Molecular systematics and historical biogeography of the green lizards (*Lacerta*) in Greece: Insights from mitochondrial and nuclear DNA

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ABSTRACT

The green lizards of the genus *Lacerta* (Sauria, Lacertidae) comprise nine recognized species, which in Europe are mainly restricted to the southern peninsulas. Four of them (*L. trilineata*, *L. viridis*, *L. bilineata* and *L. agilis*) occur in Greece. The uncertainty of morphological diversification renders the taxonomic assignment into species and subspecies problematic. In this study sequence data derived from two mitochondrial (cytochrome *b* and 16S rRNA) genes and one nuclear (NKTR) gene were used to (a) evaluate the taxonomic status of the genus *Lacerta* in Greece with emphasis on *L. trilineata* group and (b) investigate the evolutionary history of the genus through the application of phylogenetic and phylogeographic analyses, using *Gallotia* and *Timon* as outgroups. The phylogenetic analyses revealed the existence of four major clades. The first clade corresponds to *L. trilineata* group, the second to *L. media*, the third to *L. agilis* and the fourth to a complex of *L. viridis* and *L. bilineata*. However, the produced phylogenetic relationships are not congruent with the current taxonomy, especially in the first clade in which *L. trilineata* appeared to be paraphyletic in regard to *L. pamphylica*. Six distinct lineages were inferred within *L. trilineata*, despite the current recognition of nine morphological subspecies, the genetic differentiation of which exceeds that of other *Lacerta* species, imposing a thorough taxonomic revision of the species. Our results suggested a rapid diversification of *L. trilineata* group during the late Miocene. We believe that the present distribution of the genus in Greece is the result of several dispersal and vicariant events that took place during the late Miocene and early Pliocene.

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1. Introduction

The Mediterranean basin – one of the world's most geographically complex regions (Blondel et al., 2010) with remarkable paleogeographic evolution and tectonic history (Cavazza and Wexel, 2003) – has been subjected to intense phylogeographic research (Hewitt, 2004, 2011; Nieto, 2011). The Balkan Peninsula, and especially the Aegean Archipelago, is characterized by biodiversity richness and an exceptionally high percentage of endemism (Blondel et al., 2010). The intricate geological history of the Aegean Sea during the upper Tertiary and Quaternary, involving successive land connections and submergences of land bridges, together with the regional climatic changes and the intense human activity, have led to great lineage diversity in both terrestrial vertebrates (Kasapidis et al., 2005a,b; Kyriazi et al., 2013; Poulakakis et al., 2005a,c; Ursenbacher et al., 2008) and invertebrates (Klossa-Kilia et al., 2006; Kornilios et al., 2009; Kotsakiozi et al., 2012; Papadopoulou et al., 2009; Parmakelis et al., 2005, 2006; Poulakakis and Sfenthourakis, 2008; Simaiakis et al., 2012).

Lacertid lizards emerged in Eurasia and underwent rapid multiple divergence during the Early and Middle Miocene (Arnold et al., 2007; Lutz et al., 1986). The family contains two subfamilies: the Lacertinae and the Gallotinae (revision after Arnold et al., 2007). The lacertids are the most widespread and diversified group of reptiles in Europe (Arnold et al., 2007) with several cases of cryptic variation (Barata et al., 2012; Böhme et al., 2007; Harris and Sá-Sousa, 2001; Kyriazi et al., 2008; Poulakakis et al., 2003). The low interspecific morphological divergence in relation with the high degree of phenotypic variation within some species (Arnold, 2004; Harris and Sá-Sousa, 2001; Kapli et al., 2013; Poulakakis

et al., 2003) raise questions about their taxonomic status and systematic position. Thus, the phylogenetic relationships of lacertids based on morphological data have been widely discussed during the last two decades (Arnold, 1989; Arnold et al., 2007; Fu, 2000; Godinho et al., 2005; Harris et al., 1998).

The genus *Lacerta sensu stricto* (Nettman, 2001) includes large-bodied lizards that expand from the Iberian Peninsula to western Asia (Arnold et al., 2007). Its taxonomy is ambiguous and has been subjected to a series of revisions, mainly based on morphological data and hybridization experiments (Arnold, 1973; Peters, 1962; Rykena, 1991, 2001; Schmidtler, 1986). Most of the phylogenetic and phylogeographic studies that have been conducted within the genus have revealed complex phylogeographic patterns (*L. schreiberi*: Godinho et al., 2008, 2005; *L. agilis*: Kalyabina et al., 2001), cases of cryptic variation (*L. bilineata* and *L. viridis*: Böhme et al., 2007) and rapid diversification events (*L. trilineata* group: Ahmadzadeh et al., 2013a,b). In the particular case of *L. trilineata* group, Ahmadzadeh et al. (2013a,b) reached a rather intriguing conclusion: they ascribe the recent diversification of *L. trilineata* and *L. media* (4.0 Mya) and the spread of *L. trilineata* to the Balkans to multiple dispersal events in a short time scale during Pliocene.

At present the genus is considered monophyletic (Arnold et al., 2007; Godinho et al., 2005) and includes nine allopatric species with partially overlapping distributions (*L. agilis, L. bilineata, L. media, L. mostoufii, L. pamphylica, L. schreiberi, L. strigata, L. trilineata* and *L. viridis*). Within this group, *L. trilineata* and *L. viridis* appear to be paraphyletic with respect to *L. pamphylica* (Ahmadzadeh et al., 2013b; Godinho et al., 2005) and *L. bilineata* (Godinho et al., 2005; Mayer and Beyerlein, 2002), respectively. However, Böhme et al. (2007) argued for the existence of *L. bilineata* in the Pindos Mountains as a new subspecies of *L. bilineata* ssp., revoking the paraphyly of *L. viridis* to *L. bilineata*, and raising the number of *Lacerta* species in Greece to four (*L. trilineata, L. viridis, L. agilis,* and *L. bilineata*) (Böhme et al., 2007; Valakos et al., 2008). The morphological distinction between the species, and especially between *L. viridis* and *L. trilineata*, is difficult due to the very subtle morphological differentiation (high variation in the coloration and the number of dorsal, head and collar scales) (Rykena et al., 2002). *L. trilineata* is widely distributed in insular and continental Greece (Fig. 1) that hosts 9 out of the ten subspecies (Mayer and Beyerlein, 2002; Valakos et al., 2008). *L. viridis* includes five subspecies (Rykena et al.,

2002) three of which have been reported from specific locations in Macedonia (Frör, 1979; Mayer and Tiedemann, 1982; Pafilis and Maragou, 2013) (Fig. 1). Regarding the other two species, *L. agilis* reaches the northern part of Greece whereas *L. bilineata* has been found in the Pindos Mountain range (northwestern Greece; Böhme et al., 2007) (Fig. 1).

