ORIGINAL ARTICLE

A phylogenomic resolution for the taxonomy of Aegean green lizards

Panagiotis Kornilios[1,2,3] | Evanthia Thanou[1,3] | Petros Lymberakis[4] |
Çetin Ilgaz[5,6] | Yusuf Kumlutaş[5,6] | Adam Leaché[1,7]

[1]Department of Biology, University of Washington, Seattle, WA, USA

[2]Institute of Evolutionary Biology (CSIC – Universitat Pompeu Fabra), Barcelona, Spain

[3]The Molecular Ecology Backshop, Loutraki, Greece

[4]Natural History Museum of Crete, University of Crete, Irakleio, Crete, Greece

[5]Department of Biology, Faculty of Science, Dokuz Eylül University, Buca-İzmir, Turkey

[6]Research and Application Center for Fauna Flora, Dokuz Eylul University, Buca-İzmir, Turkey

[7]Burke Museum of Natural History and Culture, University of Washington, Seattle, WA, USA

Correspondence
Panagiotis Kornilios, Institute of Evolutionary Biology (CSIC – Universitat Pompeu Fabra), Passeig Marítim de la Barceloneta 37-49, Barcelona E-08003, Spain.
Emails: korniliospanagiotis@gmail.com, korniliospan@yahoo.gr

Abstract

Lacerta pamphylica and *Lacerta trilineata* are two currently recognized green lizard species with a historically problematic taxonomy. In cases of tangled phylogenies, next-generation sequencing and double-digest restriction-site-associated DNA protocols can provide a wealth of genomic data and resolve difficult taxonomic issues. Here, we generated genome-wide SNPs and mitochondrial sequences, and applied molecular species delimitation approaches to provide a stable taxonomy for the Aegean green lizards. Mitochondrial gene trees, genetic cluster delimitation and population structure analyses converged into recognizing the populations of (a) *L. pamphylica*, (b) east Aegean islands, Anatolia and Thrace (*diplochondrodes* lineage), (c) central Aegean islands (*citrovittata*), and (d) remaining Balkan populations and islands (*trilineata*), as separate clusters. Phylogenomic analyses revealed a split into two major clades, east and west of the Aegean Barrier, unambiguously showing a sister–clade relationship between *pamphylica* and *diplochondrodes*, rendering *L. trilineata* paraphyletic. Species delimitation models were tested in a Bayesian framework using the genomic SNPs: lumping all populations into a single 'species' had the lowest likelihood but the current taxonomy was also outperformed by all other models. All lines of evidence support the Pamphylian green lizard as a valid species; thus, east Aegean *L. trilineata* should also be considered a distinct species under the name *Lacerta diplochondrodes*. Finally, evidence from the mitochondrial and nuclear genomes is overwhelmingly in favour of recognizing the morphologically distinct Cycladian green lizards as a distinct species. We propose their elevation to full species under the name *Lacerta citrovittata*. All remaining insular and continental populations of the Balkan Peninsula represent the species *L. trilineata*.

KEYWORDS
ddRAD, Lacertidae, Mediterranean, molecular systematics, taxonomy

1 | INTRODUCTION

Reptiles and especially lizards have long been used as models for the study of speciation and evolutionary processes, with the family Lacertidae being the most commonly studied group (Camargo, Sinervo, & Sites, 2010). This family of 'true lizards' currently includes more than 280 species organized in 40 genera that are found in Eurasia and Africa (Arnold, Arribas, & Carranza, 2007), among them the genus *Lacerta* Linnaeus 1758, from which the family received its

name. The name *Lacerta* is as old as the scientific fields of systematics and taxonomy themselves (Schmidtler, 2010). It was used by Linnaeus in his 10th edition of Systema Naturae in 1758, as one of the four genera included in his order of Reptiles, along with *Testudo*, *Rana* and *Draco*, to describe a great variety of animal forms from salamanders to crocodiles, also including today's Lacertas. After 160 years of many taxonomic re-arrangements, the name *Lacerta* was restricted to include all present Lacertidae species (Boulenger, 1920). Since then, another 100 years that included major and minor taxonomic changes within the Lacertidae have passed. The use of molecular markers has proven to be essential for

the re-organization of the systematics and taxonomy of the family, especially after the work of Arnold et al. (2007) who defined several genera within the family providing a more stable taxonomy for the group. The systematics and taxonomy of lacertids have been continuously revisited and revised, especially with the added strength of DNA-based analytical methods and integrative species delimitation approaches, and new cryptic species have been uncovered (Bellati, Carranza, Garcia-Porta, Fasola, & Sindaco, 2014; Psonis et al., 2017; Šmíd et al., 2017; Tamar et al., 2015).

The west Eurasian genus *Lacerta*, also known as green lizards, includes eight recognized species and a plethora of
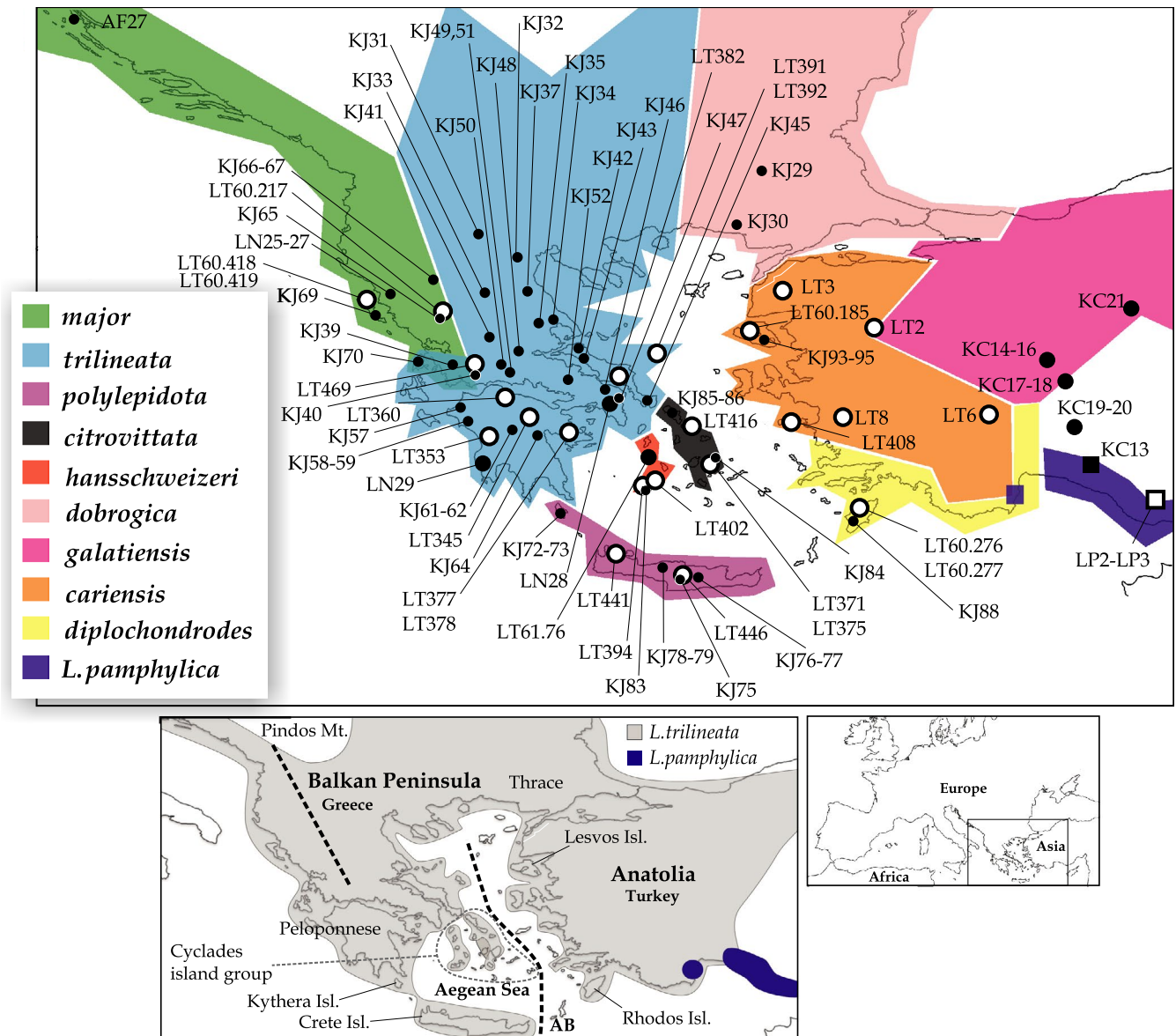

