

Phylogeography and systematics of *Algyroides* (Sauria: Lacertidae) of the Balkan Peninsula

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Abstract

The area of the south-western Balkans hosts a remarkably high species richness and has been considered a biodiversity hotspot. The significance of the Balkan Peninsula as a biodiversity refugium during the Quaternary climatic fluctuations has been repeatedly pointed out in literature, yet the area remains quite understudied in terms of phylogeography. Contributing to the biogeography and phylogeography of the Balkan area, we herein present the phylogeographic relationships within the lizards of the genus *Algyroides*, focusing on the two species that occur in the Balkans (namely, *A. nigropunctatus* and *A. moreoticus*), including representatives of *Dinarolacerta* and *Lacerta* lizards as outgroups. We combined phylogenetic, phylogeographic and species distribution modelling analyses, using both mitochondrial and nuclear DNA data, in order to uncover the phylogeographic history of the genus and evaluate the validity of the extant taxonomy. Our results reveal three major clades within *Algyroides* in southern Balkans; one corresponds to *A. moreoticus*, having sister group relationships with the genus *Dinarolacerta*, and the other two to *A. nigropunctatus*, each corresponding to one of the morphological subspecies, which seem to be monophyletic, allopatric and reproductively isolated. These results expose an unprecedented phylogeographic pattern in the area of western Greece where the two major clades of *A. nigropunctatus* meet, and can contribute to a taxonomic re-evaluation of the species. Moreover, questions are raised on the validity of *Dinarolacerta* as a genus due to its clustering within the genus *Algyroides*.

KEYWORDS

biogeography, cryptic diversity, mtDNA, nuclear DNA, phylogenetics, taxonomy

1 | INTRODUCTION

The Mediterranean Basin is one of the world's top biodiversity hotspots (Blondel & Aronson, 1999; Myers et al., 2000). The three southern European peninsulas—the Iberian, the

Italian (Apennine) and the Balkan—have higher species and genetic diversity when compared to higher latitude areas of the European continent (Hewitt, 2011a). This is due to their important geographical roles as glacial refugia during the Pleistocene climatic oscillations (glacial–interglacial periods), especially for thermophilic biota which have

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exhibited localized speciation, diversification and radiations (Hewitt, 1996, 2000, 2011b; Taberlet et al., 1998).

The Balkan Peninsula is characterized by a complicated late Tertiary geomorphological and climatological history, which has magnified speciation and so it hosts the highest species richness of all three major European Mediterranean peninsulas (Hewitt, 2011a). It is not isolated from the rest of the continent by extended mountain ranges (such as the Pyrenees and the Alps in the Iberian and the Italian Peninsula, respectively), and thus, there are no major dispersal barriers to the north (Jablonski et al., 2016). In consequence, the Balkan Peninsula has been a significant centre of post-glacial dispersal of species into central and northern Europe (Griffiths et al., 2004). Despite the high levels of biodiversity, the Balkans is the less studied area among the three southern European peninsulas, in terms of phylogeography of the species (Hewitt, 2011a). Numerous recent studies have shown that in the Balkans, many terrestrial vertebrates lie in the grey zone of speciation, forming distinct lineages, hybrid zones and species complexes that often perplex species delimitation issues and raise 'species versus subspecies' taxonomic arguments (see Speybroeck et al., 2020). Examples can be found in the literature for different groups, such as mammals (e.g. Alexandri et al., 2012, 2016; Tryfonopoulos et al., 2010), amphibians (e.g. Dufresnes, Strachinis, Suriadna, et al., 2019; Dufresnes, Berroneau, 2020; Dufresnes, Probonas, 2020; Pabijan et al., 2017; Wielstra & Arntzen, 2014) and reptiles such as lizards (e.g. Gvoždík et al., 2010; Kornilios et al., 2020; Marzahn et al., 2016; Psonis et al., 2018; Sagonas et al., 2014) and snakes (e.g. Kornilios et al., 2013; Musilová et al., 2010; Ursenbacher et al., 2008). Most of this genetic richness is probably an outcome of the high geographical and climatic heterogeneity of the Balkans which resulted in the formation of 'refugia within refugia' during the Quaternary climatic fluctuations (see Abellán & Svenning, 2014; Gomez & Lunt, 2007). However, it is still quite unclear in which cases the Quaternary fluctuations have been old enough to trigger speciation events or have simply led to ephemeral diversification that later merged, or will merge, during secondary contacts (e.g. as in Balkan Spadefoot toads, see Dufresnes, Strachinis, Suriadna, et al., 2019).

Regarding reptile species, the south-western Balkans, which are demarcated by the Dinarides and External Hellenides (Pindos Mts.), constitute one of the richest areas of Europe (Sillero et al., 2014). These mountain ranges, being major biogeographical barriers and causing a filtering effect for the species' latitudinal dispersal (Psonis et al., 2018), have significantly affected a plethora of species in continental Greece and the Peloponnese Peninsula (Lymberakis & Poulakakis, 2010). At the southernmost of the Balkan Peninsula, to wit, the Peloponnese, the reptile richness reaches the highest levels, not only in the Balkans, but in whole European continent (see Sillero et al., 2014). The long

and complex geological, biogeographical and palaeoclimatological history of the Peloponnese (Creutzburg, 1963; Zachos et al., 2001) has led to the emergence of several endemic lizards with high levels of genetic diversity, even at genus level (Psonis et al., 2018; Thanou et al., 2014).

Lizards have been extensively used as models for phylogeographic and speciation studies, with Lacertidae being the most studied family of lizards in Europe (Camargo et al., 2010). This is due to the accumulated knowledge on their demographics, life history strategies and adaptive ecomorphology, and to the fact that they are easy to find and capture in the field (Camargo et al., 2010). Herein, we focus in on the two *Algyroides* lizards of the Balkans to unravel their phylogenetic relationships and to contribute with clues in the phylogeography of the south-west and southern parts of the Balkan Peninsula, where these species currently occur.

The genus *Algyroides* Bibron & Bory, 1833, is endemic to southern Europe and consists of four species with distinct geographical distributions: *Algyroides marchi* Valverde, 1958 in south-eastern Spain, *A. fitzingeri* (Wiegmann, 1834) in Corsica and Sardinia, *A. nigropunctatus* (Duméril & Bibron, 1839) in the western Balkans and *A. moreoticus* Bibron & Bory de Saint-Vincent, 1833 in the Peloponnese and some Ionian islands (Arnold, 1973, 1987). These lizards are characterized by enlarged and markedly keeled dorsal scales and usually brownish, sombre dorsal area, often with darker markings, among other diagnostic features (Arnold, 1973, 1989; Harris et al., 1999). All *Algyroides* species are mainly associated with woodland and woodland-edge habitats and they tend to prefer cool and shady places (Arnold, 1987; Harris et al., 1999). The monophyly of the genus has been disputed in several phylogenetic studies using both mitochondrial and nuclear markers (Mendes et al., 2016; Pavlicev & Mayer, 2009; Pyron et al., 2013), revealing a paraphyly of the genus *Algyroides* with regard to the genus *Dinarolacerta*.

The Dalmatian *Algyroides* (*Algyroides nigropunctatus*) ranges from north-eastern Italy (regions of Udine, Gorizia and Trieste) down to the Gulf of Corinth in Greece, following the Adriatic and Ionian coasts including some adjacent islands (Arnold & Ovenden, 2002; Podnar & Mayer, 2006; Speybroeck et al., 2016). In the north, its distribution is restricted to the east by Dinarides, and in the south restricted to the east by the Hellenides, with some exceptions (e.g. Prespes area and Fteri, IS *pers. obs.*; Andriopoulos & Pafilis, 2016; also introduced in Athens, Deimezis-Tsikoutas et al., 2020). The species occurs on the Ionian islands of Corfu, Diapontia Islets, Paxoi, Lefkada (and adjacent islets), Kephallonia and Ithaca (Chondropoulos, 1986; Stille & Stille, 2016; Valakos et al., 2008; Wilson et al., 2014; Figure 1). Two subspecies of Dalmatian *Algyroides* have been described based on coloration distinctions: the nominate form *A. n. nigropunctatus*, which covers a large majority of the species range, and *A. n. kephallithacius*