Given the incomplete knowledge regarding the evolutionary history of the Greek green lizards, the present study aims to explore the phylogenetic relationships and phylogeographic structure of *Lacerta* species in Greece, and examine the validity of their current taxonomy. These issues were addressed by investigating *Lacerta* specimens belonging to the four aforementioned species and analyzing two mitochondrial [cytochrome *b* (cyt *b*) and the large sub-unit of ribosomal RNA (16S rRNA)] and one nuclear [natural killer-tumor recognition (NKTR)] genes.

2. Materials and methods

2.1. Specimens, DNA extraction, amplification and sequencing

The origin of the specimens used in the current study is provided in the Supplementary material (Supplementary Table S1) and illustrated in Fig. 1. Eighty nine specimens of the four focal species, covering their whole distributional range in Greece, were used to assess the phylogenetic relationships of the *Lacerta* in Greece and deposited in the collections of the Natural History Museum of Crete (NHMC), University of Crete. Ethanol-preserved tongue or tail tip tissues were homogenized using the Holmes-Bonner lysis buffer (Holmes and Bonner, 1973) and total genomic DNA was extracted following the phenol/chloroform extraction procedure with isopropanol precipitation method proposed by Hillis et al. (1996).

Partial segments of two mitochondrial (cyt *b* and 16S rRNA) and one nuclear (NKTR) genes were amplified. Single strand sequencing was conducted on an ABI3730XL automated sequencer by using Big-Dye Terminator Cycle Sequencing (v3.1) kit. The primers used in cycle sequencing were the same as in the PCR amplifications. Primers and conditions used in PCR amplifications are given in Table 1. All the sequences generated for this study were deposited in GenBank. Furthermore, 77 additional *Lacerta*
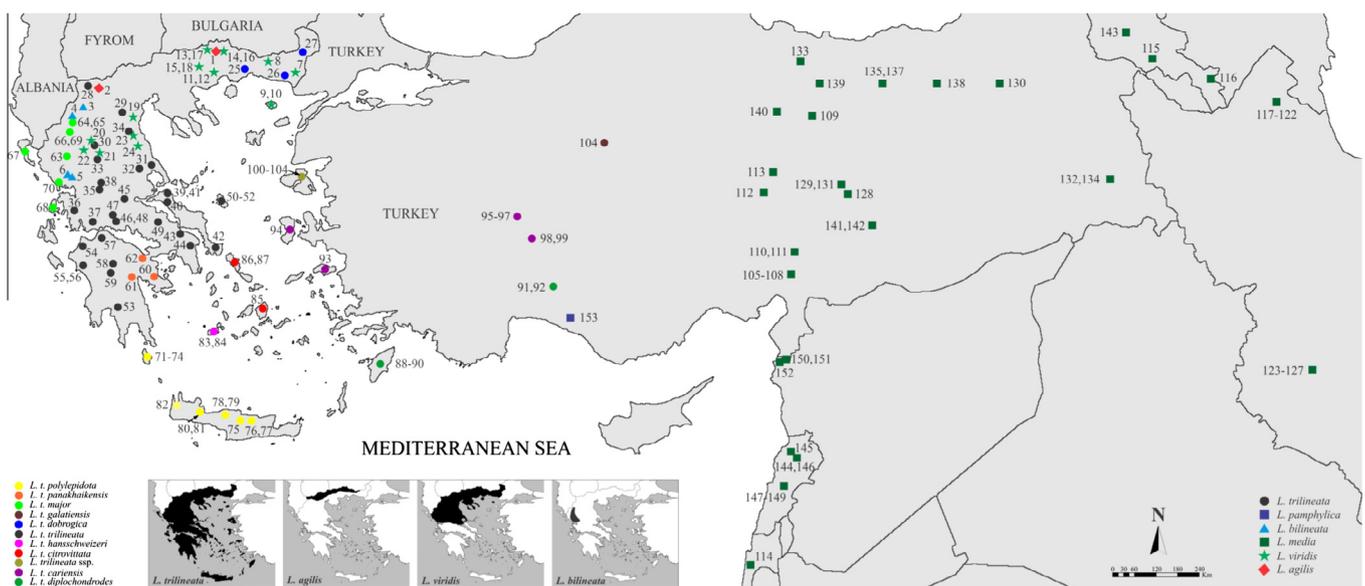

Fig. 1. Sampling localities and geographical distributions (separate inset) of the four focal *Lacerta* species in the Greek area and Turkey. IDs refer to Supplementary Table S1.

Table 1
Primers and conditions used for the PCR amplification and sequencing for the three gene segments.

Gene	Primers	Sequence (5′–3′)	Targeted size	Conditions	References
Cyt b	GLUDG-L	TGA CCT GAA RAA CCA YCG TTG	~420 bp	3 mM Mg++, 94 °C/ 1 min, 48.6 °C/ 1 min, 72 °C/ 1 min × 40 cycles	Palumbi (1996)
	CB2-H	CCC TCA GAA TGA TAT TTG TCC TCA			
16S	16Sar-L	CGC CTG TTT ATC AAA AAC AT	~550 bp	3 mM Mg++, 94 °C/ 1 min, 50 °C/1 min, 72 °C/1 min × 40 cycles	Palumbi (1996)
	16Sbr-H	CCG GTC TGA ACT CAG ATC ACG T			
NKTR	NKTR-f19	GAT GAC ATG GAG ATY TGY ACT CC	~630 bp	3 mM Mg++, 94 °C/ 1 min, 55 °C/1 min, 72 °C/1 min × 40 cycles	Townsend et al. (2011)
	NKTR-r18	CTY CTD GAY CGA CTT CTT GAG TGA CT			

specimens (13 *L. trilineata*, 2 *L. pamphylica,* 48 *L. media*, 5 *L. agilis*, 2 *L. viridis*, 5 *L. bilineata*, 1 *L. strigata* and 1 *L. schreiberi*) for cyt *b* and 16S rRNA were retrieved from GenBank and included in the phylogenetic analyses. Finally, six specimens of *Gallotia* ssp. and four from the genus *Timon*, the sister taxon to *Lacerta* (Arnold et al., 2007; Fu, 2000), were used as outgroups and for age constraint comparisons (Supplementary Table S1 presents the accession numbers and references for the sequences retrieved from GenBank).

2.2. Sequence alignment and data analysis

Sequences were viewed and edited using Codon-Code Aligner (v. 4.1.1, Codon Code Corporation). The sequence alignment was conducted with ClustalX (Thompson et al., 1997) and minor adjustments, when necessary, were performed after a visual inspection of the alignment. Coding sequences (cyt *b* and NKTR) were translated into amino acids and checked for stop codons. Genetic distances were calculated in MEGA v. 5.05 (Tamura et al., 2011) using the Tamura and Nei (TrN) model (Tamura and Nei, 1993) of nucleotide substitution.

The Akaike Information Criterion (AIC; Akaike, 1974) as implemented in Modeltest 3.7 (Posada and Crandall, 1998) was used to choose the best-fit model of DNA substitution for both the mtDNA and the nDNA datasets. However, due to the incapability of the Gamma (G) plus Invariable (I) models (I + G models) to estimate both parameters reliably, if $\alpha \leqslant 1$, only the Gamma (G) distribution model was tested (for more details see Yang, 2006).