FIGURE 1 Bottom: Map of the Aegean region showing the geographic distribution of *Lacerta trilineata* and *Lacerta pamphylica*, several geographic features mentioned in the text and the Aegean Barrier (AB). Top: Approximate geographic distribution of the *L. trilineata* subspecies (Sagonas et al., 2014 and references therein; authors' records). In the same map, the sample localities of the current study are also shown: small closed circles = short mitochondrial data set; large closed circles = long mitochondrial data set; large open circles = genomic data and both mitochondrial data sets; numbers refer to working codes given in the Table S1

morphological subspecies (*L. mostoufi* from Iran is now considered an invalid species; Khosravani, Rastegar-Pouyani, Rastegar-Pouyani, Hosseinian Yousefkhani, & Oraie, 2016). In the east Mediterranean, the *Lacerta trilineata* group comprises three species: *L. media* Lantz & Cyrén, 1920, *Lacerta pamphylica* Schmidtler, 1975 and *L. trilineata* Bedriaga, 1886. *Lacerta media* is a morphologically and genetically distinct taxon, a sister species to the other two in all phylogenetic reconstructions (Ahmadzadeh, Flecks, Rödder, et al., 2013; Godinho, Crespo, Ferrand, & Harris, 2005; Mayer & Beyerlein, 2001; Sagonas et al., 2014). It is distributed from central Turkey and eastwards, reaching Iran in the east and Jordan in the south, and it presents high levels of genetic and morphological diversity (Ahmadzadeh, Flecks, Rödder, et al., 2013). However, the taxonomic situation for the *trilineata + pamphylica* clade (Figure 1) has historically been problematic. Mitochondrial phylogenies of the past decade have placed *L. pamphylica* within *L. trilineata* (Ahmadzadeh, Flecks, Rödder, et al., 2013; Godinho et al., 2005; Sagonas et al., 2014), but the respective relationships were either poorly or moderately supported, so that a clear paraphyly of *L. trilineata* with respect to *L. pamphylica* could not be demonstrated (Ahmadzadeh, Flecks, Rödder, et al., 2013; Godinho et al., 2005). Additionally, alternative topology tests that constrained *L. trilineata* to be monophyletic, representing the currently accepted taxonomy, could not be rejected (Godinho et al., 2005). Finally, an mtDNA phylogeny that presented a relatively stronger case for the paraphyly of the group, showed a 'peculiar' sister–clade relationship between *L. pamphylica* and the central Aegean *L. trilineata* populations (Sagonas et al., 2014). In this case, the values of statistical support varied among the different analyses from very poor to very high and, once again, the monophyly of *L. trilineata* could not be rejected in alternative topology tests. Moreover, this relationship between populations from central south Turkey (*L. pamphylica*) and the central Aegean islands seems to be biogeographically inexplicable and it might actually be an artefact of long branch attraction between these two long mtDNA clades (LBA; Felsenstein, 1978). Very recently, a study that used genome-wide markers to investigate the biogeographic history of the group showed that *L. trilineata* populations east of the Aegean Barrier (AB; Figure 1) had a sister–clade relationship with *L. pamphylica*, rather than with western *L. trilineata* (Kornilios et al., 2019).

A similarly complex situation occurs for the subspecific taxonomy of this group. *Lacerta pamphylica*, and even *L. media*, had been considered as subspecies of *L. trilineata* in the past (Schmidtler, 1975) but later elevated to the species level (Schmidtler, 1986). *Lacerta trilineata* currently includes nine morphological subspecies distributed throughout the Balkan Peninsula and west Anatolia (Figure 1; Sagonas et al., 2014 and references therein; authors' records). Results from mitochondrial phylogenies also show evidence of taxonomic

problems regarding the subspecies, such as paraphylies or extremely low, almost non-existent, genetic divergence among some of them (Ahmadzadeh, Flecks, Rödder, et al., 2013; Godinho et al., 2005; Sagonas et al., 2014).

Cryptic reptile and amphibian lineages have continuously been uncovered in the circum Aegean region during the past decade (Dufresnes et al., 2018; Kornilios et al., 2014), and in many cases, new species have been described or elevated from subspecies (Kornilios, Kumlutaş, Lymberakis, & Ilgaz, 2018; Kotsakiozi et al., 2018; Psonis et al., 2017; Sindaco, Kornilios, Sacchi, & Lymberakis, 2014). This is not only the outcome of a better targeted and more thorough representation of populations, but also the extensive use of molecular and genetic tools applied in recent studies to detect divergence and infer phylogenetic relationships. Advances in molecular taxonomy are critical because they suggest that cryptic diversity may be largely underestimated. Besides being important additions to the regional and global faunal lists, cryptic taxa may be important for conservation policies that are incomplete without correct species delimitations and stable taxonomic frameworks. In most cases, mitochondrial phylogenies alone or, in few cases, combined with data from single-copy nuclear markers, have been used. Massively parallel sequencing techniques (next-generation sequencing [NGS]) can provide a wealth of genome-wide data to help resolve difficult taxonomic questions and further investigate cryptic divergence. Double-digest restriction-site-associated DNA sequencing (ddRAD; Peterson, Weber, Kay, Fisher, & Hoekstra, 2012) has proven a very efficient approach for the construction of reduced representation libraries, providing a large amount of genomic data (genome-wide single nucleotide polymorphisms [SNPs]) for non-model organisms (Leaché & Oaks, 2017).

In this study, we propose formal changes to the taxonomy of the *Lacerta trilineata-pamphylica* group to properly reflect the phylogenetic relationships. This extends previous work that relied solely on mitochondrial markers, with a more complete representation of subspecies and geography and with resolved and conclusive phylogenetic results. Our study takes advantage of genome-wide markers and the ddRAD approach, which provides more evidence for phylogenetic relationships from across the genome, and modern analytical approaches for molecular species delimitation to provide an updated taxonomy for the focal clade.

