *Pleurodeles poireti* (EU880329), *Pleurodeles waltl* (EU880330), *Euproctus platycephalus* (EU880317) and *Euproctus montanus* (EU880316). Nuclear gene fragments for *Salamandra salamandra* were included from Vences et al. (2014) and Shen et al. (2013).

*DNA extraction, PCR and sequencing*

DNA was isolated using the Qiagen Blood and Tissue Kit following the manufacturer's instructions. We sequenced fractions of the following genes: 16SrDNA short (primers 16SAL and 16SBH of Palumbi et al., 2002; initial melting for 120 s at 94°C, 33 cycles of denaturation for 30 s at 94°C, primer annealing for 30 s at 51°C, extension for 60 s at 65°C, final step at 65°C for 10 min), 16SrDNA long (primers L3002 and H4419 of Macey et al., 1997 and Macey et al., 1998; initial melting for 120 s at 94°C, 30 cycles of denaturation for 35 s at 95°C, primer annealing for 35 s at 56°C, extension for 60 s at 70°C, final step at 65°C for 10 min), 12SrDNA (12SAL and 12SBH of Kocher et al., 1989; initial melting for 120 s at 94°C, 33 cycles of denaturation for 45 s at 94°C, primer annealing for 45 s at 52°C, extension for 90 s at 72°C, final step at 72°C for 5 min), ND1 (primers L4160, L4221 and H4980 of Kumazawa and Nishida, 1993 and Macey et al., 1997; initial melting for 120 s at 94°C, 30 cycles of denaturation for 35 s at 95°C, primer annealing for 35 s at 41°C, extension for 60 s at 70°C, final step at 65°C for 10 min), ND2 (primers L4437 and H5934 of Macey et al., 1997; initial melting for 120 s at 94°C, 30 cycles of denaturation for 35 s at 95°C, primer annealing for 35 s at 54°C, extension for 60 s at 70°C, final step at 65°C for 10 min), ATPase (primers L-LYS-ML and H-COIII-ML of Veith et al., 2008; initial melting for 10 s at 94°C, 30 cycles of denaturation for 30 s at 98°C, primer annealing for 30 s at 67°C, extension for 30 s at 72°C, final step at 72°C for 1 min), Rag-1 (primers RAG1-SAL-F1 and RAG1-SAL-R1 of Vences et al., 2014 and Hauswaldt et al., 2011, respectively; initial melting for 120 s at 94°C, 33 cycles of denaturation for 30 s at 94°C, primer annealing for 30 s at 51°C, extension for 60 s at 65°C, final step at 65°C for 10 min), KIAA (primers KIAA1239F1 and KIAA 1239R1 for PCR and KIAA1239NF1 and KIAA 1239R1 for nested PCR; Shen et al., 2012, 2013; initial melting for 120 s at 94°C, 33 cycles of denaturation for 30 s at 94°C, primer annealing for 30 s at 45°C, extension for 60 s at 65°C, final step at 65°C for 10 min), and TTN (primers TTNF1 and TTNR1 for PCR and TTNNF1 and TTNNR1 for nested PCR of 16SBH of Shen et al., 2013; initial melting for 120 s at 94°C, 33 cycles of denaturation for 30 s at 94°C, primer annealing for 30 s at 45°C, extension for 60 s at 65°C, final step at 65°C for 10 min).

PCR reactions were prepared using either 5Prime Master Mix (12S, 16S, ND1, ND2, Rag1, KIAA, TTN), or the Phusion Flash Master Mix of Thermo Science (ATP6, ATP8). PCR products were purified using the High Pure PCR Product Purification Kit of Roche. Sanger reactions for all genes were run using the Big Dye Terminator (ABI) with initial melting for 60 s at 96°C, 25 cycles of denaturation for 10 s at 96°C, primer annealing for 5 s at 50°C, extension for 240 s at 60°C. We sequenced single stranded fragments from both directions each on an ABI 3500 Genetic Analyzer Serie 2 automatic sequencer using standard protocols. Altogether, we obtained sequences from three sections of the mitochondrial genome: 12S (partim), max. 393 bp, positions 498-885 of the *L. flavimembris* mitochondrial genome (EU880318); 16S(partim)-$tRNA_{Leu}$-ND1-$tRNA_{Ile}$- $tRNA_{Gln}$– $tRNA_{Met}$- ND2- $tRNA_{Trp}$- $tRNA_{Ala}$-$tRNA_{Asn}$-$tRNA_{Tyr}$(partim), max. 3373 bp, positions 1934-5274 of the *L. flavimembris* mitochondrial genome (EU880318); ATP8/ATP6(partim), max. 733 bp, positions 7732-8457 of the *L. flavimembris* mitochondrial genome (EU880318).

*Sequence alignment and phylogenetic reconstruction*

Phylogenetic analyses were done with Mega (version 6, Tamura et al., 2013), RAxML (version 8, Stamatakis, 2014) and MrBayes (version 3.2.6, Ronquist and Huelsenbeck, 2003; Ronquist et al., 2012). Sequences were aligned with ClustalW (Thompson et al., 1994) and refined by eye where necessary. We manually defined 14 subsets for the mitochondrial alignment (12S, 16S, every codon position for the protein coding genes ND1, ND2, ATP6 and ATP8, as well as nine tRNAs) and 9 subsets for the nuclear alignment (codon positions for every gene). Partition Finder (version 1.1.1, Lanfear et al., 2012) was used to determine models of nucleotide evolution and the best-fit partitioning schemes of the defined subsets, implementing the greedy algorithm, unlinked branch length across partitions and the corrected Akaike information criterion (AICc) for model selection.