2.3. Phylogenetic analyses and gene tree estimation on mtDNA

Phylogenetic analyses were performed using Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI) methods.

Maximum parsimony analysis with heuristic search using stepwise addition and performing tree-bisection-reconnection (TBR) branch swapping process (Swofford et al., 1996) was conducted in PAUP* (v.4.0b10) (Swofford, 2002). Bootstrap analysis with 1000 replicates was assessed to provide confidence estimate in the nodes (Felsenstein, 1985).

Bayesian inference was performed in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) using the GTR + G model of evolution (Rodriguez et al., 1990) for both mtDNA markers. The analysis was run simultaneously four independent times with eight chains. In each run, the number of generations was set to 10^7 with sampling every 100 generations. This generated an output of 10^5 trees. The average standard deviation of split frequencies below 0.01 was used to confirm the convergence of the two runs onto the stationary distribution (see MrBayes 3.1.2 manual). We further evaluated the 'burn-in' by plotting the log-likelihood scores and the tree length against generation number using Tracer v.1.5.0 (Rambaut and Drummond, 2008). The $-\ln L$ was stabilized after approximately 10^6 generations and the first 25% (2.5×10^4) were discarded as burn-in, in order to avoid the possibility of including random, suboptimal trees. A 50% majority rule consensus tree ('Bayesian tree') was calculated from the posterior distribution of trees. The support of the nodes was assessed with the posterior probabilities as a percentage of clade reconstruction. Recovering clades with values ⩾95% indicated significant support.

Maximum likelihood analysis was conducted using GARLI v.2.0 software (Zwickl, 2006). Heuristic search was performed to evaluate the more efficiently alternative ML topologies. The most likely GARLI tree topology was inferred from 50 independent runs, without specifying a starting tree. The remaining parameters were set to their default values. The separate partitions (the two mtDNA genes) were treated as 'unlinked' and the model parameters were estimated separately for each gene. The independent analyses were considered to have converged when the likelihood values showed a difference of less than one likelihood unit. The ML tree with the higher likelihood score was considered to be the best, and used as a starting tree in the bootstrap analysis involving 200 pseudo-replicates. Based on the trees of the bootstrap analyses, a majority rule consensus tree was created using Sum Trees in Python, as indicated by the site of GARLI (http://www.nescent.org/wg_garli/Advanced_topics# Using_SumTrees). The support values of each node on the consensus tree were depicted on the best tree found by GARLI.

2.4. Testing alternative hypotheses

Two alternative phylogenetic hypotheses were tested by using the Shimodaira-Hasegawa (SH) test (Goldman et al., 2000; Shimodaira and Hasegawa, 1999) and the RELL bootstrap with 1000 replicates as implemented in PAUP v.4.0b10 (Swofford, 2002). The first hypothesis that we tested suggests that *L. agilis* is more closely related to *L. viridis* (Godinho et al., 2005; Lutz and Mayer, 1985), whereas its alternative one is that *L. agilis* is more closely related to *L. trilineata*. The second hypothesis argues for the monophyly of *L. trilineata*, while its alternative supports that *L. trilineata* is paraphyletic with respect to *L. pamphylica*. The support for internal branches was obtained by the ML-based zero-branch length test proposed by Slowinski (2001) and as implemented in PAUP* v.4.0b10 (Swofford, 2002).

2.5. Estimation of divergence times on mtDNA

To estimate the timing of cladogenetic events for the genus *Lacerta* we used the method followed by Ahmadzadeh et al. (2013a, 2013b). Namely, we used fossils records for the splits between *Lacerta* and *Timon* (17.5 Mya, Čerňanský, 2010), European and North African *Timon* (5.3 Mya, Estes, 1983) and the diversification of *L. viridis* group (8.7 Mya, Venczel, 2006). In addition, we used as external calibration age constraint the split between *Gallotia caesaris caesaris* and *G. c. gomerae* – a well-documented divergent event – based on the formation of El Hierro island in the Canary Islands (1.05 Mya, Guillou et al., 1996). The geological ages of the Canary Islands in general, have been widely used to estimate the

divergence times of other lizards species (Carranza et al., 2004, 2008; Cox et al., 2010; Kapli et al., 2013; Poulakakis et al., 2003).

The estimation of divergence times was conducted under the Bayesian framework implemented in BEAST v1.7.5 (Drummond and Rambaut, 2007). The input file was formatted with the BEAUti utility included in the software package. The analysis was run for 10^8 generations with a 1000-step thinning from which 10% were discarded as burn-in. Models and prior specifications applied were as follows (otherwise set to default values): cyt *b* and 16S rRNA – GTR + G (Rodriguez et al., 1990); Relaxed Uncorrelated Lognormal Clock (estimate); Yule process of speciation; random starting tree. For all analyses implemented in BEAST, results were analyzed in Tracer to assess convergence and effective sample sizes (ESSs) for all parameters. The final tree (Tree with divergence estimates and their 95% highest posterior densities, HPD) was computed in TreeAnnotator v.1.7.5. Trees were visualized using the software FigTree v1.3.1 (Rambaut, 2006–2009).

2.6. Phylogenetic analyses on the complete dataset (mt and nDNA)

The same phylogenetic analyses (MP, ML, and BI) were performed on the concatenated dataset that includes both mitochondrial and nuclear gene fragments, forming a dataset of 75 specimens and 1621 bp. Due to the unavailability of nuclear data for *L. media* and *L. pamphylica*, only the four focal species (*L. trilineata*, *L. viridis*, *L. bilineata* and *L. agilis*) that are present in Greece were used in these analyses. For ML and BI analyses, GTR + G (Rodriguez et al., 1990) and HKY + G (Hasegawa et al., 1985) models of evolution were used for mtDNA and nuclear genes, respectively. The remaining parameters were set as already described.

3. Results

3.1. Data analysis on mtDNA

Of the 989 aligned sites examined (429 for cyt *b* and 560 for 16S rRNA) for the 166 *Lacerta* specimens, 305 were variable and 227 were parsimony informative (385 and 309 respectively when *Timon* and *Gallotia* spp. were included in the analysis).

The TrN genetic distances within and between *Lacerta* lineages and the outgroup taxa (*Timon* and *Gallotia*) are given in Table 2. The ingroup sequence divergence for all pairs ranged from 0% to 25.9% for the cyt *b* and from 0% to 14.3% for 16S rRNA. The high sequence divergence between *L. trilineata* lineages is intriguing (varying from 3.6% to 9.5% for cyt *b* and up to 3.5% for 16S rRNA, Table 2) and among subspecies (0.9% to 10.3% for cyt *b*, Supplementary Table S2).

3.2. Data analysis on nDNA

Regarding the nuclear (NKTR) gene, 58 out of 89 focal *Lacerta* specimens were amplified and sequenced based on the mtDNA analyses and in order to cover the whole distributional range of the four species in Greece. Of the 630 aligned sites examined for the 58 *Lacerta* specimens, 30 variable and 16 parsimony informative sites were observed. The ingroup sequence divergence for all pairs was relatively low, ranging from 0% to 2.4%, while the genetic distance between the four species ranged from 0.4% to 1.3%.