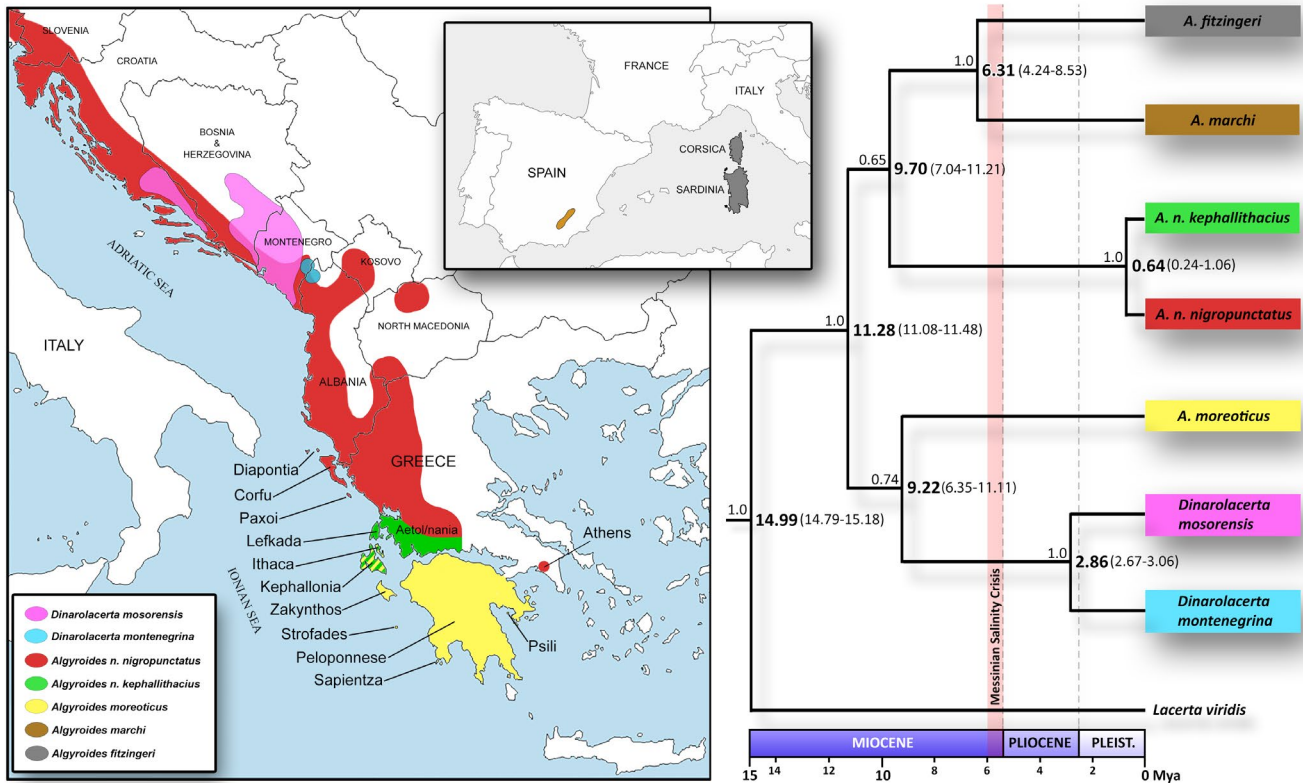


FIGURE 1 Left: Approximate range map for all *Algyroides* and *Dinarolacerta* species according to Arnold and Ovenden (2002), Andriopoulos and Pafilis (2016) and Speybroeck et al. (2016), as well as authors’ personal records. The colours represent the distinct clades revealed in all analyses. Right: Species tree including all *Algyroides* and *Dinarolacerta* species (BEAST). The estimated divergence times are shown next to the corresponding nodes in millions of years (mean heights in bold and 95% HPD intervals in brackets). Posterior probability values are shown left of each corresponding node [Colour figure can be viewed at wileyonlinelibrary.com]

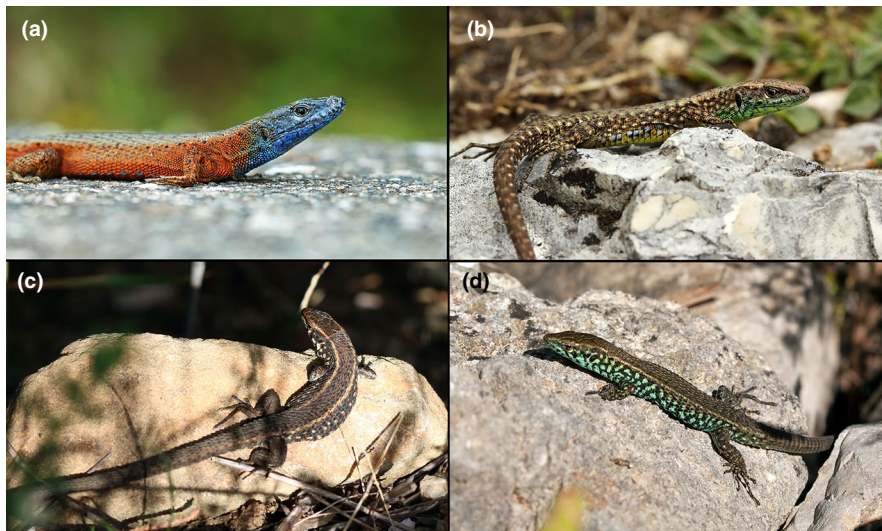


FIGURE 2 Typical male colour phenotypes of *A. n. nigropunctatus* (a), *A. n. kephallithacius* (b), *A. moreoticus* from Peloponnese (c) and *A. moreoticus* from Ithaca island, Greece (d). Photo credit: All photographs by Ilias Strachinis [Colour figure can be viewed at wileyonlinelibrary.com]

(Keymar, 1986), which was initially represented by populations from Kephallonia and Ithaca. Examining the colour morphotypes and their geographical distribution can be substantial when studying lizards’ phylogeny, given that lizards can often develop reproductive isolation as an outcome of colour assortative mating (see Pérez i de Lanuza

et al., 2012, 2013). Adult males of *A. n. kephallithacius* have green/greenish throats and yellow bellies, in contrast with the typical adult male coloration of the nominate subspecies which displays a blue throat and orange to red bellies (Figure 2). Populations from the island of Lefkada and the adjacent mainland (west Aetoloakarnania) show the

same colour morphotype as *A. n. kephallithacius* (Arnold & Ovenden, 2002; Speybroeck et al., 2016). There are also other unusual colour phenotypes that have been reported, such as totally black, melanistic individuals (e.g. Corfu [Urošević, 2014] and Slovenia [Jagar & Ostanek, 2011]), totally turquoise (Parga; Johann Mols, *pers. comm.*), and orange-throated males instead of blue-throated (prevalent phenotype on Erikoussa islet, less common on Mathraki islet, and rarely seen in north Corfu; Stille & Stille, 2016, 2017; Peter Oefinger, *pers. comm.*; Giannis Gasteratos, *pers. comm.*).

Regarding the Dalmatian *Algyroides*' intraspecific genetic diversity, the analysis of two mtDNA gene fragments (*12S* and *16S rRNA*) from only eight specimens revealed three distinct mitochondrial lineages (Podnar & Mayer, 2006): the first from Adriatic coasts and Pindos Mt., the second from Corfu and Parga, and the third from Lefkada and Kephallonia. That genetic work supports the morphological taxonomic validity of the subspecies *A. n. kephallithacius* as a separate clade and confirms its presence also on Lefkada Island. Moreover, it indicates possible cryptic diversity within the species in its southern range; however, the authors pointed out the inadequacy of their limited data set to obtain clarity of the species' phylogeography (Podnar & Mayer, 2006).

The Greek *Algyroides* (*Algyroides moreoticus*) is a Greek endemic found only in the Peloponnese and adjacent islets Sapientza and Psili, as well as the south Ionian islands of Kephallonia, Ithaca, Zakynthos and Strofades (Chondropoulos, 1986; Speybroeck et al., 2016; Valakos & Mylonas, 1992; Figure 1). The species is monotypic in that no subspecies have been described; however, insular populations seem to show a different phenotype where males have brighter and more intense colours ventrally and laterally, with greenish or even turquoise bellies, throats and flanks, while females appear darker than those from Peloponnese (IS *personal observation*; Figure 2). The species occurs in sympatry with *A. nigropunctatus* on Kephallonia and Ithaca without showing signs of competition, sharing even the same micro-habitats (IS *personal observation*; see Strachinis & Artavanis, 2017). Until now, no studies have been made concerning Greek *Algyroides*' genetic diversity.

In this study, we conducted several phylogenetic, phylogeographic and species distribution modelling analyses using both mitochondrial (mtDNA) and nuclear (nuDNA) data on a large sample set, in order to further assess genetic relationships within the genus *Algyroides*, focusing on the two *Algyroides* species of the Balkans, *A. nigropunctatus* and *A. moreoticus*. Our main objectives were (a) to infer intra- and inter-specific phylogenetic relationships, (b) evaluate the validity of the current taxonomy and (c) provide some clarity to their phylogeographic history.

2 | MATERIALS AND METHODS

2.1 | Sample collection

In total, 91 tissue samples of *A. nigropunctatus* (70) and *A. moreoticus* (21) from throughout both species' range were used in the phylogenetic analyses (Table S1). Moreover, 10 sequences of the same gene fragments from several species of Lacertinae were retrieved from GenBank and used as outgroups in the analyses (*Lacerta viridis*, *Dinarolacerta mosoensis*, *Dinarolacerta montenegrina*, *Algyroides marchi* and *Algyroides fitzingeri*).

2.2 | Laboratory procedures

Total genomic DNA was isolated using a standard ammonium acetate extraction protocol (Bruford et al., 1998) from muscle, liver or blood of specimens preserved in absolute ethanol. The sampling localities are shown in Figure 3, and the specimens are listed in Table S1. Double-stranded PCR was used to amplify partial sequences of two mitochondrial genes (mtDNA) encoding the cytochrome oxidase subunit I (COI) and the cytochrome b (*Cytb*), as well as two nuclear genes (nuDNA) encoding the melanocortin receptor 1 (MC1R) and the natural killer-tumour recognition (NKTR). Primers and conditions used in PCR amplifications and in cycle sequencing reactions are given in Table S2. Single-stranded sequencing of the PCR product was performed using the Big-Dye Terminator (v3.1) Cycle Sequencing kit[®] on an ABI3730 automated sequencer following the manufacturer's protocol and using the same primers as in the PCR.