We ran Partition Finder for each phylogenetic analysis tool to get the optimal partition scheme which could be used as a priori configuration in the single tools. The best-fitting partitioning scheme divided the mitochondrial dataset into five partitions and the nuclear data set into three partitions. Almost all partitions favor the GTR model with Γ, I or Γ + I.

For phylogenetic reconstruction, we used Maximum-Likelihood (ML) and Bayesian Inference (BI) methods using the selected partitions and substitution models. The ML tree was calculated with RAxML, running 2000 bootstrap replicates using rapid bootstrapping and the greedy algorithm (Stamatakis, 2014). BI was performed with MrBayes, with two runs with 10 million generations each. We used four independent Markov Chain Monte Carlo (MCMC) analyses, with one cold and three heated chains, and the run was sampled every 1000th generation with a burn-in of 20%. Since the amount of substitutions in the nuclear gene alignment was extremely low compared to that of the mitochondrial genes (see below), we abstained from running a combined analysis; rather we analyzed both data sets separately to compare the phylogenetic signals of these differentially inherited genomes.

*Test for hard versus soft polytomy*

For a basal polytomy of seven major lineages within *Lyciasalamandra* (see below and fig. 1), we tested the hypothesis of hard versus soft polytomy using Mesquite (version 3.10, Maddison and Maddison, 2016). A hard polytomy of the mitochondrial tree was imported as a user defined tree with the same topology as the respective best tree (non-zero internal branches) calculated under MrBayes with Bayesian Inference, but with internal branches connecting the seven major lineages collapsed into one polytomy. According to Olave et al. (2015), we first simulated 10 000 trees for the hypothesis of a hard polytomy and compared the simulated data against the hypothesis model to obtain an approximation of a probability distribution of expected deep coales-

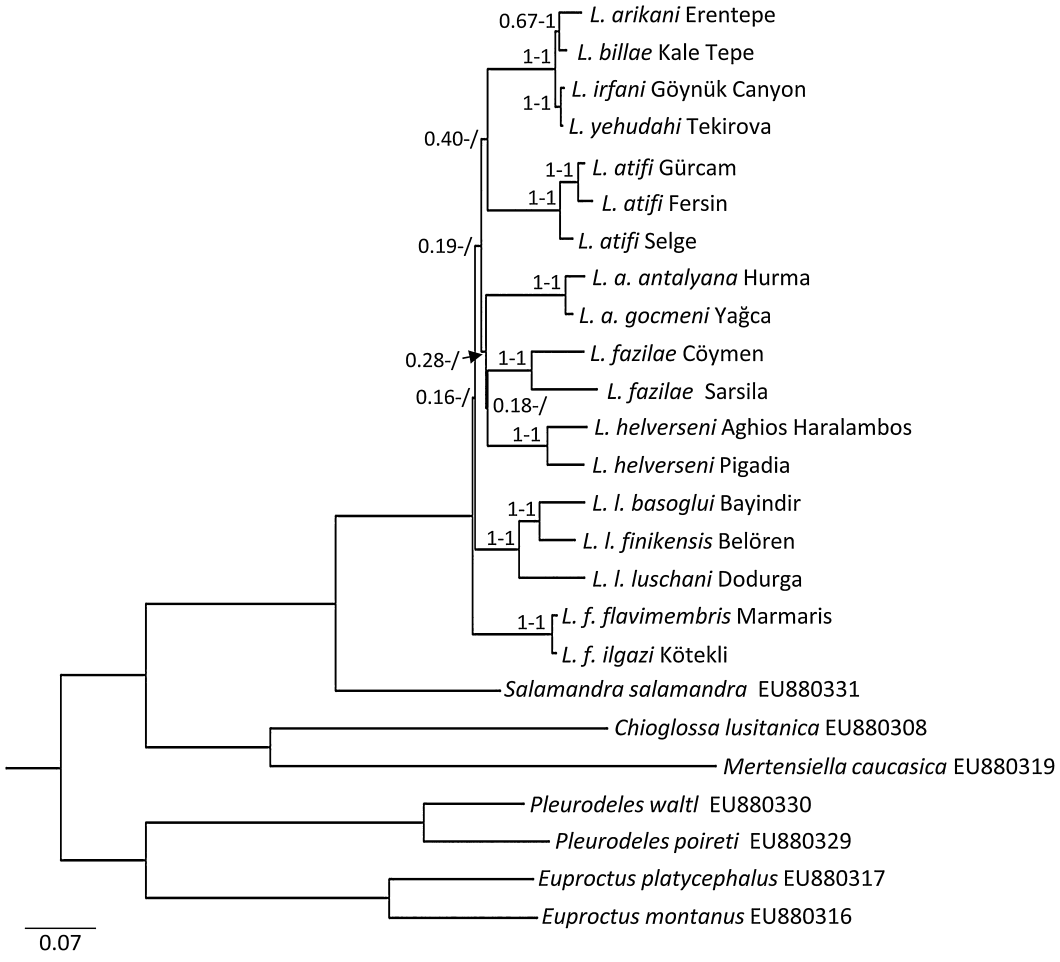


**Figure 1.** Maximum likelihood tree from RAxML based on the results of Partition Finder; numbers at nodes indicate node support values: ML bootstrap value – Bayesian posterior probability; / = no sufficient node support in the tree shown.

cence events. Second, we compared the number of deep coalescence events of the respective best tree against the probability distribution of deep coalescent events. If the number of deep coalescence events for the respective best tree is outside the 95% confidence interval, we can reject the null hypothesis for a basal polytomy of seven major lineages within *Lyciasalamandra*.