3.3. Phylogenetic analysis on mtDNA

The MP, ML and BI analyses produced trees that had similar topologies (Figs. 2 and 3). Excluding the lineages of *L. strigata* and *L. schreiberi* that each one includes only one specimen, four

major clades of *Lacerta* were revealed (Fig. 2) comprising the six different taxa distributed in Greece and Turkey. The first clade (*L. trilineata* clade, J) corresponds to *L. trilineata* and *L. pamphylica*, the second (*L. media* clade, P) to *L. media*, the third (*L. agilis* clade, O) to *L. agilis* and the fourth (*L. viridis* clade, F) to a complex involving *L. viridis* and *L. bilineata*.

Within *L. trilineata* clade, six lineages (I–VI) occupying distinct geographic regions were recognized (I: *L. t. major*, *L. t. trilineata* and *L. t. hansschweizeri* from continental Greece and southwest Cyclades, II: *L. t. trilineata* from western Peloponnisos, III: *L. t. polylepidota* and *L. t. panakhaikensis* from eastern Peloponnisos and the islands of Crete and Kythera, IV: *L. t. citrovittata* from eastern Cyclades, V: *L. t. dobrogica*, *L. t. diplochondrodes*, *L. t. cariensis* and *L. galatiensis* from Thrace, eastern Aegean islands and central Turkey and VI: *L. trilineata* spp. from the island of Lesvos; Fig. 3), while *L. trilineata* appears to be paraphyletic to *L. pamphylica*. Nonetheless, the SH test did not reject the alternative hypothesis of the monophyly of *L. trilineata* (*P* = 0.106).

In the case of *L. viridis* clade, two very well-supported lineages were inferred. The first lineage comprises specimens of *L. bilineata* from northwestern Greece, whereas the second to *L. viridis* from northeastern (group 1) and central (group 2) Greece (Fig. 3).

3.4. Estimation of divergence times

The effective sample sizes (EESs) in all BEAST analyses were high (posterior ESS values >1000) and assessment of convergence statistics in Tracer indicated that all analyses had converged. According to the inferred time estimates, the diversification of the *Lacerta* lineages in Greece started in the middle Miocene (13.9 Mya; node C; Fig. 2) with the split of *L. viridis* group from *L. trilineata* and *L. agilis*, whereas the diversification within *L. trilineata* (node J) and *L. viridis*/ *L. bilineata* (node F) seems to have occurred in late Miocene (8.63 and 6.78 Mya, respectively).

3.5. Phylogenetic analyses on the complete dataset (mt and nDNA)

The concatenated dataset of mtDNA and nDNA produced a dataset of 1621 aligned bp. The phylogenetic analyses (MP, ML and BI) on the complete dataset (72 OTUs including the outgroups and a few *Lacerta* specimens retrieved from GenBank) produced topologies that are in agreement with the topology of the mitochondrial tree (Supplementary Fig. S1).

4. Discussion

4.1. Phylogenetic relationships of the genus Lacerta in Greece

All phylogenetic analyses support the monophyly of the genus *Lacerta* and revealed that three out of the four major clades of the genus are distributed in Greece (Fig. 2); *Lacerta viridis* clade with *L. viridis* and *L. bilineata*, *Lacerta agilis* clade comprising only the homonym species, and *Lacerta trilineata* clade with *L. trilineata*.

From a phylogenetic point of view, the clade of *L. trilineata* has sister-group relationships with the clade of *L. media*, whereas *L. agilis* seems to have a basal position to this group. This is in disagreement with previous studies supporting the phylogenetic affinity of *L. agilis* to the *L. viridis* group (Godinho et al., 2005; Lutz and Mayer, 1985). Given that the alternative hypothesis (sister group relationship of *L. agilis* and *L. viridis* clades) could not be rejected (SH test; *p* = 0.086), the inconsistency could be attributed to the under-representation of the first species in our analyses. We believe that more data from the whole range of this species could shed light on its phylogenetic relationships.

Table 2
Sequence divergences (%) among the main lineages of *Lacerta* and the outgroups (*Timon* and *Gallotia*). The values for the cyt *b* (below diagonal) and 16S rRNA (above diagonal) are based on the Tamura and Nei model of evolution. Values in diagonal are the within lineages sequence divergences [cyt *b* (16S rRNA)]. n/c refers to not estimated values.

Lineage	1	2 (I)	3 (II)	4 (III)	5 (IV)	6 (V)	7 (VI)	8	9	10 (1)	11 (2)	12	13	14	15	16	17
1. *L. agilis*	**3.2(2.1)**	7.6	8.2	7.9	7.5	8.2	7.5	8.4	8.7	8.2	8.5	9.8	8.8	9.0	10.4	13.8	21.9
2. *L. trilineata* (**I**)	12.9	**1.1(0.5)**	1.7	0.5	2.4	2.6	2.8	8.6	9.0	7.9	8.7	2.6	3.9	8.6	11.5	11.9	20.0
3. *L. trilineata* (**II**)	11.9	3.6	**0.9(0.9)**	1.3	2.7	3.2	3.5	8.5	8.9	8.1	8.6	3.2	4.3	8.7	11.6	12.4	20.0
4. *L. trilineata* (**III**)	11.7	4.2	4.7	**1.8(0.3)**	2.6	2.8	3.1	9.3	9.5	8.6	9.4	2.4	4.2	8.9	11.7	11.9	19.9
5. *L. trilineata* (**IV**)	11.1	9.0	8.1	8.5	**1.8(0.6)**	2.3	2.8	8.3	8.9	8.5	9.1	3.0	3.8	9.2	11.4	13.3	20.1
6. *L. trilineata* (**V**)	12.2	9.5	8.8	9.1	6.2	**1.4(0.2)**	1.7	9.9	10.1	8.8	9.6	3.5	4.8	8.6	12.7	13.1	22.3
7. *L. trilineata* (**VI**)	11.2	8.9	8.1	9.1	5.6	3.5	**0.7(0.5)**	9.8	9.8	8.5	9.3	3.9	5.4	8.4	11.8	13.0	21.4
8. *L. bilineata* ssp.	13.1	18.6	15.3	16.9	15.6	15.8	16.5	**2.9(1.5)**	1.3	2.8	3.6	12.0	10.2	8.8	8.6	15.0	22.3
9. *L. bilineata*	12.3	17.1	13.8	15.7	13.4	13.8	14.9	3.0	**1.3(2.8)**	2.9	3.7	11.8	10.3	8.8	8.8	15.4	23.2
10. *L. viridis* (**1**)	12.4	17.2	14.0	15.0	14.1	14.3	14.9	5.6	6.3	**3.1(0.7)**	1.3	10.9	9.7	8.2	8.9	14.7	22.8
11. *L. viridis* (**2**)	13.0	16.3	15.0	14.3	12.7	12.9	13.3	6.7	6.6	5.8	**4.3(1.1)**	11.9	10.1	9.2	8.9	15.1	23.0
12. *L. pamphylica*	11.8	9.7	9.3	9.3	5.7	7.5	6.4	14.7	14.7	13.4	13.4	**0.0(0.0)**	4.9	9.3	13.9	14.1	22.6
13. *L. media*	10.2	8.8	8.2	8.6	6.6	7.8	7.4	16.1	14.5	14.7	14.2	7.7	**4.7(2.0)**	10.3	11.9	13.4	21.4
14. *L. schreiberi*	17.5	14.4	12.4	14.5	13.7	13.8	14.4	17.0	16.2	17.1	18.8	15.4	14.62	**n/c**	11.3	14.0	26.4
15. *L. strigata*	17.5	17.7	17.1	16.7	15.4	15.9	16.8	18.9	17.1	16.3	16.8	17.5	15.4	17.9	**n/c**	18.4	28.9
16. *Timon*	21.4	20.7	21.1	20.2	21.0	23.5	22.3	21.8	19.9	21.7	21.1	22.6	20.9	23.3	21.0	**17.3(8.6)**	22.0
17. *Gallotia*	27.8	28.2	27.8	27.4	25.2	27.7	26.8	26.5	26.5	26.1	26.8	26.0	25.9	29.4	25.9	28.4	**13(4.9)**