2.3 | Data analysis

Sequences were viewed and edited using CodonCode Aligner v. 3.7.1 (CodonCode Corporation[®]). The authenticity of the sequences and the homology to the targeted genes were evaluated with a BLAST search in the NCBI genetic database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). All newly determined sequences have been deposited in GenBank (Table S1). The alignment of sequences was performed separately for each amplified fragment using the algorithm ClustalW as it is implemented in MEGA v.6 (Tamura et al., 2013). All gene sequences (*Cytb*, COI, MC1R, and NKTR) were translated prior to further analysis in order to check for the existence of stop codons. Sequence divergence (*p*-distances) was estimated using MEGA.

In order to investigate the phylogenetic relationships between the two focal species (i.e. *A. nigropunctatus* and *A. moreoticus*) and their affinity with the outgroup taxa, three data sets were initially constructed:

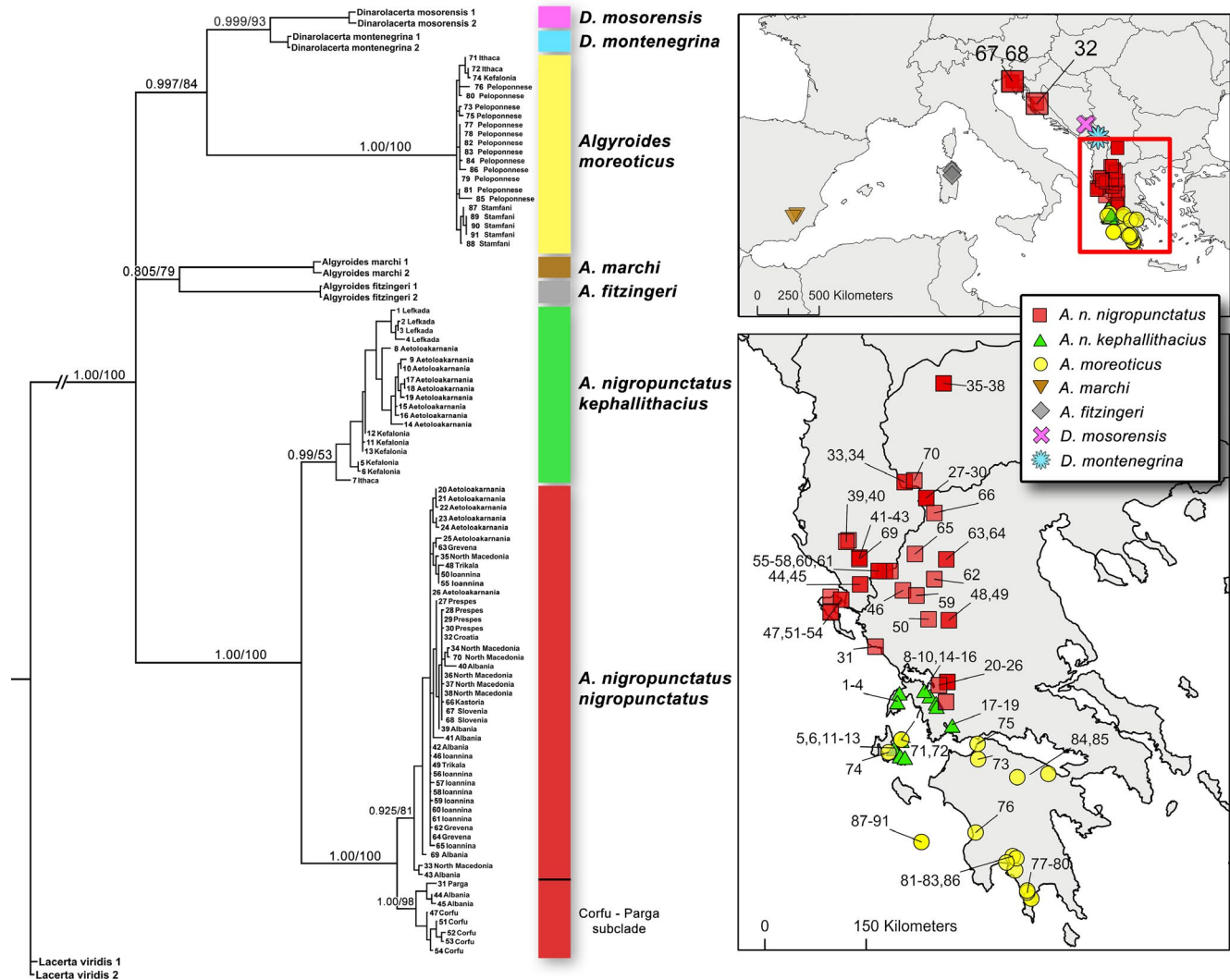


FIGURE 3 Left: Bayesian inference concatenated gene tree (*Cytb*, *COI*, *NKTR* and *MC1R* fragments). Posterior probabilities (PP) and bootstrap values (BS) resulted from the Bayesian inference and maximum likelihood analyses, respectively, are given above the branches (PP/BS). Right: Sample localities on the map. The numbers represent the samples' code numbers as used in our analyses (Table S1) [Colour figure can be viewed at wileyonlinelibrary.com]

1. a mitochondrial DNA data set that includes the two mtDNA gene fragments (*Cytb* and *COI*)
2. a nuclear DNA data set with *NKTR* and *MC1R*
3. a concatenated DNA data set with all 4 loci

The mtDNA alignment was partitioned into six blocks, including three blocks for the 1st, 2nd and 3rd codon positions for the protein-coding locus *Cytb* and three blocks for the 1st, 2nd and 3rd codon positions for the protein-coding locus *COI*. Similarly, the nuDNA alignment was also partitioned into six blocks for the 1st, 2nd and 3rd codon positions for each one of the two nuclear gene fragments (*MC1R*, *NKTR*). These initial partition schemes were loaded in PartitionFinder (PF) v.2.1 (Guindon et al., 2010; Lanfear et al., 2012, 2016) in order to calculate and select the best-fit partitioning scheme and models of molecular evolution for each downstream analysis according to the models that can

be implemented in each programme, based on the Bayesian information criterion (BIC), the greedy algorithm and considering that the blocks of each alignment have linked branch lengths. The models that include both gamma distribution and invariable sites were ignored (Yang, 2006). PHASE v.2.1.1 (Stephens et al., 2001) was used for nuclear gene fragments as it is implemented in DnaSP v.5.10.01 (Librado & Rozas, 2009) prior to alignment, in order to statistically infer the allelic sequences.

2.4 | Phylogenetic analyses

Phylogenetic reconstruction was conducted using two different approaches, maximum likelihood (ML) and Bayesian inference (BI). The same parameters were used when analysing all three of the data sets. Maximum likelihood analyses were performed

using RAxML v.8.1.21 (Stamatakis, 2014) as implemented through raxmlGUI v.1.5 (Silvestro & Michalak, 2011). The best ML tree for each data set was selected from 50 iterations, and the confidence of the branches was further assessed based on 1,000 thorough bootstrap replicates.

Bayesian inference analyses were conducted in MrBayes v.3.2.6 (Ronquist et al., 2012), with four runs and eight chains for each run. Each chain ran for 2×10^7 generations sampling every 10^3 generations. Several MCMC convergence diagnostics were used to check for convergence and stationarity (the plot of the generation versus the log probability of the data [the log likelihood values], the average standard deviation of split frequencies, the average Potential Scale Reduction Factor [PSRF], and the minimum value of minimum Estimated Sample Sizes [ESS]). The -lnL was stabilized after approximately 6×10^6 generations for the mitochondrial and the combined analysis and after 2×10^6 generations for the analysis of the nuclear data set. The first 35% (7×10^6) trees were discarded as burn-in, as a measure to sample from the stationary distribution and avoid the possibility of including random, sub-optimal trees. A majority rule consensus tree was then produced from the posterior distribution of trees, and the posterior probabilities were calculated as the percentage of samples recovering any particular clade. Posterior probabilities ≥ 0.95 indicate statistically significant support (Huelsenbeck & Ronquist, 2001).

Finally, the relationships among the DNA sequences were explored through a TCS (Clement et al., 2002) built in PopArt (Population Analysis with Reticulate Trees) (<http://popart.otago.ac.nz>).

2.5 | Divergence time estimation and demographic history

Species tree and divergence times of *Algyroides* in southern Balkans were estimated using the data set containing both mtDNA and nuDNA sequences using StarBEAST2 package as implemented in BEAST2 v.2.4.7 (Bouckaert et al., 2014). The input files (xml format) were created using BEAUti v.2.4.7, also implemented in BEAST2. The nucleotide substitution models were specified a priori according to the results of the PF analysis. As for other priors, the birth–death model was selected for speciation, the constant population model for population model and the uncorrelated lognormal or strict clock model for describing the molecular clock. We compared the marginal likelihood of the models in Tracer based on the Akaike's information criterion through a Markov chain Monte Carlo simulation (AICM) (S.E. estimated from 100 bootstrap replicates [Baele et al., 2012]). Under the AICM, an increase in the number of parameters penalizes more complex models, and models with lower AICM values are preferred over models with higher values (Leache et al., 2014).

Regarding the divergence time estimation, three calibration points retrieved from the recent study on Lacertini radiation were used (Mendes et al., 2016). In particular, the split of *Lacerta* from *Algyroides/Dinarolacerta* is estimated at ~15 Mya, the split of *Algyroides* from *Dinarolacerta* at ~11.3 Mya, and the split of *Dinarolacerta montenegrina* from *Dinarolacerta mosorensis* at 2.9 Mya. The MCMC analysis was run for 4×10^8 generations, saving the result every 5×10^3 generations. The first 25% of the saved trees were discarded after the inspection of the log files with Tracer v.1.6 (Rambaut et al., 2014). The maximum clade credibility (MCC) tree that best represented the posterior distribution was identified using TreeAnnotator v.2.4.7 (also included in BEAST2), which subsequently annotated this selected topology with the mean ages of all the nodes.