*Molecular clock analysis*

Molecular clock analyses were conducted in Beast under a Bayesian framework (version 2.4.2, Bouckaert et al., 2014) using the mitochondrial tree only. We used the topology of the BI tree to constrain four clades as monophyla (nodes 3, 4, 23, 24 in fig. 2).

The a priori distribution of the divergence times was fixed for two clades using primary and secondary calibration points in two different calibration scenarios. For calibration we used the splits between *Pleurodeles poireti* and *P. waltl* and between *Euproctus montanus* and *E. platycephalus*, respectively. Two different scenarios have each been invoked to explain the evolution of either species pair: The end of the Messinian Salinity Crises 5.33 ± 0.02 mya for both splits (Carranza and Arnold, 2004; Carranza and Amat, 2005); the Betic crises ca. 14 mya (Lonergan and White, 1997) for the split between *P. waltl* and *P. poireti* (Veith et al., 2004), and the Corsica-Sardinia microplate disjunction 15-9 mya for the split between *E. montanus* and *E. platycephalus* (Caccone et al., 1994). Altogether, this results in four different combinations of calibration points: two young splits, two old splits, and two combinations of one young and one old split, respectively.

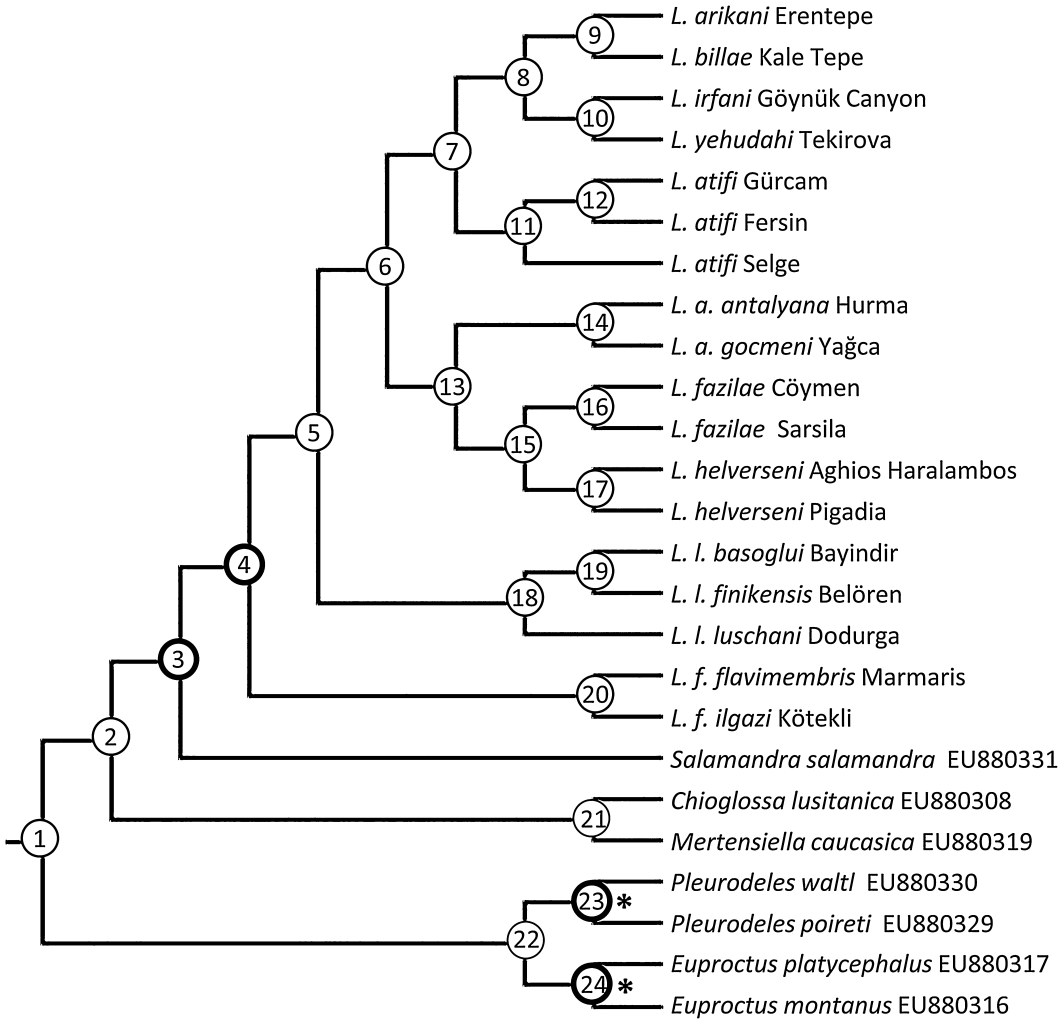


**Figure 2.** Node numbers of the Bayesian cladogram based on mitochondrial genes; numbers of nodes that were kept monophyletic are encircled with a bold line, calibration points are marked with an asterisk.