Regarding *L. trilineata*, the phylogenetic tree exhibited substantial conflicts with the current taxonomy, revealing issues that call for further investigation, whereas the results of *L. viridis* group are in consistence with the study of Böhme et al. (2007).

4.1.1. Lacerta viridis clade

Within the *Lacerta viridis* clade, two main lineages were recognized, representing the two species of this clade, *L. viridis* and *L. bilineata*. In *L. viridis* two allopatric groups of specimens were detected (Fig. 3) with low mean genetic distance (5.8% in cyt *b* and 1.3% in 16S rRNA; Table 2). The geographic distribution of these groups coincides with the geographic distribution of the two recognized subspecies of *L. viridis* [*L. v. meridionalis* (1) and *L. v. guentherpetersi* (2)] located in northeastern and central Greece, respectively]. However, the findings of Böhme et al. (2007), suggesting the occurrence of another *L. viridis* lineage in the Thessaloniki region, could not be investigated due to the lack of samples from the respective area.

The genetic distance among *L. viridis* and *L. bilineata* is relatively low (6.3% in cyt *b* and 3.2% in 16S rRNA) compared to these recorded among other *Lacerta* species (i.e. ∼12% between *L. agilis* and *L. trilineata* and ∼15.5% between *L. trilineata* and *L. bilineata*). Our results are in agreement with previous studies that reported low genetic divergence between *L. viridis* and *L. bilineata* (Böhme et al., 2007; Godinho et al., 2005). It should be noted that the average uncorrected divergence between species in the same genera is 13.6% in reptiles and 11% in lacertids (Harris, 2002). Taking into account that (a) *L. bilineata* and *L. viridis* are able to hybridize (Rykena, 2001), (b) their morphological distinction is very hard due to the subtle morphological differences that they exhibit (Brückner et al., 2001; Nettman, 2001), (c) they have low interspecific genetic differentiation, and (d) the geographic distribution of *L. bilineata* is also doubtful [most of the studies, except Böhme et al. (2007), suggest that the Apennine Peninsula is the eastern edge of its occurrence (Godinho et al., 2005; Joger et al., 2001; Keller and Vassilakaki, 2002; Nettman, 2001)], it seems that the distinction in species-level for *L. viridis* and *L. bilineata* is difficult. Due to these difficulties it is not unreasonable that previous authors referred to them as a single species (Arnold, 2002; Godinho et al., 2005).

4.1.2. Lacerta trilineata and Lacerta media clades

The phylogenetic relationships among these major clades, which include three morphological species (*L. media*, *L. trilineata*

and *L. pamphylica*), revealed several taxonomic issues in species and subspecies level for *L. trilineata* populations located in Greece. The six recognized lineages of *L. trilineata* (I–VI, see Fig. 3) could be divided into two main groups, but with relatively weak support (nodes K and M; Fig. 3), that are mainly distributed west and east of the mid-Aegean trench (hereafter MAT), respectively. The first group includes the lineages I, II, and III from continental Greece, Peloponnisos, Cyclades, and Crete, and the second group comprises the lineages IV, V and VI from northeastern Greece, east Aegean islands and northeastern Cyclades. *L. trilineata* appears to be a paraphyletic taxon with respect to *L. pamphylica* (lineage IV; Fig. 3). This is a very interesting relationship that shows the strong affinity of *L. pamphylica* from southern Turkey with *L. trilineata* from Cyclades and not with *L. media* or *L. trilineata* from Turkey. Although the SH test could not reject the alternative hypothesis of the monophyly of *L. trilineata*, these phylogenetic relationships stress the necessity for the re-evaluation of the status of *L. trilineata*.

Paraphyly can be observed in many species (Funk and Omland, 2003 and references therein) and has multiple potential causes, such as the introgressive hybridization through interspecific mating, the incomplete lineage sorting due to recent speciation events and the imperfect taxonomy caused by misidentification of intraspecific variation (Funk and Omland, 2003). In our case it seems that the paraphyly of *L. trilineata* might be the result of inaccurate taxonomy and could be resolved by changing the current taxonomic status of *L. pamphylica* to subspecies level. The other two causes (introgressive hybridization and incomplete lineage sorting) could be rejected due to the allopatric distribution and the very old divergence (5.7 Mya, Fig. 3) of *L. pamhpylica* and the populations from northeastern Cyclades (lineage IV). This is in agreement with previous studies (Ahmadzadeh et al., 2013b; Schmidtler, 1975), but the small number of specimens of *L. pamphylica* used in this study constrains the interpretation of the observed paraphyly.