2 | MATERIAL AND METHODS

2.1 | Sampling

Samples were selected to represent both currently recognized species of the target group (*L. trilineata* and *L. pamphylica*), all known morphological subspecies (*L. t. galatiensis* and *L. t. dobrogica* could only be included in the mtDNA

analyses) and the geographic variation of the studied taxa. Our complete mtDNA data set included a total of 93 *L. trilineata* and *L. pamphylica* samples, with 30 samples included in the genomic data set, including two *Lacerta viridis* as outgroup. Specimen data, including working codes, sampling localities and GenBank Accession Numbers, are given in Table S1. Additionally, the geographic and subspecific origin of the samples is shown in the map of Figure 1.

2.2 | Mitochondrial DNA: single-locus cluster delimitation

We combined sequences of the complete cytochrome *b* gene, generated in our previous work (Kornilios et al., 2019), with published ones (Ahmadzadeh, Flecks, Carretero, et al., 2013; Ahmadzadeh, Flecks, Rödder, et al., 2013; Brückner et al., 2001; Godinho et al., 2005; Marzahn et al., 2016; Pavlicev & Mayer, 2009; Sagonas et al., 2014) and aligned them in ClustalX v.2.0.12 (Larkin et al., 2007) using default parameters. Two mtDNA data sets were constructed: one including 41 longer ingroup sequences (1,137 bp—mtDNA-L data set) and a second one with 93 shorter ingroup sequences (399 bp—mtDNA-S).

Delimitation of mtDNA genetic clusters included the Bayesian implementation of the Poisson tree processes model (bPTP; Zhang, Kapli, Pavlidis, & Stamatakis, 2013) and the multi-rate PTP (mPTP: Kapli et al., 2017), both of which use non-ultrametric phylogenetic trees, as input. In this context, we built maximum-likelihood (ML) trees using both mtDNA data sets and ran each species delimitation analysis with both trees, after cropping the outgroups. For each bPTP analysis, we performed five independent runs on the PTP server (http://species.h-its.org/ptp/) with 5×10^5 generations, a thinning of 100 and a burn-in of 10%, while mPTP ran locally.

The ML trees were constructed with IQ-TREE 1.4.3 (Chernomor, Haeseler, & Minh, 2016; Nguyen, Schmidt, Haeseler, & Minh, 2015; Trifinopoulos, Nguyen, Haeseler, & Minh, 2016). Analyses ran under a single partition scheme for the mtDNA-S data set due to the smaller size of the segment, and with the 'partitionfinder' and 'Auto' options to determine the best partitioning scheme and best-fit substitution model for each partition (codon position) for the mtDNA-L data set. Nodal support was tested via SH-aLRT tests with 10,000 replicates (Guindon et al., 2010), an approximate Bayes test (aBayes), 10,000 ultrafast bootstrap alignments (Minh, Nguyen, & Haeseler, 2013) and 1,000 standard bootstrap alignments (Felsenstein, 1985). We included *L. media* and rooted the tree with *L. agilis*.

Since it has been suggested that independent mtDNA haplotype networks, using the statistical parsimony algorithm and the 95% connection limit, represent distinct evolutionarily significant units (ESUs) (Fraser & Bernatchez, 2001), we performed this analysis in TCS v.1.21 (Clements et al.,

2000) to infer the number of independent networks within the studied group, using both mtDNA data sets. Finally, we estimated genetic divergence among population clusters by calculating pairwise F_{ST} values (Weir & Cockerham, 1984) with DnaSP v.6 (Rozas et al., 2017), using the mtDNA-L data set.

2.3 | Mitochondrial DNA: testing for long branch attraction

In order to test for the possibility of LBA, we followed two common approaches: long-branch exclusion and alternative topology testing. For the first, we ran two ML analyses excluding the branches that might be subject to LBA, which were *L. pamphylica* and *L. t. citrovittata*, respectively, and compared the topologies to the ML tree that included all lineages. For the second, we tested two alternative topologies by constraining (a) all *L. trilineata* populations to be monophyletic and (b) the populations east and west of the Aegean to be reciprocally monophyletic. The constrained trees were compared to the unconstrained topology, using the topology tests and the RELL (resampling estimated log-likelihood) bootstrap (10,000 replicates) included in the IQ-TREE package: bootstrap proportion (BP), Kishino–Hasegawa test (Kishino & Hasegawa, 1989), Shimodaira–Hasegawa test (Shimodaira & Hasegawa, 1999), expected likelihood weights (Strimmer & Rambaut, 2002) and approximately unbiased (AU) test (Shimodaira, 2002). Analyses ran with the mtDNA-L data set.

2.4 | ddRAD bioinformatics and genomic SNPs

We processed raw Illumina reads (Kornilios et al., 2019) using the program iPyRAD v.0.7.8 (Eaton, 2014). We demultiplexed samples using their unique barcode and adapter sequences and reduced each read to 39 bp, after removal of the 6 bp restriction site overhang and the 5 bp barcode. Sites with Phred quality scores under 99% (Phred score = 20) were changed into 'N' characters and reads with ≥10% N's were discarded. Within the iPyRAD pipeline, the filtered reads for each sample were clustered using VSEARCH v.2.4.3 (Rognes, Flouri, Nichols, Quince, & Mahé, 2016) and aligned with MUSCLE v.3.8.31 (Edgar, 2004). We assembled the ddRADseq data using a relatively stringent clustering threshold of 92%, in order to reduce the risk of combining paralogs, while still accommodating a realistic level of sequence variation. As an additional filtering step, consensus sequences that had low coverage (<10 reads), excessive undetermined or heterozygous sites (>4) or too many haplotypes (>2 for diploids) were discarded. The consensus sequences were clustered across samples using the within-sample clustering threshold (92%). Again, alignment was done with MUSCLE, applying a paralog filter that removes

loci with excessive shared heterozygosity among samples (paralog filter = 200). The maximum number of SNPs per locus was set to 15 (default value 20) to minimize the possibility of returning paralogs.

We generated final data sets with no missing data (all loci were present for all samples) for several types of phylogenomic analyses. Population structure, species tree and species delimitation (see below) were performed on data matrices that included one random SNP from each putatively unlinked locus ('uSNP' data sets). The population structure analysis did not include the outgroup samples, the species tree analysis did and the species delimitation analysis had a smaller total number of individuals for computational reasons. In this context, we ran the iPyRAD pipeline separately for the reconstruction of each data set. Finally, the concatenated tree was based on the combined sequence of all loci, again with no missing data, no admixed individuals and including the outgroup (details on the samples included in each analysis are shown in Table S1).

2.5 | De novo population structure, coalescent species tree and concatenated tree

The genetic structure within our study system was inferred with the discriminant analysis of principal components (DAPC; Jombart, Devillard, & Balloux, 2010) implemented in the R package Adegenet (Jombart, 2008). DAPC identifies genetic clusters using the k-means clustering algorithm according to the Bayesian information criterion (BIC) and describes the relationship between these clusters, optimizing the variance between groups while minimizing variance within groups. The optimal number of clusters (from 1 to 12) was estimated with the find.cluster function in ADEGENET. We used the a-score function to determine the number of principal components and avoid overfitting. The data set used in DAPC included 28 individuals (no outgroup) and 1,085 uSNPs.