The ML tree (fig. 1) showed nearly identical lengths for the branches of the *Pleurodeles* and *Euproctus* clades, suggesting equal substitution rates. If so, this would allow us to reduce the molecular clock calibration to only two scenarios, one with two old splits and one with two younger splits. Therefore, and prior to conducting the molecular clock analyses, we performed in Mega Tajima's relative rate test (Tajima, 1993) and a maximum likelihood molecular clock test (Kumar and Filipski, 2001) to test for rate constancy between the *Pleurodeles* and the *Euproctus* lineage. We tested for equal evolutionary rate over a tree consisting of both *Euproctus* and *Pleurodeles* taxa as well as one outgroup (*Chioglossa lusitanica*) by analyzing all possible combinations of taxa of *Pleurodeles* and *Euproctus* with the given outgroup under Tajima's relative rate test with the null hypothesis of equal rates between lineages (supplementary table S2); we used the default settings in MEGA. For all taxa combinations the null hypothesis of equal rates could not be rejected. Furthermore, we conducted a molecular rate test with the same taxa set under a maximum likelihood approach, with the null hypothesis of equal evolutionary rates in Mega (General Time Reversible model; default settings). The likelihoods of the trees with ($\ln L = -13\,317.889$) and without ($\ln L = -13\,317.502$) the molecular clock constraint again showed that the null hypothesis of equal evolutionary rates throughout the tree was not rejected at the 5% significance level ($p = 0.993$). This justified a reduction of the available calibration scenarios to only two (table 1).

For Beast analysis, we linked tree models across partitions since all partitions share the same topology and branching times. According to ClockstaR (Duchene et al., 2013) the best number for partitions for the clock model is one partition, so we linked the clock models across all partitions, too. For the site models of the molecular clock application we used the results of the best-fitting substitution models from Partition Finder as a priori distribution. We changed the options for the prior ucld.mean (mean rate under uncorrelated log-normal relaxed molecular cock) from the default options into a hyperprior, with a mean of 10.0 and an offset of 0.0 and the prior ucld.stdev from the default gamma distribution to an exponential distribution with a mean of 0.3337. Hyperpriors are allowed to vary and have no consequences on the log-normal prior distribution on branch-specific substitution rates. We also altered the gamma priors on the exchangeability rates for the shape parameter ($\alpha = 2$) and scale parameter ($\beta = 0.5$) due to MCMC mixing problems with default gamma priors (Heath, 2015). At the end of the analysis, if the prior of ucld.stdev $<$ 0.1 (standard deviation $\sigma$ of the uncorrelated log-normal relaxed clock; see Brown and Yang, 2011), then there is no variation in rates among branches. In this case, molecular clock estimation could be conducted under the assumption of a strict clock. In our analysis the ucld.stdev $> 0.1$, thus a log-normal relaxed clock had to be applied.

The tree prior was selected according to the Beast tutorial "Tree priors and dating" (http://beast2.org/tree-priors-and-dating/). The coalescent tree prior to assuming a constant population size back through time and the Yule tree prior that assumes a (unknown) constant lineage birth rate for each branch in the tree, both with the Jeffreys prior on population size or on birth rate respectively for the Yule tree prior, came to nearly the same results. Since the Yule tree prior is most suitable for trees describing the relationship between individuals from different species, we used it for further analyses.

Three independent MCMC tests were run for 50 million steps for each of the two different calibration approaches, sampled every 1000th step. Analyzing the results in Tracer (version 1.6, Rambaut et al., 2014), the three independent runs had similar posterior distributions, after the burn-in was set to 20%. Effective sample size (ESS) values in calibration II were greater than 200 for all parameters in each run; in calibration I the ESS values were smaller than 200 for some parameters, but the combination of all runs resulted in ESS values greater than 200. Thus, we combined the three different runs and resampled the combined runs to have 10 001 trees in total using LogCombiner (version 2.1.3, Bouckaert et al., 2014). We used TreeAnnotator (version 2.1.3, Bouckaert et al., 2014) with mean heights for node heights summarizing the information of 10 001 trees into a single target tree.