In order to investigate the demographic history of the two focal species (i.e. *A. nigropunctatus* and *A. moreoticus*), we performed an extended Bayesian skyline plot (EBSP) analysis (Heled & Drummond, 2008), as implemented in BEAST, which uses multi-locus data to increase accuracy of inferences. In particular, EBSPs were performed for each revealed major clade (one clade for *A. moreoticus* and two clades for *A. nigropunctatus*, see results section) on the combined data set of mtDNA and nuDNA. All operator parameters were set following that suggested in the EBSP manual. Analyses were run for 100 million generations, sampled every 10,000. A 10% was discarded as burn-in. Stationarity was assessed by analysing the effective sample sizes of all parameters in Tracer. The plots were made in RStudio 1.2.1335 (RStudio Team, 2018) using plotEBSP.r script available at <https://github.com/CompEvol/beast2/tree/master/doc/tutorials/EBSP/scripts>.

Moreover, three types of neutrality tests were performed: (a) R_2 statistics (Ramos-Onsins & Rozas, 2002), (b) Fu's F_s (Fu, 1997) and (c) Tajima's D (Tajima, 1989), with 10,000 coalescent simulations to calculate the significance using DnSp 6.12.03 (Rozas et al., 2017). Significantly large negative values of Tajima's D and Fu's F_s and significantly positive R_2 values can be interpreted as evidence of population expansions, and negative values of Fu's F_s indicate an excess of recent mutations and reject population stasis.

2.6 | Species distribution modelling (SDM) and past time projections

Presence-only data sets for the two *Algyroides* species were analysed. The presence points used in the analyses were extracted from the collections of Natural History Museum of Crete and Zoological Research Museum Alexander Koenig, iNaturalist (2020) public database, authors' and authors' colleagues' records. We obtained 515 occurrence records for *A. nigropunctatus* and 164 points for *A. moreoticus*. The area of the modelling was based on the current species distribution (Figure 1).

TABLE 1 Mean genetic *p*-distances (%) among the major clades, revealed by the phylogenetic analyses, for each amplified fragment (*Cytb*, COI, MC1R and NKTR)

	1	2	3	4	5	6	7	8
1. <i>A. moreoticus</i>	0.310.710.110.07							
2. <i>A. n. nigropunctatus</i>	19.7 15.9 1.2 4.3	1.410.210.510.3						
3. <i>A. n. kephallithacius</i>	19.9 16.1 11.0 4.3	3.2 10.6 0.7 0.3	1.511.210.610.05					
4. <i>A. marchi</i>	22.2 N/A 1.4 N/A	16.2 N/A 1.6 N/A	16.2 N/A 1.6 N/A	1.01N/A 0.01N/A				
5. <i>A. fitzingeri</i>	18.4 N/A 1.4 N/A	15.1 N/A 1.6 N/A	15.4 N/A 1.7 N/A	15.5 N/A 1.0 N/A	0.01N/A 0.01N/A			
6. <i>D. mosorensis</i>	19.4 N/A 0.9 N/A	18.1 N/A 1.4 N/A	18.6 N/A 1.3 N/A	15.3 N/A 1.4 N/A	17.0 N/A 1.4 N/A	0.41N/A 0.21N/A		
7. <i>D. montenegrina</i>	15.6 N/A 0.8 N/A	15.5 N/A 1.3 N/A	15.8 N/A 1.2 N/A	13.7 N/A 1.0 N/A	14.9 N/A 1.3 N/A	6.2 N/A 0.3 N/A	0.01N/A 0.01N/A	
8. <i>L. viridis</i>	17.4 17.9 N/A 4.9	16.2 18.1 N/A 3.7	16.1 16.3 N/A 3.7	16.4 N/A N/A N/A	15.6 N/A N/A N/A	16.3 N/A N/A N/A	16.4 N/A N/A N/A	1.010.01N/A 0.4

Note: The intra-clade divergences are presented in diagonal (bold), while values for each gene are separated by vertical line. Genetic distances that are not available are marked as 'N/A'.

Environmental data at 5×5 km spatial resolution from PALEOCLIM and CHELSA database were used (Bobrowski & Schickhoff, 2017; Karger et al., 2017). The spThin R package (Aiello-Lammens et al., 2015) with an occurrence thinner radius of 5km was used for the minimization of the effects of sampling bias and match with the environmental data (Boria et al., 2014). The USDM R package (Naimi et al., 2014) was used for the calculation of variance inflation factor (VIF) for the set of selected predictors in order to exclude the highly correlated variables from the set through a stepwise procedure (VIF values < 10).

The Wallace R package (Kass et al., 2018) was used for the modelling, allowing the fine tune (Hao et al., 2020) of the MaxEnt algorithm using the ENMeval R package (Muscarella et al., 2014). The 'block' partition scheme of ENMeval was selected for all the analyses (Muscarella et al., 2014) for training/validation since it is more suitable for modelling when projections in past/future climate conditions are among the aims of the analysis. ENMeval allowed us to evaluate models using a spatial partitioning scheme and to 'fine-tune' two parameters of MaxEnt that affect model complexity and predictive power. These parameters are the regularization multiplier (RM) or beta values and the feature classes (FCs). The RM penalizes overly complex models, whereas the FCs are functions of the raw environmental data (Morales et al., 2017). All FCs (L = Linear, Q = Quadratic, H = Hinge, P = Product) were selected and the RM was set between 1 and 5 with steps of 0.5 allowing for model complexity and model tuning for each clade. Basically, all predictor variable coefficients were shrunk progressively until some reach 0, when they drop out of the model. Only those variables with the greatest predictive contribution were retained in the model. Model selection was based on the average test area under the curve value (avg. test.AUC) along with the lowest delta corrected Akaike information criterion (delta.AICc) (Leroy et al., 2018), calculated for each model following the method by Warren and Seifert (2011). In total, 45 different models were built, run and tested for species.

3 | RESULTS

Regarding the mtDNA data set, a total of 1,144 base pairs (bp) were obtained (*Cytb* 430 bp and COI 614 bp). Out of the 430 sites comprising the *Cytb* sequences, 165 were variable when examining the complete data set, while this number dropped to 118 when the outgroups were excluded (*Lacerta*, *Dinarolacerta* and the two *Algyroides* species not distributed in the Balkans). For the COI sequences, the corresponding numbers were 186 and 152 sites, respectively. A total of 1,228 bp were obtained from the nuDNA markers (MC1R 628 bp and NKTR 597 bp). Out of the 628 sites comprising the MC1R sequences, 32 were variable when examining the complete data

set and 23 when excluding the outgroup. The corresponding numbers for NKTR were 49 and 37 sites, respectively.

Pairwise genetic distances (p -distances) for the mtDNA loci ranged from 0% to 24.3% for *Cytb* and from 0% to 21.4% for *COI*. For the nuDNA loci, sequence divergence varied from 0% to 2.1% in *MC1R* and from 0% to 8.7% in *NKTR*. The mean genetic distances among the major clades (as revealed by the phylogenetic analyses, see below), for each individual locus, are given in Table 1. The best-fit partitioning schemes for each downstream analysis as well as the selected nucleotide substitution models are presented in Table 2. Maximum Likelihood and Bayesian inference analyses produced fairly similar topologies for all three examined data sets regarding the major lineages (Figure 3; also Figures S1 and S2). Considering the mtDNA data set, the ML and BI analyses resulted in topologies with $\ln L = -3991.12$ and -4059.79 , respectively. For the nuDNA data set, the corresponding numbers were $\ln L = -2361.71$ and $\ln L = -2432.81$, while for the combined data set, the likelihood values were as follows: $\ln L = -6763.40$ for the ML analysis and $\ln L = -6740.72$ for the BI analysis.

In all phylogenetic analyses, all *Algyroides* and *Dinarolacerta* species consist of monophyletic groups, in which three subgroups with unresolved phylogenetic relationships were recognized: the first corresponds to *A. nigropunctatus*, the second to *Algyroides* species from Spain (*A. marchi*) and Corsica/Sardinia (*A. fitzingeri*), and the third to *A. moreoticus* and the two species of *Dinarolacerta* (*D. mosorensis* and *D. montenegrina*). For the focal species, *A. moreoticus* and *A. nigropunctatus*, three major clades were recognized: the first corresponds to *A. moreoticus*, which appeared to have sister group relationships with *Dinarolacerta* lineages, and the other two to *A. nigropunctatus* with mean p -distances between them at 6.9% and 0.5%, for mitochondrial and nuclear concatenated fragments, respectively.

Moreover, a subclade with shallow divergence from Corfu and adjacent mainland branched within the clade of *A. nigropunctatus* (Figure 3). The TCS haplotype network clearly separates all the above (sub)clades for the focal *Algyroides* species in the Balkans (Figure S4).

In the chronophylogenetic analyses on the combined data set, the model comparison based on AICM favoured the relaxed molecular clock model versus the strict clock model. According to the results of the chronophylogenetic analysis ($\ln L = -6856.90$), the divergence of the *A. nigropunctatus* from *A. moreoticus* was estimated to about 11.28 Mya (early late Miocene), whereas the divergence of the two *A. nigropunctatus* clades was estimated at around 0.64 Mya in the middle Pleistocene (Figure 1).