The high intraspecific divergence of the six lineages of *L. trilineata* (i.e., 10.3% in cyt *b* between lineages I and V, Supplementary Table S2), which is comparable to that recorded among lacertid species (Böhme et al., 2007; Crochet et al., 2004; Godinho et al., 2005), raises concerns regarding its taxonomic status and the question of whether or not it is a single species. It is obvious that a similar and in many ways deeper revision of the current taxonomy is needed in subspecies level. With regard to the morphological subspecies of *L. trilineata*, it is clear that they do not represent monophyletic units (the ten morphological subspecies of *L. trilineata*
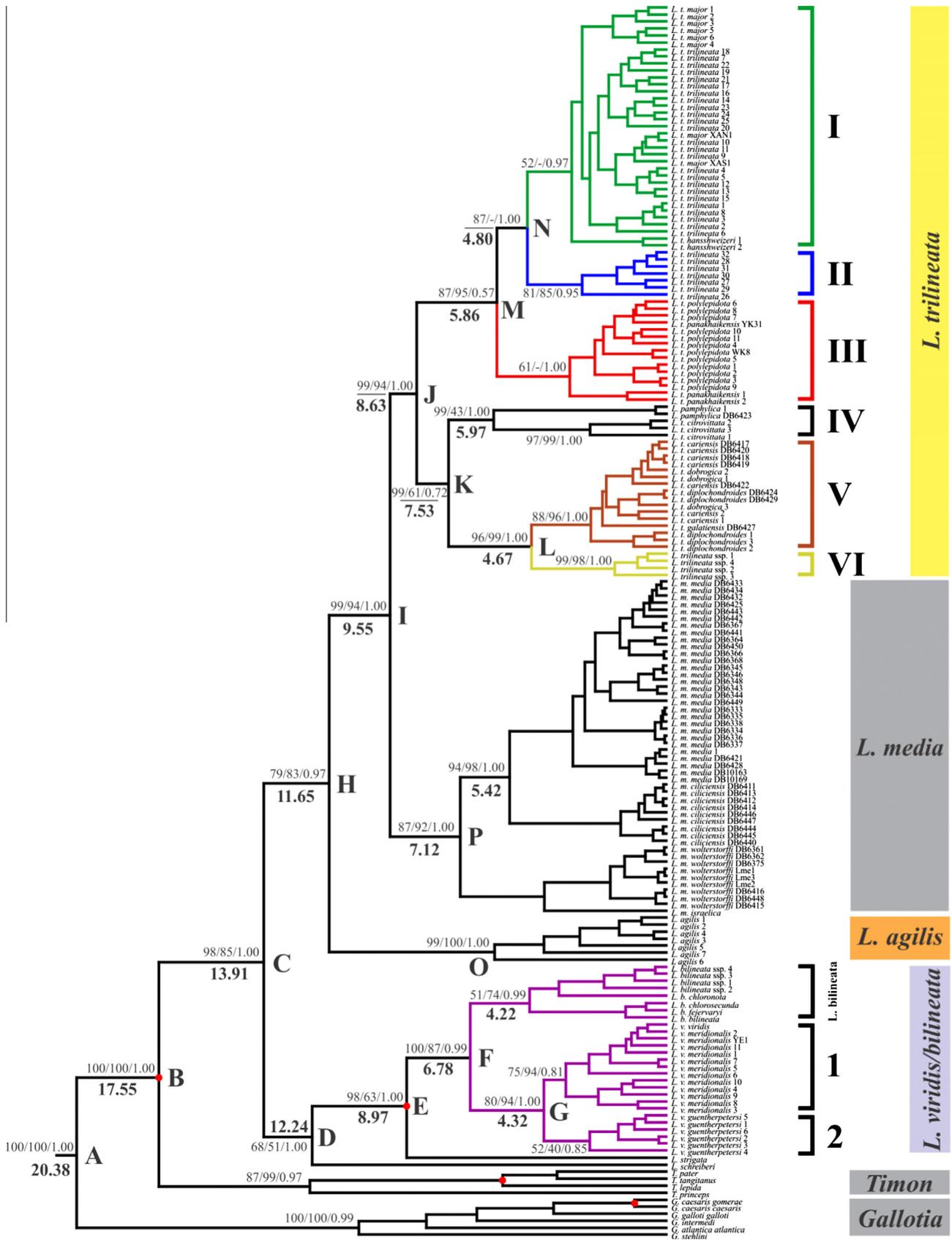
Fig. 2. Bayesian Inference tree for *Lacerta* species reconstructed from the mtDNA dataset. Numbers on branches indicate the bootstrap support of the Maximum Parsimony and Maximum Likelihood and the posterior probabilities of the Bayesian Inference (MP/ML/BI). The bold numbers below the branches are the divergence times estimated with BEAST. The four red dots indicate the calibration points. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
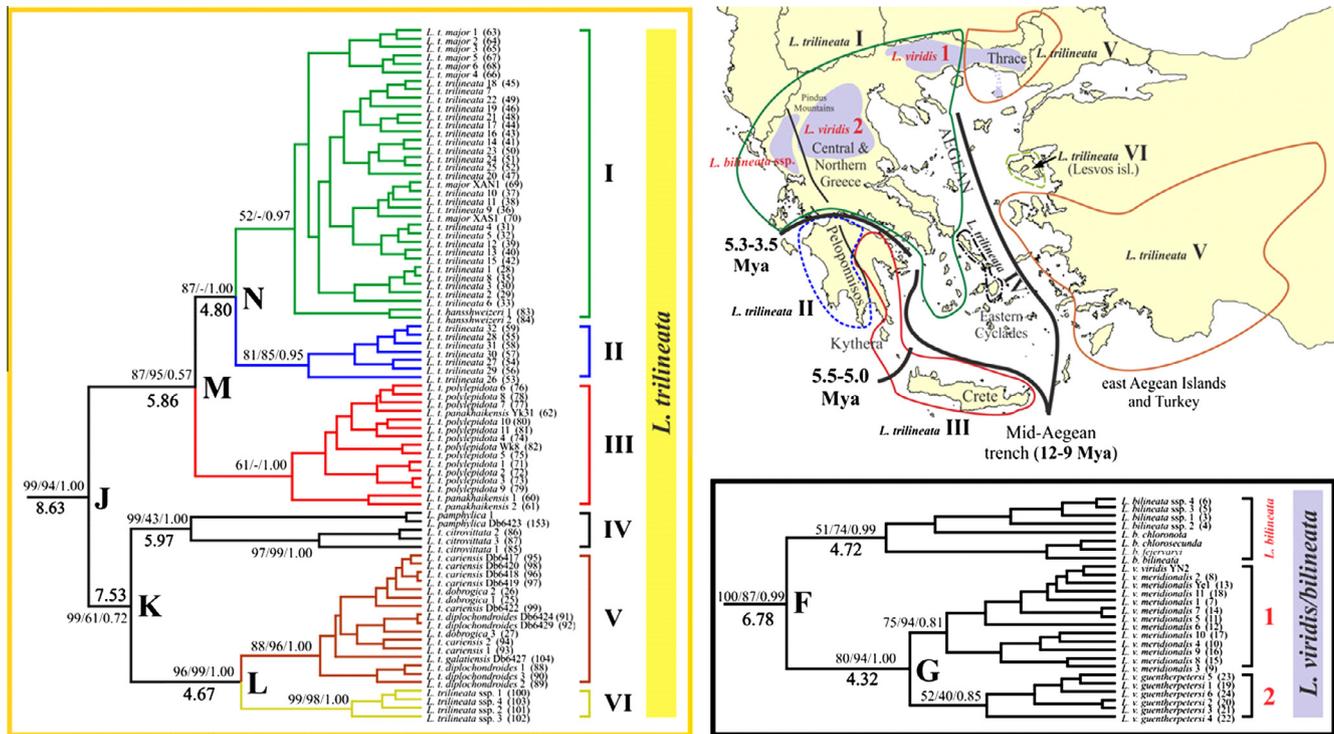
Fig. 3. The two main clades of the Bayesian Inference tree as illustrated in Fig. 2 for the three focal *Lacerta* species (*L. viridis*, *L. bilineata* and *L. trilineata*). The numbers in parentheses refers to IDs of samples as they are given in Fig. 1 and Supplementary Table S1. The embedded map has been re-drawn after Lymberakis and Poulakakis (2010). It illustrates the distribution pattern of the *Lacerta* clades/lineages in Greece, obtained from the analysis and the basic barriers of geological origin in the Aegean and continental Greece. Numbers on branches indicate the bootstrap support of the Maximum Parsimony and Maximum Likelihood and the posterior probabilities of the Bayesian Inference (MP/ML/BI). The bold numbers below the branches are the divergences time estimated with BEAST.