## Results

### *Phylogenetic reconstruction*

ML tree and Bayesian inference of the mitochondrial genes produced almost identical mtDNA tree topologies, at least concerning

**Table 1.** Molecular clock scenarios for calibrations I and II (nodes 23 and 24 in fig. 2); types of priors and settings for the priors on the age of the calibrated nodes are given.

| Node | Time of Divergence | | | |
|---|---|---|---|---|
| | Calibration I | Type of prior | Calibration II | Type or prior |
| (23) *P. waltl* – *P. poireti* | 5.33 ± 0.02 mya (Carranza and Arnold, 2004) | normal prior (mean 5.33; sigma 0.02; offset 0.0) | ca. 14 mya (Veith et al., 2004) | exponential prior (mean 1.0; offset 14) |
| (24) *E. montanus* – *E. platycephalus* | 5.33 ± 0.02 mya (Carranza and Amat, 2005) | normal prior (mean 5.33; sigma 0.02; offset 0.0) | 9-15 mya (Caccone et al., 1994) | uniform prior (lower 9; upper 15; offset 0.0) |

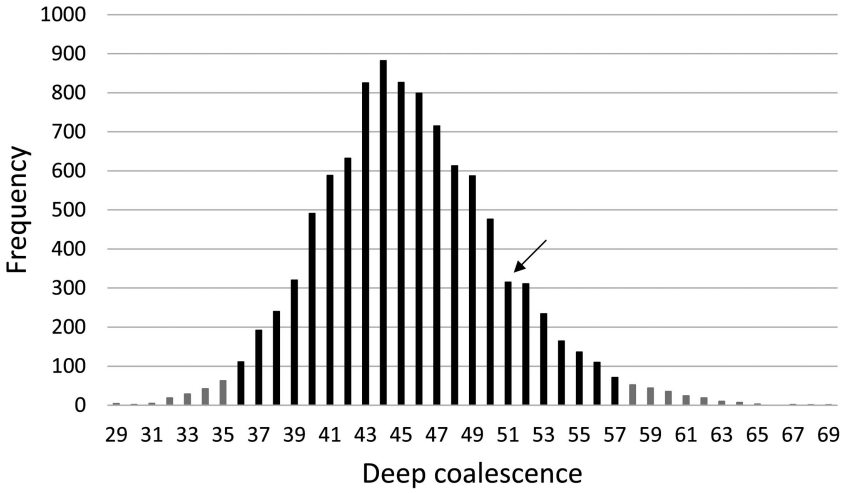


**Figure 3.** Probability distribution of expected deep coalescence for the 1000 simulated trees. Grey bars indicate deep coalescence numbers, which fell outside the 95% confidence interval. The arrow shows the number of deep coalescence events of the respective best tree (non-zero internal branches).

well supported nodes (fig. 1). Within *Lyciasalamandra*, seven lineages seem to diverge in a basal polytomy; there is no support for any internal dichotomy among these seven lineages (bootstrap values < 60% and Bayesian Posterior Probabilities < 80%). Our test for soft versus hard polytomy did not reject the null-hypotheses of a basal polytomy of seven major lineages within *Lyciasalamandra*; the deep coalescence events for the respective best tree are inside the 95% confidence interval of the probability distribution of expected deep coalescence events for the 10 000 simulated trees (fig. 3).

Six major lineages within *Lyciasalamandra* correspond to species described before 2011: *L. antalyana*, *L. atifi*, *L. fazilae*, *L. flavimembris*, *L. helverseni* and *L. luschani*. The seventhes major clade (we herein call it *L. billae*-group) is formed by *L. billae* and the newly described species *L. arikani*, *L. irfani* and *L. yehudahi*. Intraspecific differentiations are strongest in *L. fazilae*, *L. helverseni* and *L. luschani*; within the latter species three subspecies have been described.

The topology of the nuclear tree is completely unresolved (see figs S1 and S2 in the online supplementary material). This may be due to the fact that only few mutations have been found at the ca. 3000 sites analyzed (10 mutations for the RAG1, 2 for the KIAA, and 6 for the TTN fragment), with the uncorrected nuclear *p*-distance among *Lyciasalamandra* species ranging from 0.1 to 0.4 only. Therefore, we abstained from merging all information into one analysis, since the inconclusive signal inherent in the nuclear data set would have been masked by the high amount of variability found in the mitochondrial DNA.

### *Molecular clock analysis*

The two calibrations produced mutually exclusive scenarios with almost no overlap between the upper 95% CI border of calibration I and the lower 95% CI border of calibration II (table 2). Based on calibrations I and II the sister genera *Salamandra* and *Lyciasalamandra* diverged ca. 9.7 (95% CI: 6.3-13.4) or 25.1 (95% CI: 15-36), respectively. Major lineages within *Lyciasalmandra* diverged between 3.9 and 4.7 mya (2.7-6.3 mya) according to scenario I, and between 10.2 and 12.3 (6.2-17.3) mya according to scenario II. Further intraspecific divergence started in *L. luschani* (2.7 or 7 mya for calibration I and II, respectively), *L. fazilae* (2.3 or 5.9 mya) and *L. helverseni* (1.6 or 4.2 mya), followed by *L. atifi* (1.0 or 2.7 mya), *L. antalyana*

**Table 2.** Estimated times of divergence with mean values and lower and upper 95% credible interval (CI) for calibrations I and II (for node numbers see fig. 2); nodes 3, 4, 23 and 24 were kept monophyletic, calibration points are marked with an asterisk.