The Extended Bayesian skyline plots (EBSPs) rejected a constant size model for *A. moreoticus* but not for the two clades of *A. nigropunctatus*, because the 95% highest posterior density (HPD) excluded the value 0. In the case of *A. moreoticus*, the EBSP showed a very recent and rapid population growth (Figure S4).

Summary statistics of DNA polymorphism, Fu's F_s , Tajima's D , and Ramos-Onsins & Rozas's R_2 for *A. moreoticus* and the two clades of *A. nigropunctatus* are given in Table 3. Among them, only *A. moreoticus* showed negative significant value for Fu's F_s , indicating a population expansion ($p < .01$). However, the values of Tajima's D and R_2 , though negative and a small positive, respectively, are not significant.

3.1 | Species distribution modelling (SDM) and past time projections

From the initial 515 and 164 occurrence records for *A. nigropunctatus* and *A. moreoticus*, respectively, we ended up

TABLE 2 Partitioning schemes and best-fit models of sequence evolution selected in PartitionFinder (PF) for downstream analyses

RAxML (ML)		BEAST		MrBayes (BI)	
Partition subset	Best model	Partition subset	Best model	Partition subset	Best model
cytbpos2, COI pos3	GTR + I	cytb pos2, COI pos3	HKY + I	cytb pos2, COI pos3	HKY + I
cytb pos3, COI pos1	GTR + G	cytb pos3, COI pos1	TRN + G	cytb pos3, COI pos1	GTR + G
cytbpos1, COI pos2	GTR + G	cytb pos1 COI pos2	TRNEF + G	cytb pos1 COI pos2	K80 + G
mc1r pos3	GTR + I	mc1r pos3	HKY + I	mc1r pos3	HKY + I
mc1r pos2	GTR	mc1r pos2	HKY	mc1r pos2	F81
Nktr pos1, Nktr pos2, Nktr pos3, mc1r pos1	GTR + I	Nktr pos1 Nktr pos2, Nktr pos3 mc1r pos1	TRN + I	Nktr pos1 Nktr pos2, Nktr pos3 mc1r pos1	GTR + I

TABLE 3 Summary statistics and population expansion test for the three focal clades in Greece (the clade of *A. moreoticus* and the two clades of *A. nigropunctatus*) based on the concatenated data set

Clade	<i>n</i>	<i>nh</i>	<i>h</i>	π (%)	<i>k</i>	Tajima's D	Fu's F_s	R^2
<i>A. moreoticus</i>	21	14	0.938	0.004	3.662	-1.2820	-6.592**	0.095
<i>A. n. nigropunctatus</i>	51	20	0.885	0.008	7.757	-0.1916	-1.889	0.101
<i>A. n. kephallithacius</i>	19	18	0.994	0.008	15.216	-0.2056	-5.811*	0.118

Note: Numbers of individuals (*n*), number of haplotypes (*nh*), haplotype diversity (*h*), nucleotide diversity (π), mean number of pairwise nucleotide differences (*k*).

* $p < .05$.

** $p < .01$.

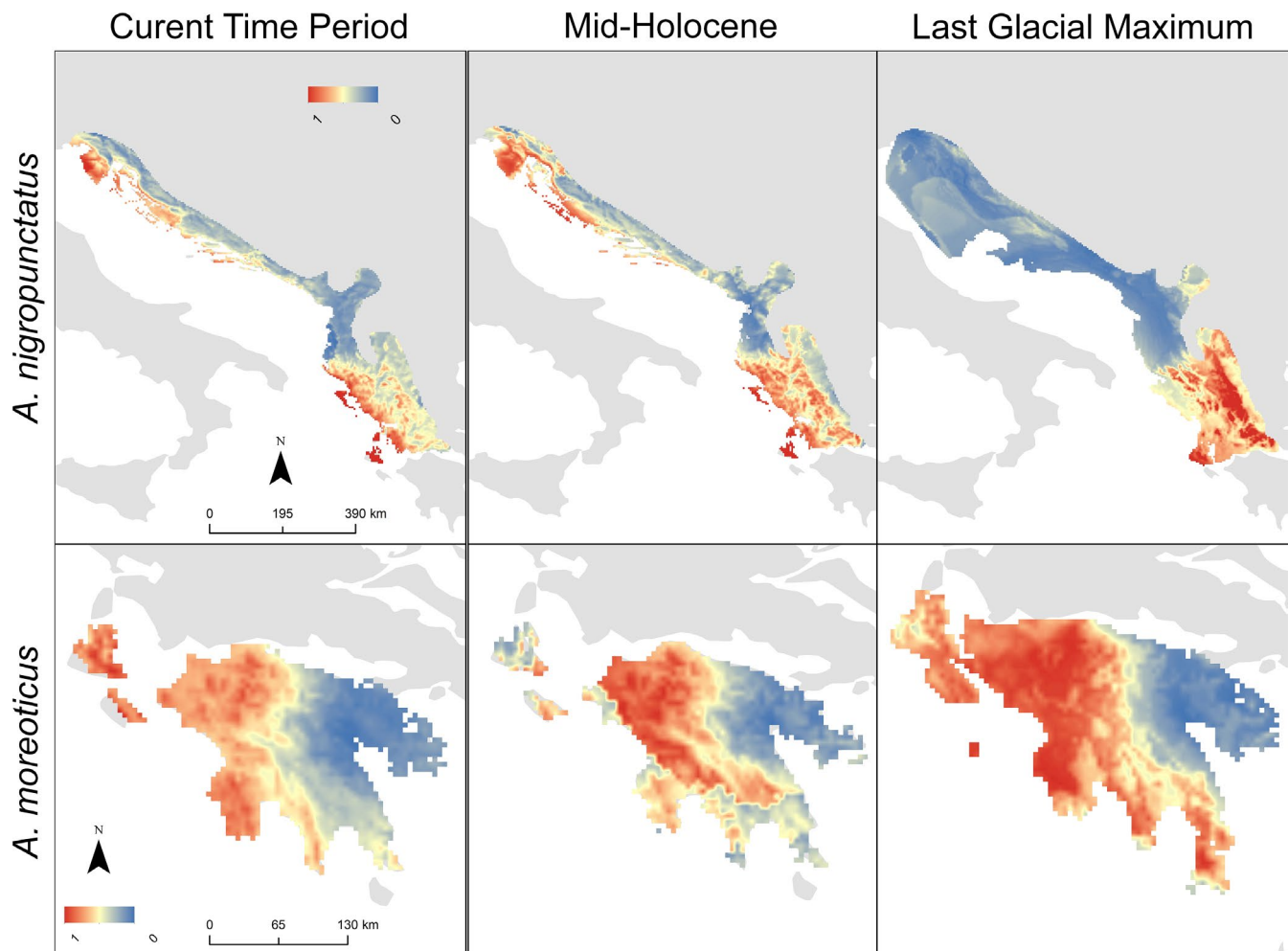


FIGURE 4 Species distribution modelling (SDM) using the MaxEnt model for the focal species (top: *Algyroides nigropunctatus*; bottom: *A. moreoticus*). The SDM projections correspond to current time period (left), mid-Holocene (middle) and last glacial maximum (right) [Colour figure can be viewed at wileyonlinelibrary.com]

with 173 and 78 occurrence points for *A. nigropunctatus* and *A. moreoticus*, respectively. Due to the different distribution range of those species, the VIF analysis was performed separately for each species. Seven and five out of the initial 19 predictors were finally selected for *A. nigropunctatus* and *A. moreoticus*, respectively (Table S3).

For each species, 45 models have been run consecutively; the model with the lowest delta.AICc for *A. nigropunctatus* was the one that uses all FCs along with a regularization

multiplier of 3.5 (LQHP_3.5). AUC was 0.72 for train.AUC and 0.69 for avg.test.AUC. The model for *A. moreoticus* was the one that uses L, Q and H feature class along with a regularization multiplier of 4 (LQH_4). AUC was 0.69 for train.AUC and 0.59 for avg.test.AUC. Most important variables were isothermality (bio3), temperature of wettest quarter (bio8) and the seasonality of precipitation (bio15) for *A. nigropunctatus* and the precipitation of coldest and warmest Quarter (bio19) for *A. moreoticus* (Table S3).

For *A. nigropunctatus*, the projections in the current and mid-Holocene climate periods showed a steady overall spatial coverage (where the species might occur), but with a large reduction of its distribution in the last glacial maximum (LGM) and a slow spread in the current period (Figure 4). For *A. moreoticus*, all three projections show a steady overall spatial coverage (where the species might occur) but with a minor reduction of its distribution in the mid-Holocene and a slow expansion in the current period (Figure 4).

4 | DISCUSSION

Herein we present the first robust analyses using comprehensive sampling to assess the phylogenetic relationships within *A. nigropunctatus* and *A. moreoticus*, using both mtDNA and nuDNA markers. Our analyses provide very interesting results, revealing the intraspecific genetic diversity of both *Algyroides* species of the Balkans. Once more, the importance of the southern Balkans as a glacial refugium for thermophilic fauna is highlighted through our findings which indicate the presence of sub-refugia in western Greece and Peloponnese. Those glacial sub-refugia became centres of diversification and starting points of postglacial range expansions, contributing to the formation of the extant distribution of lineages. Both familiar and novel phylogeographic patterns revealed within the two *Algyroides* species in the wider area of south-western Greece, which might also provide clues on the geological history of the region. Our results indicate a complex evolutionary history within the genus *Algyroides* and could contribute to taxonomic revisions, regarding the *A. nigropunctatus*' (sub)specific status and the monophyly of the genus.