A coalescent species tree was estimated using SVDquartets v.1.0 (Chifman & Kubatko, 2014) implemented in PAUP* v.4.0a (Swofford, 2003). This method infers trees for subsets of four samples using unlinked multilocus data, assigning a score to each of the three possible quartet topologies and estimates the species tree using a quartet assembly method. We evaluated all possible quartets of samples with prior assignment of individuals to the population clusters as resulted from DAPC. We used nonparametric bootstrapping with 1,000 replicates for the statistical support. The analysed data set included 27 individuals (with *L. viridis* as the outgroup) and 826 uSNPs.

A phylogenomic ML tree was also constructed using the concatenated ddRAD loci with IQ-TREE, with the 'Auto' option and with nodal support via 10,000 SH-aLRT, an aBayes test and 10,000 ultrafast bootstrap alignments. The data set included 27 individuals and a total of 38,601 bp, and the tree was again rooted with *L. viridis*.

2.6 | Bayesian testing of species delimitation models using genomic SNPs

We conducted a Bayesian model comparison using the genomic SNPs under the BFD* protocol (Leaché, Fujita, Minin, & Bouckaert, 2014). For each species delimitation model (SDM), we estimated a species tree and calculated marginal likelihoods with SNAPP V1.3 (Bryant, Bouckaert, Felsenstein, Rosenberg, & Roy Choudhury, 2012) implemented in BEAST2 V2.6 (Bouckaert et al., 2014). To estimate the marginal likelihood for each SDM, we used path sampling with 40 steps (50,000 iterations, 25% burn-in). We repeated each analysis twice using random starting seeds to ensure stable marginal likelihood estimation.

For this analysis, the data set included 18 individuals and 853 unlinked biallelic SNPs. Using the same number of SNPs in all SDM comparisons provides more accurate results that reflect the differences in sample assignments and not levels of missing data (Leaché, McElroy, & Trinh, 2018). We used *L. viridis* as outgroup in all SDMs, which allowed us to also test a single-species model for *trilineata-pamphylica* (two species including *L. viridis*) against the other SDMs, which would not be possible without an outgroup. As in Kornilios et al. (2019), mutation rates (u, v) were both fixed at 1.0, the λ prior we set as a broad gamma distribution with a mean value of 1,000 ($\alpha \times \beta$, with $\alpha = 2$ and $\beta = 500$), and the θ prior was set as a gamma distribution with a mean value of .001 (α/β, with $\alpha = 25$ and $\beta = 25,000$).

Since SNAPP is computationally intensive each 'species' included at least two individuals and up to 16. We tested seven SDMs, which included from one to five 'species' for our target-system, described in Table 1. The one-species model (Sp1) lumped all *trilineata-pamphylica* populations in one taxon. Two two-species models represented (Sp2.1) the current taxonomy (*L. pamphylica* and *L. trilineata*) and (Sp2.2) the recognition of two 'species' east and west of the Aegean, respectively. Two three-species models represented (Sp3.1) one 'species' in the east (lumping *L. pamphylica* and east *L. trilineata*) and two in the west (splitting *L. t. citrovittata* of the central Aegean islands from the others) or (Sp3.2) two 'species' in the east (*L. pamphylica* and east *L. trilineata*) and one in the west. The combination of all four 'species', two in the east and two in the west was used in Sp4. Finally, Sp5 further split the populations west of the Aegean Barrier into a southern 'species' (Crete and Peloponnesos) and a northern one (Balkan Peninsula and West Cyclades).

TABLE 1 The species delimitation models (SDMs) tested in the current study with a short description, the number of 'species' included, their marginal likelihood, the Bayes factor values between each model and the current taxonomy (Sp2.1) and their rank (1–7) from the best to the least supported model. The investigated models include combinations from one to five taxa for the target group (two to six including the outgroup *Lacerta viridis*)

SDM	Description	Number of species	Marginal likelihood	Bayes factor (2lnBF) with current taxonomy	Rank
Sp1	Lump all populations into one 'species'	1	−8,485	−1,046	7
Sp2.1	Current taxonomy: *Lacerta pamphylica* and *Lacerta trilineata*	2	−7,962	—	6
Sp2.2	Lump east and west populations, respectively	2	−7,103	+1,718	5
Sp3.1	*L. pamphylica*; east *L. trilineata* (*diplochondrodes*); west *L. trilineata*	3	−6,951	+2,022	4
Sp3.2	Lump east *L. trilineata* and *L. pamphylica*; *L. trilineata* from central Aegean (*citrovittata*); west *L. trilineata* (*trilineata*)	3	−6,561	+2,802	3
Sp4	*L. pamphylica*; east *L. trilineata* (*diplochondrodes*); *L. trilineata* from central Aegean (*citrovittata*); west *L. trilineata* (*trilineata*)	4	−6,405	+3,114	2
Sp5	*L. pamphylica*; east *L. trilineata* (*diplochondrodes*); *L. trilineata* from central Aegean (*citrovittata*); south-west *L. trilineata*; north-west *L. trilineata*	5	−6,125	+3,674	1

Finally, similarly to the mtDNA, we estimated genetic divergence among population-clusters by calculating pairwise F_{ST} values with DnaSP v.6, using the unlinked SNPs data set (28 individuals, 1,085 uSNPs).

3 | RESULTS AND DISCUSSION

3.1 | Mitochondrial and genomic clusters

The ML analysis of the longer mtDNA data set ran with each codon position as a separate partition. The resulting mtDNA gene tree shows four major clades (Figure 2 and Figure S1). These correspond to the three clades discussed in Ahmadzadeh, Flecks, Rödder, et al. (2013), named there and here *pamphylica* (for *L. pamphylica*), *trilineata* (for west Aegean *L. trilineata*) and *diplochondrodes* (for east Aegean *L. trilineata*), with the addition of the clade *citrovittata* (central Aegean islands *L. t. citrovittata*) that was not represented in that study. The pairwise F_{ST} values among these four major mtDNA clades are extremely high and similar to each other, ranging from .74 to .89. The F_{ST} value between the southern and northern subclades of the *trilineata* lineage was significantly lower at .46.

The mtDNA clades corresponding to *pamphylica* and *citrovittata* form a monophyletic unit, as shown before (Sagonas et al., 2014; Kornilios et al., 2019), but the support for this node is ambiguous: SH-aLRT and standard bootstrap values are >80, which imply significant support, but aBayes and ultrafast bootstrap values are <0.95, which is considered weak support, according to the developers' guidelines. Our investigation regarding this particular

relationship showed that none of the different topologies that we tested was rejected by the topology tests. Hence, once again the monophyly of *L. trilineata* cannot be rejected in the mitochondrial phylogeny, neither does the distinction of two monophyletic units east and west of the Aegean. Additionally, when we excluded *pamphylica* or *citrovittata* from the mtDNA ML analysis, that is the potentially 'problematic' long branches, the remaining three clades always formed a polytomy. This biogeographically strange relationship is most probably artifactual, a result of LBA. Such artefacts can be resolved with the analysis of independent genetic markers and species tree approaches, rather than gene trees. The results from the population clustering and tree analyses of the genomic markers (see below) clearly reject any relationship between *pamphylica* and *citrovittata* and show an east–west divergence for the group, reinforcing the conclusion of LBA in the mtDNA tree. The ML tree from the short mtDNA-S data set showed the same groupings and relationships, but with generally weaker support values (Figure S2).