| Nodes | Calibration I | | | Calibration II | | |
|---|---|---|---|---|---|---|
| | mean | lower 95 CI | upper 95 CI | mean | lower 95 CI | upper 95 CI |
| 1 | 20.85 | 14.89 | 27.12 | 53.83 | 35.44 | 72.1 |
| 2 | 17.93 | 12.11 | 24.08 | 46.06 | 28.73 | 63.89 |
| 3 | 9.66 | 6.28 | 13.44 | 25.12 | 14.99 | 35.98 |
| 4 | 4.73 | 3.31 | 6.34 | 12.34 | 8.01 | 17.31 |
| 5 | 4.61 | 3.27 | 6.17 | 11.96 | 7.87 | 16.79 |
| 6 | 4.3 | 3.02 | 5.74 | 11.19 | 7.0 | 15.39 |
| 7 | 3.92 | 2.66 | 5.27 | 10.21 | 6.19 | 14.23 |
| 8 | 0.69 | 0.42 | 1.01 | 1.79 | 1.0 | 2.67 |
| 9 | 0.52 | 0.29 | 0.76 | 1.35 | 0.71 | 2.09 |
| 10 | 0.17 | 0.07 | 0.29 | 0.45 | 0.16 | 0.79 |
| 11 | 1.03 | 0.6 | 1.53 | 2.72 | 1.44 | 4.09 |
| 12 | 0.44 | 0.22 | 0.67 | 1.14 | 0.54 | 1.82 |
| 13 | 4.08 | 2.86 | 5.47 | 10.58 | 6.62 | 14.64 |
| 14 | 0.61 | 0.32 | 0.95 | 1.58 | 0.74 | 2.53 |
| 15 | 3.95 | 2.77 | 5.27 | 10.24 | 6.56 | 14.32 |
| 16 | 2.28 | 1.36 | 3.29 | 5.9 | 3.2 | 8.8 |
| 17 | 1.63 | 0.89 | 2.42 | 4.24 | 2.19 | 6.52 |
| 18 | 2.7 | 1.75 | 3.86 | 7.01 | 4.07 | 10.04 |
| 19 | 1.71 | 0.96 | 2.52 | 4.44 | 2.33 | 6.69 |
| 20 | 0.24 | 0.09 | 0.43 | 0.64 | 0.22 | 1.14 |
| 21 | 13.72 | 7.91 | 19.98 | 35.13 | 18.77 | 52.5 |
| 22 | 15.36 | 10.79 | 20.33 | 39.68 | 26.02 | 53.78 |
| 23* | 5.33 | 5.29 | 5.37 | 14.69 | 14.0 | 16.06 |
| 24* | 5.33 | 5.29 | 5.37 | 12.96 | 10.04 | 15 |

(0.6 or 1.6 mya) and the *L. billae* species group (0.7 or 1.8 mya). The most recent divergence was estimated for the two subspecies of *L. flavimembris* (0.2 or 0.6 mya).

## Discussion

### *Evolution of major intrageneric lineages*

Despite the extended genetic sampling of ca. 4500 bp from five mitochondrial genes we were not able to even tentatively solve the intrageneric phylogeny of major lineages within *Lyciasalamandra*. Already Weisrock et al. (2001) and Veith et al. (2008) found a basal polytomy for all species. They both concluded that more or less contemporary speciation events must have occurred and are responsible for the lack of sufficient accumulation of synapomorphic base substitutions along internal branches. Our test on hard versus soft polytomy did not reject the hypothesis of a hard polytomy, showing that all seven lineages of *Lyciasalamandra* may in fact have evolved synchronously, which in consequence may have to be explained by one and the same paleo-historical event. This is supported by the fact that under both calibration scenarios the respective 95% credible intervals of the splits among the seven major lineages broadly overlap, making it even more likely that a single period in the history of the genus could have accounted for their emergence.

Unfortunately, there are no objective criteria to decide which of the two calibration scenarios may be preferred relative to the other. Fromhage et al. (2004) applied three different calibration scenarios to a combined phylogeny of the anuran genera *Alytes*, *Bombina* and *Discoglossus* in the western Mediterranean. They compared divergence time estimates of numerous splits within these three genera to a

comparatively large set of reliably dated paleogeographical and paleoclimatological events. This allowed them to statistically test combined evolutionary scenarios for temporal and spatial plausibility. Nevertheless, and despite a strong signal for one scenario to be superior over the others, Fromhage et al. (2004) could not unambiguously exclude any of the two less plausible scenarios. In western Anatolia and the Aegean Sea such explanatory events are rare, and their lower and upper temporal limits are not as precisely set as many of those known from the western Mediterranean region.

The key to the understanding of what accounts for the simultaneous evolution of major *Lyciasalamandra* lineages may be the island endemic *L. helverseni*. The Aegean islands, including the Karpathos archipelago, had reached their current position by 10-9 mya (Creutzburg, 1963; Dermitzakis and Papanikolaou, 1981). No further tectonic events have been reconstructed that can be made responsible for a burst of species number in this region. Therefore, and under scenario II, the only tectonic event that may have caused the synchronous development of seven *Lyciasalamandra* lineages (and possibly for speciation events in many other taxa) must have been the tectonic events that led to the emergence of the Mid Aegean trench 13-9 mya (Creutzburg, 1963; Dermitzakis, 1990).