4.1 | Phylogenetic relationships within *A. nigropunctatus*

Our phylogenetic results, in both gene trees and species tree analyses, revealed the presence of two major clades within *A. nigropunctatus* (6.9% mean mtDNA *p*-distance), with high posterior probabilities and bootstrap values. Those two clades are depicted with green and red colour in Figure 3. The green clade is geographically restricted in west Aetoloakarnania and some of the south Ionian islands (Lefkada, Kephallonia and Ithaca), whereas the red clade represents the rest of the species' range (Figure 1). Moreover, two subclades were clearly recognized within the red clade, showing shallow divergence during the late Pleistocene (Figure 3; 4.4% mean mtDNA *p*-distance): one from the island of Corfu and the adjacent Greek and Albanian mainland, and one from the rest of the range (these two subclades present no apparent colour

or other morphological differences). These three groups (the green clade and the two red subclades) seem to correspond to the mitochondrial clades of 'Southern Ionian', 'Northern Ionian' and 'Adria-Pindos', respectively, revealed by Podnar and Mayer (2006).

From a morphological point of view, the Dalmatian *Algyroides* presents two main location-specific colour phenotypes, described as two different subspecies, *A. n. nigropunctatus* and *A. n. kephallithacius*. The *kephallithacius* morphotype (Figure 2b) is localized on the islands of Lefkada, Kephallonia, Ithaca, the adjacent islets and the adjacent mainland in west Aetoloakarnania, whereas the typical morphotype of the nominate subspecies (Figure 2a) occupies the rest of the species' range. The two subspecies meet at Agrinio valley, with *A. n. kephallithacius* being restricted in the west part of Aetoloakarnania, occurring on both sides of Acheloos River (which is the only major geographical barrier in the area), and demarcated by Amvrakikos Bay, Petalas Mountain and Trichonida Lake (Figure 5). The two major clades (red and green) of the produced phylogenetic tree fully correspond to the two extant *A. nigropunctatus* subspecies defined by colour phenotypes. Hence, it seems that these two genetically and phenotypically different lineages correspond to two distinct, allopatric and monophyletic groups, showing no signs of introgression in spite of the absence of a significant geographical barrier between them.

The above pattern could be a case of the competitive exclusion principle (or Gause's principle; Hardin, 1960), where 'complete competitors cannot coexist', and could indicate a reproductive isolation between the two subspecies, despite their recent divergence (0.64 Mya) and close phylogenetic relationship. It has been shown that reproductive isolation in closely related *taxa* can be an outcome of intrinsic isolation mechanisms, such as colour assortative mating (Kindler et al., 2017; Pérez i de Lanuza et al., 2012, 2013 and references therein), as well as differentiated non-visual reproductive signals (e.g. pheromones and scents; see Bickford et al., 2006; Carazo & Font, 2011; Cooper & Pérez-Mellado, 2002; López & Martín, 2005). The aforementioned data might suggest the presence of two different species within *A. nigropunctatus*. However, more genetic/genomic data and other species delimitation analyses, which demand plethora of different loci (i.e. BPP), as well as comprehensive morphological approaches, are required to confirm this.

The estimated time of the divergence of the two major clades of *A. nigropunctatus* is quite recent at 0.64 Mya (Figure 1), a fact that could explain the bush like tree produced by our nuclear data (Figure S1). Unresolved phylogenetic topologies with polytomies between recently diverged lineages can be due to incomplete lineage sorting and low evolutionary rate of nuclear loci (see Funk & Omland, 2003; Hudson & Turelli, 2003).

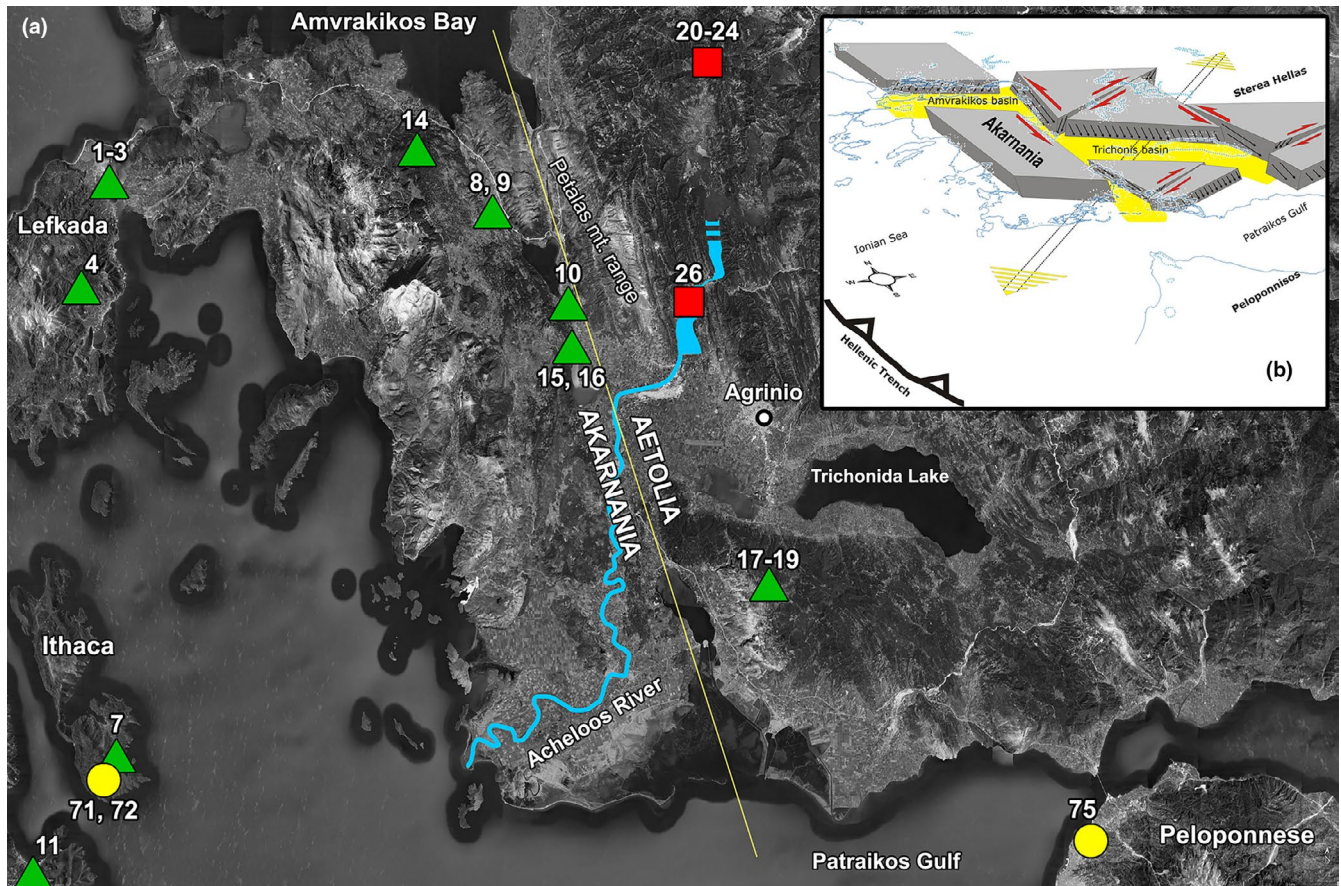


FIGURE 5 (a) Close-up of the region of Aetoloakarnania prefecture. Sample locations are depicted with red squares for *A. n. nigropunctatus*, green triangles for *A. n. kephallithacius* and yellow circles for *A. moreoticus*, as in Figures 1 and 3. The yellow line shows the approximate division between Aetolia and Akarnania, and Acheloos River is sketched with light blue colour. (b) Three-dimensional simplified block diagram in which the relative movement of the major fault blocks in the area of Aetoloakarnania is shown. The yellow areas represent active postalpine basins. Horizontal component of slip is shown with red arrows, and the yellow arrows show the kinematic indicators. Redraw after Vassilakis et al. (2006) [Colour figure can be viewed at wileyonlinelibrary.com]

4.2 | Phylogeographic patterns of *A. nigropunctatus*

Based on a higher mtDNA diversity in the southern part of *A. nigropunctatus*' range, Podnar and Mayer (2006) hypothesized a postglacial northwards dispersal of the species from a southern glacial refugium. Despite the low number of samples used in their work, their suggestion does hold true, given our results which show a relatively high genetic variation in the south, opposed to lower genetic diversity in the northern populations of the species' range, following the widely known biogeographical pattern of 'southern richness - northern purity' (Hewitt, 2000). This pattern has also been observed in other amphibian and reptile species that occur in the area of western Greece and south-western Balkans, for example *Vipera ammodytes* (Ursenbacher et al., 2008), *Elaphe quatuorlineata* (Kornilios et al., 2013), *Anguis graeca* (Jablonski et al., 2016), *Ablepharus kitaibelii* (Skourtanioti et al., 2016), *Lacerta viridis* (Marzahn et al., 2016), *Podarcis (tauricus) ionicus* (Psonis et al., 2018) and *Pelobates balcanicus*

(Dufresnes, Strachinis, Suriadna, et al., 2019, Dufresnes, Strachinis, Tzoras2019).