correspond to six phylogenetic lineages). This brings into question the practice of subspecies recognition and subsequent assignment of local populations into these subspecies on the basis of an exclusive or limited collection of characters, be that morphological, behavioral or molecular. Considering the six lineages of *L. trilineata* and their phylogenetic relationships, the position of IV (northeastern Cyclades) and VI (Lesvos isl.) lineages are extremely interesting. As we mentioned above, the six lineages of *L. trilineata* are divided into two main groups that are mainly distributed west and east of the hereafter MAT. Although the lineage IV is geographically located west of the MAT is more closely related with lineages from Turkey (east of the MAT), indicating a peculiar biogeographic history (see below). On the other hand, *L. trilineata* specimens from Lesvos Island (lineage VI) formed a monophyletic group with comparatively high genetic divergence (for cyt *b* 3.5−5.6% and for 16S rRNA 1.7−2.8%, Table 2) compared to the rest of *L. trilineata* specimens from east Aegean islands (V) (see also Supplementary Table S2). It could be probably represent another case of hidden diversity. The occurrence of hidden genetic lineages within reptilian or amphibian species that have a continuous geographic distribution in Anatolia is confirmed in other taxa as well (*Ophisops elegans, Pelophylax* spp., *Typhlops vermicularis*; Akin et al., 2010; Kornilios et al., 2011, 2012; Kyriazi et al., 2008). Although more samples from the northwest part of Turkey will shed more light on the evolutionary history of this lineage, the existence of cryptic evolutionary lineages within *L. trilineata* calls for further attention on the preservation of the intraspecific varieties and the respective geographic areas that hold them.

4.2. Phylogeography of Lacerta in southern Balkans

Reliable phylogenetic reconstructions are essential prerequisites in historical biogeography and for the proposal of a robust phylogeographic scenario. Since, not all *Lacerta* species are included in the biogeographic analysis, the following discussion will mainly focus on the *Lacerta trilineata* clade and in a less extent on the clade of *Lacerta viridis*.

Within the clade of Greek green lizards the first branch-off occurred during the middle Miocene (13.91 Mya, node C; Fig. 2) and led to the separation of the *Lacerta viridis* clade from the other two *Lacerta* clades that comprises species distributed in Greece and Turkey (*Lacerta agilis* and *Lacerta trilineata* clades). The next major cladogenetic event took place at late Miocene (11.65 Mya, node H) and led to the separation of *L. trilineata* and *L. agilis*, clades. These results are in accordance with the suggestions of Yablokov et al. (1981), who suggested that *L. agilis* arose in the Caucasian region in late Miocene (Kalyabina et al., 2001).

4.2.1. Lacerta viridis clade

The evolutionary history in the Greek area for this group started in the late Miocene (6.78 Mya, node F; Fig. 3) with the split of *L. viridis* and *L. bilineata*, a time estimate that fits very well with the late Miocene fossils of *Lacerta cf. viridis* in Hungary (Venczel, 2006) and Austria (Tempfer, 2005). Almost two million years later (4.32 Mya, node G) another cladogenetic event caused the separation of the two geographically distinct genealogical lineages of *L. viridis* in Greece (*L. v. meridionalis* and *L. v. guentherpetersi*; 1 and 2 respectively as illustrated in Fig. 3).

The proposed scenario for the origin of *L. viridis* was based on the mtDNA data and suggested its origin from one refugial area rich to microgeographical patterns in southern Balkans (Böhme et al., 2007; Godinho et al., 2005). We assume that several orogenetic events in continental Greece (i.e. the formation of Pindos mountain range from southern Albanian to northwestern Greece, and Rhodope mountain chain between Macedonia and Maritsa valley, etc.) provided several isolated refugia (Hughes, 2004) that

could explain the allopatric distribution and differentiation of the lineages of *viridis* clade and could have played a significant role in the expansion of the species in the rest of Europe (see Böhme et al., 2007; Godinho et al., 2005).

Due to the limited representation of *L. bilineata* in our study, it is risky to assume any phylogeographic scenario. However, two different hypotheses have been proposed in the past. The first claimed that the species originated from central and northern populations of *L. viridis,* while a geological event in the southern Balkans limited the distribution range of the ancestral *viridis* form, leading to the restriction and diversification of *L. bilineata* only in the Apennine Peninsula (Godinho et al., 2005). The second scenario suggests the rising of *L. bilineata* from two highly distinct refugia areas, one in the Apennines and the second in the southern Balkans (Pindos Mountain chain) (Böhme et al., 2007). The reason for this discrepancy lies in the assumption of Godinho et al. (2005) that green lizards from the western slope of the Dinaric (Croatia) and Pindus (Greece) mountains represent *L. viridis* populations. Given that the alternative hypotheses are many and the taxonomic status of these species is not very clear (see above) we claim that more data and analyses are required to evaluate the phylogeographic history of those taxa in southern Europe.

4.2.2. Lacerta trilineata and media clades

The complex geological history of the east Mediterranean region and especially of the Aegean have influenced the biogeographic history for a plethora of terrestrial animals (Lymberakis and Poulakakis, 2010 and references therein). The palaeogeographic evolution of the Aegean has been described in detail in Poulakakis et al. (2005b). One of the major geological events was the formation of MAT (Fig 3) at the late Seravallian to early Tortonian (12−9 Mya) (Creutzburg, 1963; Dermitzakis, 1990; Dermitzakis and Papanikolaou, 1981), which caused the separation of the eastern Aegean islands from the western region.