From the Aquitanian (23.0-20.4 mya) until the Langhian (20.0-13.8 mya) period, a continental arch connected the Pindic cordillera (part of today's Greece) and Anatolia (Dermitzakis and Papanikolaou, 1981). During the Late Tortonian (around 8 mya), immediately after the formation of the mid-Aegean trench, intense fracturing of the central Hellenic arc caused the invasion of the sea into the former land and the beginning of a very pronounced subsidence of the Cretan basin (Dermitzakis and Papanikolaou, 1981). At this time, Karpathos was separated from both Crete and Rhodes (the latter was probably connected to the Anatolian mainland). This vicariance was used to explain the splitting of the Karpathos clade II within the Gecko *Cyrtopodion kotschyi* (Kasapidis et al., 2005). Calibration II is fully consistent with a separation of *L. helverseni* from Anatolian species (node 15 in fig. 2 and table 2) at this time (median: 10.2 mya).

According to calibration I, the splitting of *L. helverseni* from the Anatolian lineages (mean: 4.0 mya; 95% CI: 5.3-2.8 mya) does not coincide with the Messinian Salinity Crisis; at this time Karpathos was probably connected with Anatolia via Rhodes. The re-flooding of this land bridge potentially could have initiated the separation of the Karpathos and the Anatolian populations. In the Pleistocene (starting 2.6 mya) Karpathos got its present shape and was probably again connected with Kasos, which made faunal exchange possible. The interruption of such a connection potentially could have initiated the separation of the Karpathos and the Kasos populations of *L. helverseni*, which is dated by calibration I to 1.6 (2.4-0.9) mya. However, the estimate provided by calibration II for the split between the populations of these two islands (4.2 (6.5-2.2) mya) may easily be explained by the Messinian Salinity Crisis when the two island were connected. Hence, the separation of *L. helverseni* from Anatolian lineages, as well as the separation of the Kasos and Karpathos populations, is better explained by calibration II. It is therefore sound to assume that the other more or less simultaneous splits between major lineages may have been triggered by one and the same event, the emergence of the mid-Aegean trench (fig. 4).

Lymberakis and Poulakakis (2010) stated that a simple vicariant event may not sufficiently explain the situation within *Lyciasalamandra*. They refer to Veith et al. (2008) who found a sister relationship between *L. billae* and *L. helverseni*. These authors brought rafting into play as one possible explanation for this geographically less plausible relationship. However, our largely extended data set now no longer shows any support for this relationship; rather it strengthens the idea of a synchronous

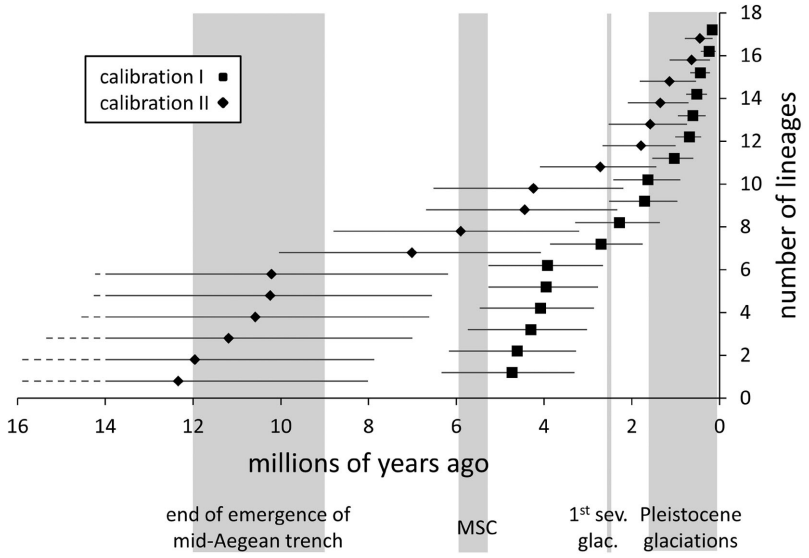


**Figure 4.** Lineage-through-time plot of *Lyciasalamandra* based on ca. 4500 bp from five mitochondrial gene fragments; horizontal bars show the 95% credible intervals of the time estimates (dashed lines to the left indicate that the upper 95% CI lies outside the time scale shown; for exact dates refer to table 2); time and duration of historical events are indicated by grey bars.

evolution of all seven lineages, which is expressed in the basal polytomy and which has already been put forward by Weisrock et al. (2001) and Veith et al. (2008).

*Nuclear sequence data*

Despite having sequences ca. 3000 bp of nuclear genes for major lineages within *Lyciasalamandra*, our resulting phylogeny was completely inconclusive. The amount of base substitutions among lineages was extremely low, and even between *Lyciasalamandra* and its sister genus *Salamandra*, the *p*-distance was only slightly above 1%. This corresponds to the observations of Vences et al. (2014) who found only low degrees of variability between species of *Salamandra*, with often even phylogenetically different species sharing one and the same haplotype for single nuclear genes. We therefore recommend that much more nuclear data need to be collected in the future (e.g., via next-generation sequencing such as transcriptome analyses) in order to contrast the mitochondrial tree with a conclusive nuclear phylogeny.

*Intraspecific evolution*

Under scenario II, further speciation within *Lyciasalamandra* lineages can be attributed to varying events, such as the MSC and the successive ice ages of the Late Pliocene and the Pleistocene. The MSC falls within the 95% credible intervals of intraspecific speciation events of the *L. luschani*, *L. fazilae* and *L. helverseni* lineages.