Our study is yet another indicator of the important role the Balkans play as a glacial refugium, and furthermore, a region of refugia within refugia, where populations were restricted during unfavourable conditions during the Pleistocene, diversified even more, and then expanded in the interglacial periods. Refugial areas, during the Pleistocene glacial periods, have been crucial for the maintenance and distribution of biodiversity, especially for species that shift their range north (Barbosa et al., 2017). Western Greece's role as a glacial refugium for *A. nigropunctatus* is clearly shown in the last glacial maximum (LGM) projection of our SDM analysis. The species' most favourable areas during the LGM are projected mainly alongside Pindos mountains (on the west) and at the south-westernmost part of its range (Figure 4). Our SDM analysis also indicates the species' expansion to the north after the LGM, during the Holocene and up to present time, as the species' genetic diversity pattern points out as well.

The divergence between *A. n. nigropunctatus* and *A. n. kephallithacius* dates back to upper middle Pleistocene, probably caused by the geohistorical events of that period and the geomorphological features of the area of west Aetoloakarnania. Akarnania is the western part of the Aetoloakarnania prefecture, bordered by the Ionian Sea, Amvrakikos Bay and Acheloos River (Figure 5). Akarnania consists of a separate, independently moving fault block, surrounded by postalpine sediments mostly of Pleistocene age, and it belongs to a group of neotectonic structures in the area of Aetoloakarnania caused by extensional deformation within the upper plate of the Hellenic arc (Figure 5b; Vassilakis et al., 2006). Given the tectonic activity in the wider area (Vassilakis et al., 2006, 2011), in conjunction with the Pleistocene sea-level fluctuations (Rohling et al., 2014), there is a chance that the appropriate conditions and barriers were created approximately 0.64 Mya, between Aetolia and Akarnania, to segregate and isolate a part of the species' ancestral population. It is probable that the two lineages were already significantly differentiated, both genetically and morphologically, when a secondary contact occurred.

The dispersal of *kephallithacius* clade to the islands of Kephallonia and Ithaca, as well as that of *A. n. nigropunctatus* to the island of Corfu and adjacent islets, has taken place later in Pleistocene, possibly during glacial periods. The island of Corfu repeatedly connected and disconnected with the mainland through land bridges during the glacial and postglacial periods (Creutzburg, 1963; Gkioni et al., 2013; Perissoratis & Conispoliatis, 2003), allowing animals to actively disperse. Lefkada was connected to Akarnania most of the time during the Pleistocene (see Creutzburg, 1963; Dermitzakis, 1990; Dermitzakis & Papanikolaou, 1981), also allowing active dispersal, and it could have possibly played a significant role as a glacial refuge in the south-western edge of the species' range. In contrast, Kephallonia and Ithaca are separated from the mainland by isobaths deeper than 200 m and they were most probably never connected to the mainland during the whole Pleistocene (see Dermitzakis & Papanikolaou, 1981; Perissoratis & Conispoliatis, 2003; Poulakakis et al., 2005 and references therein; Ferentinos et al., 2012; Gkioni et al., 2013; Rohling et al., 2014). The clade of *A. n. kephallithacius* probably dispersed to those two islands through rafting. Transmarine dispersal of reptiles through rafting, favoured by raging rivers, sea currents and winds, has been documented for several cases (Hawllitschek et al., 2017; Lymberakis & Poulakakis, 2010). During the last glacial maximum (~21,500 ya), when the sea level was about 120 m lower than present, Kephallonia, Ithaca and Zakynthos were connected as one large island and separated from the mainland by narrow straits (Ferentinos et al., 2012). Thus, the absence of the species from Zakynthos could indicate an even more recent dispersal to Kephallonia and

Ithaca than LGM period, or a later extinction of the species on Zakynthos.

As resulted herein, the phylogeography of Dalmatian *Algyroides* presents some familiar features when compared to other phylogenetic studies of reptile species of the wider area. In particular, as in the case of 'Corfu - Parga subclade' (see Figure 3), differentiated haplotypes and lineages of shallow divergence from Corfu and adjacent mainland have also been unveiled within the lizards *A. graeca* (Jablonski et al., 2016), *L. viridis* (Marzahn et al., 2016) and *P. ionicus* (Psonis et al., 2018), as well as within the snakes *E. quatuorlineata* (Kornilios et al., 2013) and *V. ammodytes* (Ursenbacher et al., 2008). On the contrary, to date, we are not aware of any other case that resembles the characteristic phylogeographic pattern formed by the two major clades of *A. nigropunctatus* (green and red) in the area of Aetoloakarnania (Figure 1). In that sense, this pattern seems unique at the moment, although the wider area is largely understudied and not adequately sampled in most relative studies.

4.3 | Phylogenetic relationships of *A. moreoticus*

The effect of the Pleistocene climatic fluctuations on the differentiation of other Balkan herpetofaunal species (e.g. *Triturus* [Wielstra et al., 2013], *Vipera ursinii* [Zinenko et al., 2015], *Podarcis muralis*, *Dalmatolacerta oxycephala* and *D. mosorensis* [Podnar et al., 2014]) has led to the detection of important climatic refugia in the southern Balkans, that gave rise to the hypothesis of 'refugia within refugia' (Abellán & Svenning, 2014; Gomez & Lunt, 2007). Dispersal ability, combined with the opportunities for range expansion after the end of a glacial period, determines the destiny of a species, which either recolonizes to the north (Hewitt, 1999, 2000), or remains as a refugial endemic with limited expansion potentials to the south (Bilton et al., 1998; Kryštufek et al., 2007), as seems to be the case here for *Algyroides moreoticus*.

According to the phylogenetic results, *A. moreoticus* consists of an old clade that diverged from its relative lineages at least 9 Mya. However, its origin is quite ambiguous. We assume that its evolutionary differentiation is related to the geological history of Peloponnese, whose geographical isolation from continental Greece has been considered as the main cause of allopatric speciation for several endemic lizards (Psonis et al., 2018 and references therein), such as *Podarcis peloponnesiacus* (Bibron & Bory de Saint-Vincent, 1833), *Hellenolacerta graeca* (Bedriaga, 1886) and *Anguis cephalonica* Werner, 1894. This has also been documented for other animal groups, such as the mammal *Talpa stankovici* (Tryfonopoulos et al., 2010), the land snails

Codringtonia (Kotsakiozi et al., 2012) and *Josephinella* (Psonis et al., 2015), and the beetle *Gnaptor boryi* (Gkontas et al., 2017).

Remarkably, the intraspecific differentiation of *A. moreoticus* is trivial, revealing low diversity or bifurcations within the species trees, even between insular and Peloponnese populations. This probably indicates that its extant distribution is the result of a recent population expansion, initiated from a refugium in Peloponnese where the species was restricted during the unfavourable conditions of the Pleistocene's climatic oscillations, as has also been proposed in the case of *P. ionicus* (Psonis et al., 2018). This is supported by the results of demographic analyses, in which Fu's F_s and EBSP showed a constant growth of population, approximately right after the LGM (Figure S4). On the other hand, according to our SDM analysis the species' population contracted in the mid-Holocene from a wider distribution in the LGM period, and subsequently slightly expanded, and in some cases shifted its range even western, forming the current distribution (Figure 4). This dissimilarity between EBSP and SDM is probably due to the poor distributional data of *A. moreoticus* from the east part of the Peloponnese that affects the SDM analysis.

As already mentioned, the islands of Kephallonia, Ithaca and Zakynthos, where *A. moreoticus* occurs, were interconnected during the LGM, but never connected to Peloponnese or continental Greece during the Pleistocene. Given the negligible genetic diversification between Peloponnese and insular populations, the presence of *A. moreoticus* on these islands is probably due to recent passive dispersal by rafting or/and by human-mediated dispersal, as in the case of *A. cephallonica* (Thanou et al., 2014). Unfortunately, we failed to amplify the target gene fragments from the individuals from Zakynthos, so this island population is not represented in our data set. Considering the geological connection of Zakynthos with Kephallonia and Ithaca during the LGM that allowed animals to actively move and disperse both sides, it is not expected that Zakynthos population would significantly vary from those of Kephallonia and Ithaca and consequently from the rest populations. However, a supplementary phylogenetic analysis with samples from Zakynthos is necessary for this to be clarified.

Transmarine dispersal by rafting or human translocation also best explains the species' occurrence on Strofades, which are small islets located approximately 45 km south of Zakynthos and 50 km west of Peloponnese coasts (Figure 1). From a biogeographical point of view, the young age of Strofades islets (no older than 350,000 years), and the fact that they were never connected to Peloponnese or other islands (Valakos & Mylonas, 1992), exclude the vicariance as a potential scenario for their colonization by *A. moreoticus* or any other species in general.