The single-locus species delimitation analyses bPTP, mPTP and parsimony networks, performed on the longer and the shorter mtDNA data sets (six analyses in total), returned similar results. All of them identified seven mtDNA clusters (Figures S1 and S2), which are (a) *L. pamphylica*, (b) *L. t. cariensis* from Lesvos island, (c) all remaining east Aegean *L. trilineata* (*cariensis*, *diplochondrodes*, *galatiensis*, *dobrogica*), (d) *L. t. citrovittata* from the central Aegean islands, (e) *L. t. trilineata* from the west Peloponnesos, (f) *L. t. trilineata* from the east Peloponnesos together with *L. t. polylepidota* from Crete and Kythera islands and (g) all *L. t. trilineata* and *L. t. major* from the northern parts

FIGURE 2 A schematic representation of the species tree based on genome-wide SNPs derived from SVDquartets is shown in light grey (and dark blue for the *pamphylica* lineage). The direct result from SVDquartets is presented in Figure S4. Embedded within the species tree is the mitochondrial gene tree, derived from the maximum-likelihood analysis of cytochrome *b* with IQ-TREE. The direct result from this analysis is presented in Figure S1. The names next to the terminal clades are the working codes of the analysed samples (Table S1; Figure 1) with the respective subspecies they belong to (colours as in Figure 1). Black closed circles indicate absolute statistical support values of the respective nodes
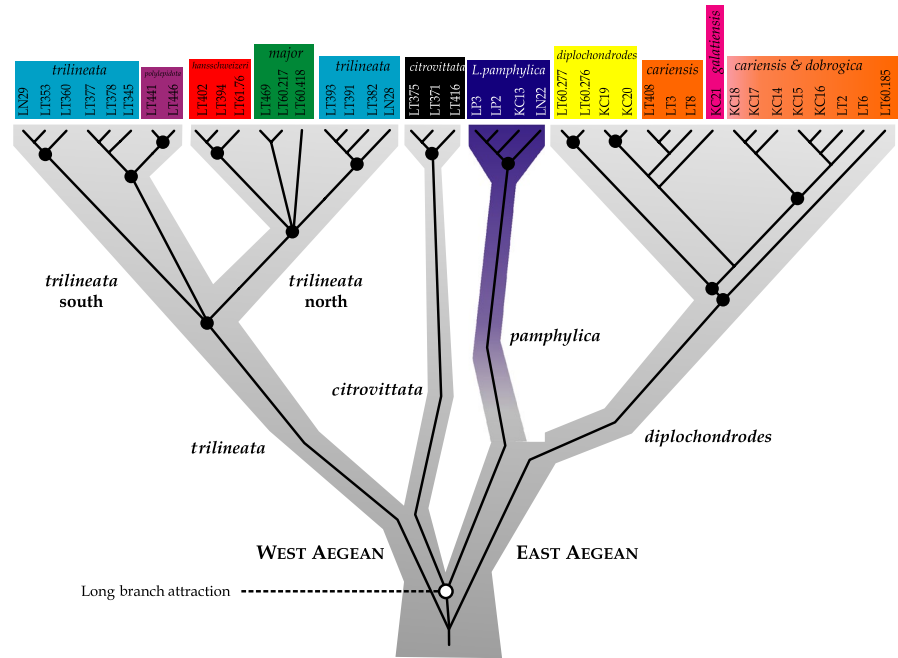
of the Balkan Peninsula together with *L. t. hansschweizeri* from the west Aegean islands. Four of the six analyses (bPTP, mPTP and network on the mtDNA-L and bPTP on the mtDNA-S) identified *L. t. citrovittata* from the north Cyclades as an additional mtDNA cluster, while one analysis (bPTP on the mtDNA-S) further identified *L. t. galatiensis* (Turkey) as a separate cluster. The independent networks from the analysis of both mtDNA data sets are shown in Figure S3.

The iPyRAD pipeline ran separately for the data sets used in downstream analyses. For the population-structure data set, the total number of prefiltered loci was 66,227, while the filtered were 1,356 loci (54,387 bp) with 2,544 SNPs and 1,085 uSNPs. For the data set used in SVDquartets and concatenated ML, there were 64,395 prefiltered loci and 963 filtered (38,601 bp) with 2,232 SNPs and 826 uSNPs. Finally, for the species delimitation data set, the prefiltered loci were 59,077 and the filtered were 1,709 (68,479 bp) with 3,570 SNPs and 1,421 uSNPs.

The population cluster analysis DAPC on the genomic data returned $K = 5$ as the optimal number of clusters, with two discriminant functions (dimensions) describing the relationships. The DAPC scatter plots for the first and second discriminant functions are shown in Figure 3, with the first differentiating the populations east and west of the Aegean Barrier and the second the *citrovittata* lineage (central Aegean islands) from all others. The scatter plot with all functions is also shown in Figure 3. The five phylogenomic clusters from DAPC match, to some extent, the results from the mtDNA gene-tree analysis and the mtDNA cluster analyses. They converge into recognizing *pamphylica*, *citrovittata* and *diplochondrodes* as distinct units, that is the three of the four mtDNA clades (Figure 2 and Figure S1), but further

recognize two groups within the fourth lineage *trilineata*, a southern and a northern one.

The coalescent species tree from SVDquartets and the concatenated ML tree present a split into two major clades, east and west of the Aegean Barrier (Figures 2 and 3, Figures S4 and S5). They unambiguously show a sister–clade relationship between the *pamphylica* and *diplochondrodes* lineages, rendering *L. trilineata* paraphyletic. In the west, *citrovittata* from the central Aegean splits very early, in agreement with the genomic clustering results that show that these populations are highly divergent (Figure 3). Finally *trilineata* further splits into two clades, a southern (*L. t. trilineata* from Peloponnesos and *L. t. polylepidota* from Crete) and a northern one (*L. t. trilineata* from the remaining parts of the subspecies' distribution in the Balkan Peninsula, *L. t. major* from the west Balkan Peninsula and *L. t. hansschweizeri* from the west Cyclades islands). These relationships also render the nominotypical subspecies paraphyletic, agreeing with the mtDNA results. Once again, pairwise F_{ST} values among clusters derived from the genomic SNPs were high, the highest ones being between *citrovittata* and all others (.26–.45). The southern and northern groups of the *trilineata* lineage presented the lowest divergence (.13), while the F_{ST} between *diplochondrodes* and *pamphylica* was .17. All other values varied between .24 and .33.