Until now, the MSC has predominantly been invoked as a trigger for vicariance events for speciation processes in the Mediterranean due to the separation of populations that had spread through a no longer submerged sea floor, and that were subsequently separated by the reflooded basin region (e.g., Busack and Lawson, 2008; Bidegaray and Arnedo, 2011; Rato et al., 2012; Vences et al., 2014). However, there is indication that it also must have had a tremendous impact on the climate of the surrounding land masses, and even on areas far away from the Mediterranean basin. Recent computer simulations based on a Community Atmosphere Model (CAM), in combination with a Community Land Model (CLM3),

a Slab Ocean 177 Model (SOM) and a thermodynamic sea ice model, demonstrated multifaceted consequences of the MSC (Murphy, 2010). They were for the first time able to link previously unexplained phenomena during the last Messinian to this enigmatic event (Murphy, 2010), such as enhanced productivity in the Atlantic Ocean (due to increased dust generation from the now exposed Mediterranean Sea floor; Wagner, 2002), increased aridity in Central Asia, and successive glaciations in the Northern Hemisphere (Hodell et al., 2001), with three peaks of glaciation occurring just after the initiation of the MSC (Vidal et al., 2002). The impact of the enormous sea level change on the regional climate must have been dramatic. The basin itself must have experienced a substantial warming due to the change in elevation alone. Changes in dust load and precipitation regimes may have caused alterations to desert vegetation inside the basin itself, and open woodlands and steppic ecosystems developed in the regions surrounding the dried basin (Murphy, 2010). These effects may have triggered climate changes even outside the region, which in turns acted synergistically back to the basin and its surrounding areas, e.g. through Northern Hemisphere sea-ice formation and climatic oscillation comparable in strength to those starting roughly in the Pliocene ca. 3 mya (Moran et al., 2006). Altogether, a significant effect of the MSC on the local climate (Murphy et al., 2009) must have had a severe impact on the flora and fauna living at that time around the Mediterranean basin.

Especially for amphibians, these climatic alterations must have been dramatic. Intra-Anatolian divergence during or immediately after the MSC was reconstructed for several genera, such as *Salamandra* (Veith et al., 1998), *Rana* (Veith et al., 2003), *Pelophylax* (Akin et al., 2010) and *Hyla* (Stöck et al., 2012). Species with a specialized drought-adapted life history such as Lycian salamanders must have been pre-adapted to survive such periods.

It is widely accepted that the repeated glaciations of the northern hemisphere were accompanied by severe climate changes in the Mediterranean region (e.g., Blondel et al., 2010; Lymberakis and Poulakakis, 2010) and triggered diversification in numerous taxa. Given the large number of successive cold and warm phases in the late Pliocene and the Pleistocene, and with regard to the broad 95% credible intervals of the Bayesian time estimates, it is not possible to assign any intraspecific split within *Lyciasalamandra* to a specific glacial cycle.

*Taxonomic implications*

Genetic pairwise uncorrected *p*-distances between members of different major lineages of *Lyciasalamandra* showed very little variation, with a median of 0.0803 (fig. 5). This once more underpins that all seven lineages accumulated more or less same amounts of mutations since their origin. Sub-specific differentiation within species varied a lot, although even the maximum value of 0.062 still being significantly lower than the lowest genetic distance between species (0.072). The lowest value between two conspecific subspecies was found for *L. f. flavimembris* and the newly described *L. f. ilgazi* (0.006). Between members of the *L. billae* species group values of between 0.004 and 0.020 (median: 0.015) were found, and indicated a low degree of genetic differentiation. Given the fact that the allopatrically distributed and recently described species *L. arikani*, *L. irfani* and *L. yehudahi* show presumably stable morphological characters that discriminate them from each other and from *L. billae* (Göçmen et al., 2011; Göçmen and Akman, 2012), we suggest to keep their taxonomic integrity, though on the subspecific level within the species *L. billae*. In the same way, the described morphological differences (Üzüm et al., 2015) and the allopatric distribution between *L. f. flavimembris* and *L. f. ilgazi* may justify their treatment as separate subspecies.

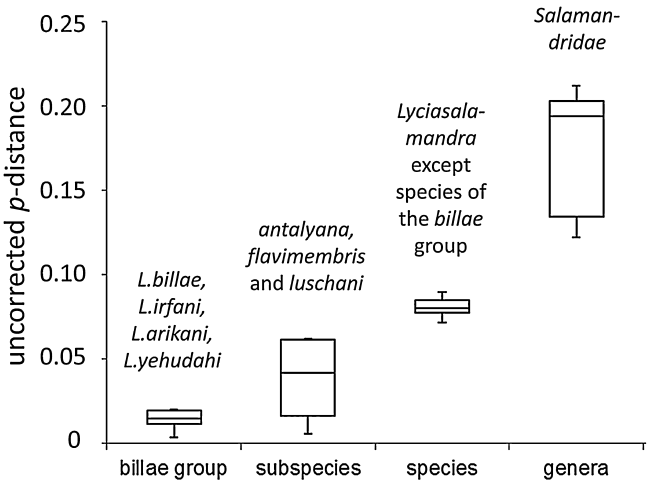


**Figure 5.** Average uncorrected pairwise p-distances for subspecies and species within *Lyciasalamandra* (as taxonomically pre-defined), between genera of the Salamandridae and between members of the *L. billae*-group.

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