Contrary to *L. viridis* that originated and differentiated in southern Balkans, *L. trilineata* seems to originate and diverged in western Anatolia (Ahmadzadeh et al., 2013b). Although we agree with the latter authors regarding the spatial origin of *L. trilineata* clade, our results revealed a different tempo pattern that fits very well with the documented palaeogeography of the Aegean. Our data supports that the first split occurred at the early Tortonian (9.55 Mya here, 4 Mya in Ahmadzadeh et al., 2013b) and produced the two taxa recognized today as *L. trilineata* (Balkans and eastern Turkey) and *L. media* (central and western Anatolia). The aforementioned discordance, however, was not due to the lower phylogenetic power of our dataset [2 mtDNA gene fragments (cyt *b* and 16S rRNA) here *vs.* 3 (cyt *b*, 16S rRNA and 12S rRNA) in Ahmadzadeh et al. (2013a,b)], but due to the underrepresentation of *Lacerta* haplotypes from localities west of the MAT in the latter study (see Supplementary Information 1 for more details). Even though the latter species remained and further diversified in Turkey, the ancestor of *L. trilineata* dispersed from Turkey before the formation of the MAT (Fig. 3), colonized Greece and then spread to the rest of the Balkans. Given the phylogeny of *L. trilineata* in the Aegean [the separation of western (I, II and III) from eastern (IV, V and VI) group lineages] and the geological history of the Greek area, there are two possible scenarios to explain its distribution. The first (vicariant scenario) suggests a long history of *L. trilineata*, in which the ancestral form of this species invaded the area from Anatolia before the complete formation of the MAT (9 Mya). The forming of this barrier (vicariant event) gave the two groups of lineages of *L. trilineata* (I, II and III at the west and IV, V and VI at the east of the trench). Within each group, some other vicariant and dispersal events have produced the present distribution of the evolutionary lineages of *L. trilineata* (e.g., the isolation of the island of Crete during the last 5–5.5 My, the isolation of Peloponnisos in Pliocene). This scenario

has been confirmed in another lizard species of the Aegean (*Ophiomorus punctatissimus*, Poulakakis et al., 2008). The second scenario (dispersal model) suggests a more recent colonization, in which the ancestral form invaded the contemporary region of the Aegean yet again from Anatolia after the trench was formed, but via two distinct routes. The first followed the path of Asia Minor – northern Greece, and evolved in continental Greece, Peloponnisos, and Cyclades, producing the present complex of western group. The second route of invasion was from Anatolia to the East Aegean islands, and evolved in this area to produce the present complex of eastern group. This pattern is similar with the distribution pattern of *Ablepharus kitaibelii* (Poulakakis et al., 2005a). Our data support that the separation of the western (I, II and III) and eastern (IV, V and VI) group lineages occurred at 8.63 Mya (node J), which coincides with the complete formation of the MAT, indicating that the first scenario is more reliable compared to the second one. The Nestos river and Rodopi mountains that cross Bulgaria and western Thrace probably created a barrier that limited the distribution of the eastern *L. trilineata* haplotypes to the western populations. Similar phylogeographic patterns have been also found in the European snake-eyed skink (*A. kitaibelii*) (Poulakakis et al., 2005a) and several other non-reptilian vertebrate taxa such as the brawn hare (*Lepus europaeus*) (Kasapidis et al., 2005b) and water frogs (*Pelophylax* spp.) (Lymberakis et al., 2007). Within the eastern group of lineages the distinction of the populations from Lesvos Island is quite interesting and difficult to explain. The east lineages include haplotypes from Thrace and the east Aegean islands (Chios, Lesvos, Rodos and Samos) that were until very recent connected to Anatolia (Perissoratis and Conispoliatis, 2003). According to this palaeogeographic history, the isolation of the lineage from Lesvos island (VI) in early Pliocene (4.67 Mya, node L) is difficult to explain. However, the absence of *L. trilineata* specimens from the northwestern part of Turkey could be masking the phylogeographic history of the lineage of Lesvos. It is possible that the populations of northwestern Turkey form a distinct lineage that could probably coincide with lineage VI from Lesvos island and the colonization of this island occurred from a Turkish lineage more recently. However, this will remain a hypothesis until specimens of *L. trilineata* from northwestern part of Turkey are included in a future study.

Interestingly, three *L. trilineata* specimens collected from the northern and central Cyclades (Andros, Naxos) occupy an unexpected position on the phylogenetic tree. Based on the geography, one would expect that *L. trilineata* from Andros and Naxos (lineage IV) to be more closely related to *L. trilineata* from other Cyclades and mainland Greece. However, they have a sister-group relationship, but with weak support, with the lineages from the eastern Aegean islands and Turkey (lineages V and VI). This unexpected pattern could be explained either as a human translocation or an overseas dispersal or geodispersal from Anatolia. However, the time of divergence of the lineage IV from the sister-group lineages V and VI is estimated to have taken place in the late Miocene (5.97 Mya, Fig. 3), during the Messinian Salinity Crisis (MSC) when the Mediterranean water levels fall and overland migration between islands and mainland was possible. This timing implies that any scenario of human translocation is impossible. It is quite interesting that several well-documented species have passed the MAT by dispersal (overseas or geodispersal), most probably not aided by humans, such as *Podarcis erhardii*, *A. kitaibelii* and *P. bedriagae* (Lymberakis et al., 2007; Poulakakis et al., 2003, 2005a). On the other hand, a similar phylogenetic pattern, bringing together Anatolian and Cyclades lineages, has been also observed in other Aegean taxa such as geckos (~6 My, *Cyrtopodion kotschyi*, Kasapidis et al., 2005a) and scorpions (~8 My, *Mesobuthus gibbosus*, Parmakelis et al., 2006).

Another dispersal event concerns the relationship among the populations from Crete, Kythera and east Peloponnisos within

lineage III (Fig. 3). The island of Crete was isolated from Peloponnisos before 5–5.5 Mya (Schüle, 1993), which is in disagreement with the low genetic divergence between the populations from Crete and east Peloponnisos. The explanation of this pattern would involve an anthropogenic translocation or a recent overseas dispersal or a combination of human translocation and overseas dispersal from Crete to NE Peloponnisos and Kythera. These areas (Peloponnisos, Crete and Kythera Island) were the birthplace of many ancient civilizations (e.g., Minoan, Mycenaean). Plentiful evidence of Minoan influence on the island of Kythera, island and cases of plant translocation and potteries exists (Coldstream, 1978) where lizards' eggs could hide in the soil. One scenario, regarding the relationships of Crete, Kythera island and the Peloponnisos, has also been proposed for the snake species *Zamenis situla* where the island of Kythera may have acted as a stepping stone for the colonization of Crete from Peloponnisos during Pleistocene (Kyriazi et al., 2013).

5. Conclusions

In summary, our results highlighted the difficulties that classical taxonomy is faced with, when attempting to infer the relationships at species and subspecies level, and stress the need for a reconsideration of the taxonomy of the genus *Lacerta* in Greece. Moreover, our data revealed that several dispersal and vicariant events resulted in the present day distribution of *Lacerta* species in Greece. In the case of *Lacerta viridis* clade, the phylogenetic analyses revealed the occurrence of two main lineages that correspond to the two species *L. viridis* and *L. bilineata*, while within *L. viridis* two allopatric groups of specimens were detected. However, more data and analyses are required to evaluate the phylogeographic history of those taxa. In the case of *L. trilineata* a taxonomic revision in species and subspecies level is required. Furthermore, we propose a different evolutionary scenario for *L. trilineata*, in contrast to Ahmadzadeh et al. (2013b), who suggested a recent radiation for the group of *trilineata* and an expansion through multiple dispersal events out of Anatolia during the late Pliocene/early Pleistocene. The high levels of genetic divergence and the estimated diversification dates, provide clues to resolve the evolutionary history of the species based on the paleogeographic history of the area.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2014.03. 013.

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