3.2 | Bayesian species delimitation with genome-wide SNPs

The first conclusion to be drawn from the results of the Bayesian comparison of SDMs (Table 1) is that Sp2.1 that reflects the two-species current taxonomy of *L. pamphylica* and *L. trilineata* is outperformed by all other SDMs, with the
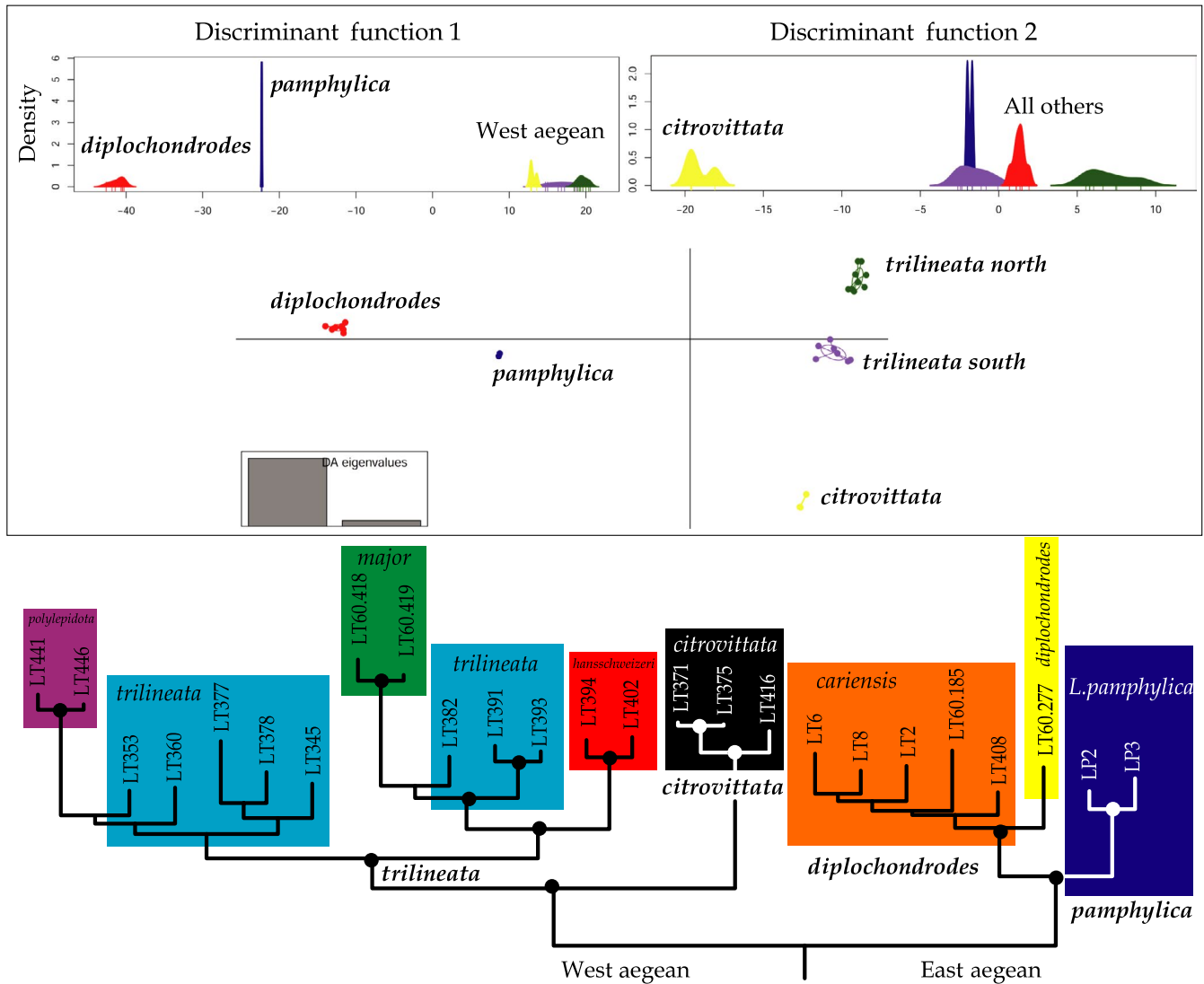
FIGURE 3 Top: The outcome from the discriminant analysis of principal components (DAPC) (optimal *K* = 5), based on the unlinked genomic SNPs, showing the population clustering results from each of the two discriminant functions and the final results from both discriminant functions. Bottom: The phylogenomic maximum likelihood tree, produced with IQ-TREE, based on the concatenated ddRAD loci. The direct result from this analysis is presented in Figure S5. The names next to the terminal clades are the working codes of the analysed samples (Table S1, Figure 1) with the respective subspecies they belong to (colours as in Figures 1 and 2). Black closed circles indicate absolute statistical support values of the respective nodes

exception of Sp1 that lumps all populations into a single 'species'. The latter had the lowest likelihood of all models, even though the better-performing SDM of the current taxonomy represents a paraphyletic phylogeny.

Several researchers have remarked that coalescent-based methods of species delimitation may be prone to over-splitting, since it seems that a larger number of 'species' included in an SDM leads to higher marginal likelihoods (Bryson et al., 2014; Nieto-Montes de Oca et al., 2017). Simulation studies have shown that over-splitting SDMs may be harder to distinguish from the true model compared to lumping 'species' (Leaché et al., 2014). However, in a recent study, the performance of SDMs did not improve with the increase of the 'species' number, but the correct identification of

'species' boundaries (the correct assignment of samples into 'species') was more influential (Leaché et al., 2018). Here, we observe a relationship between the likelihood of the models and the number of 'species' included in them (Table 1) and, since the application of Bayesian comparison in species delimitation with genomic data is in the early stage of development, this outcome must be treated with caution and conclusions should be drawn using multiple lines of evidence and not be based solely on the SDM comparisons. Additionally, this approach does not consider gene flow and treats incongruence among loci as a result of incomplete lineage sorting (Leaché et al., 2014). Analyses of genetic admixture did not detect any gene flow between the tested 'species' (Kornilios et al., 2019).

Comparisons are more straightforward when SDMs have the same number of 'species' but different sample assignments into those 'species'. In our study, there are two occasions where SDMs with the same number of 'species' are compared, with very interesting and insightful results, regarding the taxonomic situation in the study group: (a) When Sp2.1 (current taxonomy) and Sp2.2 (populations appointed into two 'species' west and east of the Aegean Barrier) were compared, the second had a profoundly better performance (+1,718 BF). (b) When the three-species SDMs, Sp3.1 and Sp3.2, were compared, the second was significantly better (+780 BF). This means that the model that identifies *citrovittata* from the central Aegean as a distinct 'species' but lumps *diplochondrodes* and *pamphylica* is strongly favoured compared to the one that keeps *pamphylica's* status but does not differentiate the central Aegean populations. The Bayesian SDM comparison, based on the genomic markers, supports *citrovittata's* validity as a species more than it does for *pamphylica*, when populations are constrained to form three-species schemes. However, they are both outranked compared to Sp4 which identifies both entities as distinct species (+1,092 BF and +312 BF, respectively). Finally, Sp5 that recognizes a south and a north species within the *trilineata* lineage is the SDM with the highest likelihood (+560 BF from Sp4).

3.3 | Species limits within the *trilineata-pamphylica* group

Previous molecular phylogenies, based on mitochondrial DNA, have demonstrated the problematic situation for the *pamphylica-trilineata* group. Despite the placement of *L. pamphylica* within *L. trilineata*, alternative topology tests and statistical support values could not reject the monophyly of all *L. trilineata* populations and, thus, the validity of the current taxonomy. In this sense, the need of a taxonomic re-evaluation of the group was argued in several studies but no formal changes were made.

The analysis of genome-wide markers has clearly shown that *L. trilineata* is a paraphyletic species, demonstrated by clustering and tree-building analyses. In order to bring stability to the systematics and taxonomy of the group, we are first presented with two basic options, with *L. pamphylica* being the key species: we can either consider *L. pamphylica* an invalid species or maintain its status as a valid one, inevitably rendering the east *L. trilineata* (*diplochondrodes* lineage) a separate species. The first is the most parsimonious solution; it does not further split the group into new species but lumps two currently recognized species into one. But is the most 'simple' solution the correct one?

3.4 | Green lizards east of the Aegean Barrier

The Pamphylian green lizard was first described as a subspecies of *L. trilineata* by Schmidtler (1975), an endemic of the central south coastal region of Turkey. A decade later, the same author published a very thorough investigation on the morphology of Anatolian green lizards to conclude that *L. pamphylica* was in fact a distinct species, exhibiting great morphological differences from the other two Anatolian species, namely *L. trilineata* in the western parts of Turkey and *L. media* in the eastern (Schmidtler, 1986). The validity of *L. pamphylica's* specific status was reinforced with biochemical analyses of blood serum proteins, as the three morphological species returned distinct profiles with notable differences (Üçüncü, Tosunoğlu, & İşisağ, 2004). Pamphylian green lizards also exhibit differences in immunoserological patterns compared to other Anatolian green lizards (Engelman & Schaffner, 1981). In mitochondrial phylogenies, *L. pamphylica* is a very divergent lineage, representing the longest or one of the longest branches in the group's mtDNA gene tree and it is one of the four major mtDNA clades (Ahmadzadeh, Flecks, Rödder, et al., 2013; present study, Figure 2 and Figure S1). All mtDNA cluster delimitation analyses performed here identified *L. pamphylica* as a distinct cluster (Figure S1), while population clustering using genomic data agree with these results (Figure 3). The Bayesian comparison of SDMs, based on genome-wide SNPs, returned a very low likelihood for the current taxonomy, but the performance of the model that rejected the validity of *L. pamphylica* by lumping it with *L. trilineata* was even worst, ranking last among all SDMs (Table 1).

The Anatolian *Lacerta* species also show extensive differentiation in their realized niche space, with *L. pamphylica* showing small niche overlap with the Anatolian *L. trilineata* (Ahmadzadeh, Flecks, Carretero, et al., 2013). Pamphylian green lizards were considered to have a parapatric distribution with the other two Anatolian green lizards, with only one population found so far within the *L. trilineata* range (Geniez, Geniez, & Viglione, 2004; Figure 1). Since *L. trilineata* has not been reported from that area, the two species probably present small-scale parapatry, rather than sympatry (Ahmadzadeh, Flecks, Rödder, et al., 2013), and the morphology of the *L. pamphylica* individuals from the specific population does not show any signs of hybridization (Geniez et al., 2004). Additionally, based on the sample tested so far, *L. trilineata* and *L. pamphylica* do not share mtDNA haplotypes and, most important, there are no signs of any genetic admixture between them, after analysing thousands of genome-wide SNPs (Kornilios et al., 2019). All lines of evidence (morphology, biochemical and immunological analyses, mitochondrial genealogies, genomic data, ecology, distribution, field observations) leave no justification whatsoever for sinking *L. pamphylica* to synonymy with *L. trilineata*.

This leads to a straightforward conclusion regarding the *L. trilineata* populations east of the Aegean Barrier: they are sister to *L. pamphylica* rather than to their conspecifics from the west part of the Aegean and should, therefore, be

considered a distinct species. Besides the clear paraphyly, these populations also form the second major clade of the four mitochondrial clades (Figure 2 and Figure S1), they are grouped in a distinct cluster in the population clustering analysis of genomic markers (Figure 3) and show no signs of mitochondrial or genomic introgression either with *L. pamphylica* or the west Aegean populations. In fact, this lineage probably does not even form a contact zone with the west Aegean *L. trilineata* (Figure 1) and its origin in the easternmost parts of the Balkan Peninsula (subspecies *L. t. dobrogica*) is very recent (Kornilios et al., 2019). Anatolian *L. trilineata* are also morphologically very different from the western ones; the eastern morphological subspecies are confined to the *diplochondrodes* lineage and restricted within its geographic range (Figure 2). Finally, they also demonstrate small niche overlap with the other green lizard lineages (Ahmadzadeh, Flecks, Carretero, et al., 2013). Following previous workers (Ahmadzadeh, Flecks, Rödder, et al., 2013) and based on name availability in the literature, we propose the name *Lacerta diplochondrodes* Wettstein, 1952 (common name: Anatolian green lizards), as the oldest available name, for the east Aegean green lizards that are now recognized as *L. trilineata*.

What needs to be decided is the taxonomic fate of the populations found west of the Aegean Barrier. These could be regarded as one species, *L. trilineata*, or further split into two by elevating *L. t. citrovittata* from the central Aegean islands to the species level.

3.5 | Green lizards west of the Aegean Barrier

One of the most interesting results from our genomic analyses is that the recognition of *citrovittata* as a distinct species has much stronger support than that of the key-species *L. pamphylica*. In our mitochondrial phylogeny, *citrovittata* is the third of the four major mtDNA clades, the fourth one being the remaining populations west of the Aegean Barrier (Figure 2 and Figure S1). All mtDNA cluster delimitation analyses and population clustering using genomic data identified *citrovittata* as a distinct cluster (Figure 3 and Figure S1). The Bayesian comparison of SDMs largely favoured the four-species model compared to the three-species ones but more importantly when the two three-species models were compared, the one that identified *citrovittata* as a distinct species largely outranked the one that identified *pamphylica* (Table 1). In terms of genetic divergence based on genome-wide SNPs, even the lowest F_{ST} value between *citrovittata* and any other cluster (.22 with *trilineata*) was higher than the one found between *pamphylica* and *diplochondrodes* (.17). As expected, this largely allopatric lineage shares no mtDNA haplotypes with any other green lizard and presents no sign of genetic admixture, based on genome-wide data (Kornilios et al., 2019).

The central Aegean green lizards have caught the attention of zoologists since very early in the history of herpetological studies in the Aegean region. Bedriaga (1881 '1882') was the first to describe these lizards as a distinct subspecies of *L. viridis* at a time that lacertid systematics was much different and perplexed than today. However, central Aegean green lizards continued to be recognized as a distinct unit within *L. viridis* and not *L. trilineata* for many years to come, despite the fact that the distinction between the latter two species had been clarified at the time (Werner, 1938; Wettstein, 1953). This was mostly because of the Cycladian lizards' unique morphology and especially their remarkable colouration patterns which related them more to *L. viridis* than to *L. trilineata*. Ultimately, Buchholz (1962), published an elaborate discussion regarding these lizards and moved them to *L. trilineata*. Unfortunately, until now, these morphologically divergent green lizards have not been included in comparative studies (morphological, biochemical, immunological, ecological), contrary to the other lineages of the group. In the very few studies that included them, they were not assigned to a distinct phylogenetic unit and were grouped either with other *trilineata* (Ahmadzadeh, Flecks, Carretero, et al., 2013) or with other non-related insular populations (Sagonas et al., 2019), as those studies aimed to answer questions aside from phylogeny or taxonomy. As a consequence, the extent of their differentiation had not been assessed and their taxonomic status remained unchanged. However, their morphological distinctiveness and the genetic evidence from both the mitochondrial and the nuclear genomes are overwhelmingly in favour of their recognition as a distinct species.

In this context, we propose to elevate this taxon to full species level under the name *Lacerta citrovittata* Werner, 1938 (common name: Cycladian green lizard). In turn, all remaining insular and continental populations of the Balkan Peninsula (with the exception of the north-easternmost parts) represent the species *L. trilineata* Bedriaga, 1886 (common name: Balkan green lizard).

4 | CONCLUSIONS AND NOTES ON SUBSPECIFIC TAXONOMY

The utility of genome-wide molecular markers, in combination with mitochondrial data, resulted in a clearer picture of the phylogenetic groupings and relationships of Aegean green lizards and provided a more stable taxonomic framework. Besides the taxonomic stability and the identification of two new species for the group and the region, this work provides a better representation of biodiversity that will greatly benefit conservation management. In particular, *L. citrovittata* is an endemic species of the central Aegean island archipelago, known to occur on eight islands so far. Despite the widely acknowledged role that the Aegean palaeogeography and formation of islands has

TABLE 2 The current and proposed taxonomy for the Aegean green lizards of the *trilineata-pamphylica* clade

Current taxonomy	New taxonomy	Distribution—observations
Lacerta pamphylica Schmidtler, 1975	*Lacerta pamphylica*	Central south coast of Turkey
Lacerta trilineata citrovittata Werner, 1935	*Lacerta citrovittata*	Central Aegean islands (Greece): Andros, Tinos, Syros, Mykonos, Naxos, Paros, Antiparos, Ios, possibly other islands. Reported occurrence in south Evvoia is doubtful (personal observations) and because animals from Evvoia were found to be genetically pure *L. t. trilineata*
Lacerta trilineata diplochondrodes Wettstein, 1952	*Lacerta diplochondrodes diplochondrodes*	South-east coast of Turkey, Rhodos and Kos islands (Greece)
Lacerta trilineata cariensis Peters, 1964	*Lacerta diplochondrodes cariensis*	West Turkey, Samos, Chios, Lesvos islands (Greece)
Lacerta trilineata dobrogica Fuhn & Mertens, 1959	*Lacerta diplochondrodes dobrogica*	North-west (European) Turkey, northeast Greece, Bulgaria, Romania. Genetically indistinguishable from *cariensis*
Lacerta trilineata galatiensis Peters, 1964	*Lacerta diplochondrodes galatiensis*	North-west and central Turkey
Lacerta trilineata polylepidota Wettstein, 1952	*Lacerta trilineata polylepidota*	Crete island, Kythera island (Greece)
Lacerta trilineata hansschweizeri Müller, 1935	*Lacerta trilineata hansschweizeri*	West Cyclades islands (Greece): Milos, Kimolos, Polyegos, Kithnos, Serifos, Sifnos
Lacerta trilineata major Boulenger, 1887	*Lacerta trilineata major*	Croatia, Montenegro, Bosnia-Herzegovina, Albania, western Greece (west of Pindos Mt) including Corfu and Paxos islands
Lacerta trilineata trilineata Bedriaga, 1886	*Lacerta trilineata trilineata*	North Macedonia, Bulgaria, Serbia, Pelopponesos and eastern Greece (east of Pindos Mt), including adjacent Aegean and south Ionian islands. Polyphyletic subspecies. Genetically admixed individuals between *major* and *trilineata* occur in south-west Greece

played on regional speciation and biodiversity richness, this is the only endemic herpetofaunal species of the central Aegean islands and merely the third for the entire Cyclades island group, besides the Milos viper and Milos wall lizard.

The taxonomic changes proposed in this work are summarized in Table 2, together with the distributions of the respective species and subspecies and other notes, while the geographic distributions of species and subspecies are also presented in the map of Figure S6.

There are currently nine recognized subspecies in the studied group. The name 'panakhaikensis' has also been used to describe individuals from the northernmost part of Peloponnesos (Böhme, 1974; Buchholz, 1960), but without a formal description, rendering the name nomen nudum. This name was erroneously used later in mtDNA phylogenies for populations from the easternmost parts of Peloponnesos where animals belong to the subspecies *L. t. trilineata* (Mayer & Beyerlein, 2001; Sagonas et al., 2014). In this context, we have treated our samples from this region as *L. t. trilineata* and not 'panakhaikensis'.

For *L. diplochondrodes*, none of the analyses and the types of markers used here and previous studies (Ahmadzadeh, Flecks, Rödder, et al., 2013; Sagonas et al., 2014) could find any differentiation and/or relationships to justify the recognition of the current morphological subspecies (*cariensis*,

diplochondrodes, *galatiensis*, *dobrogica*; Figure 1). Only one of the six mtDNA cluster analyses showed that *galatiensis* might be genetically distinct (Figure S1), but we could not evaluate this result with genomic data. It should be noted that the populations from Lesvos island and adjacent Turkey that are currently assigned to *cariensis* might represent a distinct subspecific entity as demonstrated by mtDNA (Sagonas et al., 2014; present study, Figure 2 and Figure S1) and genomic SNPs (Kornilios et al., 2019). We agree with Ahmadzadeh, Flecks, Rödder, et al. (2013) that the subspecific taxonomy within *L. diplochondrodes* has been largely inflated and needs to be revised. For the time being and since the sample analysed so far is not large, we will leave the subspecific taxonomy of east Aegean green lizards as it is, pending further investigation.

Contrary to *L. diplochondrodes*, the currently accepted subspecies within *L. trilineata* were corroborated by the genetic results. Genomic population structure analyses (Kornilios et al., 2019) and the phylogenomic tree (Figure 3) distinguished groups that agree to great extent with the current morphological subspecies *L. t. polylepidota* (Crete), *L. t. hansschweizeri* (west Cyclades) and *L. t. major* (west Balkan Peninsula), with the latter also presenting genetic admixture with *L. t. trilineata* (east Balkan Peninsula) (Kornilios et al., 2019). Additionally, they all form distinct

monophyletic clades in the mtDNA gene tree (Figure 2). On the other hand, the Peloponnesian *L. t. trilineata* populations form their own separate cluster from the mainland populations of the same subspecies, rendering it paraphyletic (Ahmadzadeh, Flecks, Rödder, et al., 2013; Godinho et al., 2005; Sagonas et al., 2014; present study, Figures 2 and 3). Additionally, mtDNA gene trees show a sister–clade relationship of the *polylepidota* subspecies from Crete and the *trilineata* populations from the east parts of Peloponnesos (Figure 2), rendering the Peloponnesian populations paraphyletic themselves, but this is not backed up by the genomic analyses (Figure 3). None of the solutions that would help resolve the apparent paraphyly for *L. t. trilineata* and the complex taxonomic situation regarding the Peloponnesian green lizards is desirable. Eliminating all *L. trilineata* subspecies would go against the morphological and genetic results that highly support their validity. Additionally, lumping Peloponnesian *L. t. trilineata* with *L. t. polylepidota*, lumping non-Peloponnesian *L. t. trilineata* with *L. t. hansschweizeri* or *L. t. major*, or splitting *L. t. trilineata* and elevating one of the lineages to a new subspecific unit would cause additional taxonomic problems, since the type locality given by Bedriaga (1886) for *trilineata* (*L. viridis trilineata* to be precise) is 'Greece', while there is no holotype or paratypes and no information in his written work to help us restrict the type locality of '*trilineata*'.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

ORCID

Panagiotis Kornilios 🆔 https://orcid.org/0000-0002-1472-9615

Evanthia Thanou 🆔 https://orcid.org/0000-0002-5008-6012

Petros Lymberakis 🆔 https://orcid.org/0000-0002-5067-9600

Çetin Ilgaz 🆔 https://orcid.org/0000-0001-7862-9106

Yusuf Kumlutaş 🆔 https://orcid.org/0000-0003-1154-6757

Adam Leaché 🆔 https://orcid.org/0000-0001-8929-6300

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