4.4 | On the parphyly of the genus *Algyroides*

The genus *Dinarolacerta* consists of only two species with a restricted distribution (Figure 1), that is *D. montenegrina* Ljubisavljević, Arribas, Džukić, & Carranza, 2007 and *D. mosorensis* (Kolombatović, 1886). In all our trees, the genus *Dinarolacerta* appears to have a sister relationship with *A. moreoticus*, clustering within the genus *Algyroides* (Figures 1 and 3; also Figures S1 and S2), making the genus of *Algyroides* paraphyletic. Other similar published studies come in congruence with our results, albeit having different tree topologies. More precisely, in the study of Pavlicev and Mayer (2009) *Dinarolacerta* clade branches next to *A. marchi* and *A. fitzingeri* as a sister group, within the clade of *Algyroides*, in a concatenated gene tree of three mtDNA and two nuDNA markers. The exact same topology was generated in a large-scaled concatenated tree in Pyron et al. (2013), made of 12 molecular markers in total. In contrast, in Mendes et al. (2016) *Dinarolacerta* is shown as a sister group of the genus *Algyroides*, branching outside of *Algyroides* clade in both nuclear-only (five markers) and mtDNA + nuDNA (seven markers in total) concatenated trees. However, in the same work, *Dinarolacerta* clutches within *Algyroides* clade in their mitochondrial-only tree, as a sister group of *A. nigropunctatus*. Despite the relative variety regarding the phylogenetic topologies shown in the above studies, the fact that *Dinarolacerta* clusters within *Algyroides* clade so 'often' should be taken seriously into account regarding the consistency of *Algyroides* monophyly. However, given the extant facts, it is too soon to make any safe conclusions, which would be probably drawn by a genome-wide analysis approach.

5 | CONCLUSIONS

Our study provides a clearer picture into the phylogenetic relationships between the four species of the genus *Algyroides*, and more specifically on the relationships within *A. nigropunctatus* and *A. moreoticus*, in the Balkan Peninsula. Our results suggest that the two main phenotypes of *A. nigropunctatus*, currently described as subspecies, represent two different monophyletic and allopatric groups that meet on the mainland in western Greece, yet seem to be reproductively isolated. These results can contribute to a taxonomic re-evaluation, leading to a split of *A. nigropunctatus* into two different species, elevating the extant subspecies to species level. Furthermore, the phylogeographic pattern revealed within *A. nigropunctatus*, regarding its two major clades meeting at the valleys of Aetoloakarnania, is unfamiliar and unprecedented to our knowledge. In regard to *A. moreoticus*,

we found that the species originates from an older lineage that diverged at least 9 million years ago, albeit it was shown that there is low genetic intraspecific diversity throughout its whole distribution, a fact that indicates a very recent species dispersal. In addition, the genus *Algyroides* appears to be paraphyletic considering the genus *Dinarolacerta* in our phylogenetic trees, as the genus clusters as a sister group to *A. moreoticus*, nestled within the *Algyroides* clade. Similar phylogenetic studies have also disputed *Algyroides* monophyly in the same way, though resulting in slightly different topologies, an argument that could be possibly solved by genome-scale analyses. Overall, we conclude that *Algyroides* is a well-differentiated genus with a significantly complicated evolutionary history, with species from Greece and the Balkan Peninsula being the outcome of ancient cladogenetic events, yet very recent differentiations.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Supporting Information

Table S1. List of samples used in our analyses with localities and GenBank accession numbers.

Code number	N.H.M.C. code	Species	Locality	LAT	LON	GenBank Accession Numbers			
						Cytb	COI	MC1R	NKTR
1		<i>A. n. kephallithacius</i>	Greece: Lefkada	38,816400	20,714180	MW373190	MW373100	MW373281	MW373369
2		<i>A. n. kephallithacius</i>	Greece: Lefkada	38,816400	20,714180	MW373191	MW373101	MW373282	MW373370
3		<i>A. n. kephallithacius</i>	Greece: Lefkada	38,816400	20,714180	MW373192	MW373102	MW373283	MW373371
4		<i>A. n. kephallithacius</i>	Greece: Lefkada	38,724520	20,686450	MW373193	MW373103	MW373284	MW373372
5		<i>A. n. kephallithacius</i>	Greece: Kephallonia	38,238130	20,642630	MW373194	MW373104	MW373285	MW373373
6		<i>A. n. kephallithacius</i>	Greece: Kephallonia	38,238130	20,642630	MW373195	MW373105	MW373286	MW373374
7		<i>A. n. kephallithacius</i>	Greece: Ithaca	38,324050	20,740620	MW373196	MW373106	MW373287	MW373375
8		<i>A. n. kephallithacius</i>	Greece: Katouna Xiromerou	38,787730	21,118980	MW373197	MW373107	MW373288	MW373376
9		<i>A. n. kephallithacius</i>	Greece: Katouna Xiromerou	38,787730	21,118980	MW373198	MW373108	MW373289	MW373377
10		<i>A. n. kephallithacius</i>	Greece: Amvrakia lake	38,714580	21,202380	MW373205	MW373115	MW373296	MW373382
11		<i>A. n. kephallithacius</i>	Greece: Kephallonia	38,238296	20,642626	MW373211	MW373121	MW373302	MW373388
12		<i>A. n. kephallithacius</i>	Greece: Kephallonia	38,163786	20,723476	MW373212	MW373122	MW373303	MW373389
13		<i>A. n. kephallithacius</i>	Greece: Kephallonia	38,147343	20,786557	MW373213	MW373123	MW373304	MW373390
14		<i>A. n. kephallithacius</i>	Greece: Loytra Tryfou	38,842769	21,045761	MW373214	MW373124	MW373305	MW373391
15		<i>A. n. kephallithacius</i>	Greece: Ozeros lake	38,677472	21,208944	MW373215	MW373125	MW373306	MW373392
16		<i>A. n. kephallithacius</i>	Greece: Ozeros lake	38,677472	21,208944	MW373216	MW373126	MW373307	MW373393
17		<i>A. n. kephallithacius</i>	Greece: Ellinika Panetolikos	38,481673	21,416717	MW373217	MW373127	MW373308	MW373394
18		<i>A. n. kephallithacius</i>	Greece: Ellinika Panetolikos	38,481673	21,416717	MW373218	MW373128	MW373309	MW373395
19		<i>A. n. kephallithacius</i>	Greece: Ellinika Panetolikos	38,481673	21,416717	MW373219	MW373129	MW373310	MW373396
20		<i>A. n. nigropunctatus</i>	Greece: Nea Malesiada	38,924000	21,352830	MW373199	MW373109	MW373290	MW373378
21		<i>A. n. nigropunctatus</i>	Greece: Nea Malesiada	38,924000	21,352830	MW373200	MW373110	MW373291	MW373379
22		<i>A. n. nigropunctatus</i>	Greece: Nea Malesiada	38,924000	21,352830	MW373201	MW373111	MW373292	MW373380
23		<i>A. n. nigropunctatus</i>	Greece: Nea Malesiada	38,924000	21,352830	MW373202	MW373112	MW373293	-
24		<i>A. n. nigropunctatus</i>	Greece: Nea Malesiada	38,924000	21,352830	MW373203	MW373113	MW373294	-
25		<i>A. n. nigropunctatus</i>	Greece: Sardinia, Amfilochia	38,889030	21,240450	MW373204	MW373114	MW373295	MW373381
26		<i>A. n. nigropunctatus</i>	Greece: Matsouki	38,717080	21,334970	MW373206	MW373116	MW373297	MW373383
27		<i>A. n. nigropunctatus</i>	Greece: Prespes	40,802164	21,072402	MW373207	MW373117	MW373298	MW373384
28		<i>A. n. nigropunctatus</i>	Greece: Prespes	40,802164	21,072402	MW373208	MW373118	MW373299	MW373385
29		<i>A. n. nigropunctatus</i>	Greece: Prespes	40,802164	21,072402	MW373209	MW373119	MW373300	MW373386
30		<i>A. n. nigropunctatus</i>	Greece: Prespes	40,802164	21,072402	MW373210	MW373120	MW373301	MW373387
31		<i>A. n. nigropunctatus</i>	Greece: Parga	39,28734	20,39732	MW373220	MW373130	MW373311	MW373397
32		<i>A. n. nigropunctatus</i>	Croatia: Velika Paklenica, Velebit	44,29874	15,46420	MW373221	MW373131	MW373312	MW373398
33		<i>A. n. nigropunctatus</i>	North Macedonia: Trpejca	40,96292	20,78396	MW373222	MW373132	MW373313	MW373399
34		<i>A. n. nigropunctatus</i>	North Macedonia: Trpejca	40,96292	20,78396	MW373223	MW373133	MW373314	MW373400
35		<i>A. n. nigropunctatus</i>	North Macedonia: Matka	41,94688	21,30219	MW373224	MW373134	MW373315	-
36		<i>A. n. nigropunctatus</i>	North Macedonia: Matka	41,94688	21,30219	MW373225	MW373135	MW373316	MW373401
37		<i>A. n. nigropunctatus</i>	North Macedonia: Matka	41,94688	21,30219	MW373226	MW373136	MW373317	MW373402
38		<i>A. n. nigropunctatus</i>	North Macedonia: Matka	41,94688	21,30219	MW373227	MW373137	MW373318	MW373403
39		<i>A. n. nigropunctatus</i>	Albania: Schendli Mts.	40,37371	20,03697	MW373228	MW373138	MW373319	MW373404
40		<i>A. n. nigropunctatus</i>	Albania: Bylysh	40,36142	20,00727	MW373229	MW373139	MW373320	MW373405
41		<i>A. n. nigropunctatus</i>	Albania: Cajupit pass	40,19980	20,18170	MW373230	MW373140	MW373321	MW373406
42		<i>A. n. nigropunctatus</i>	Albania: Cajupit pass	40,19980	20,18170	MW373231	MW373141	MW373322	-
43		<i>A. n. nigropunctatus</i>	Albania: Cajupit pass	40,19980	20,18170	MW373232	MW373142	MW373323	MW373407
44		<i>A. n. nigropunctatus</i>	Albania: Syri i Kalter	39,92603	20,19191	MW373233	MW373143	MW373324	MW373408
45		<i>A. n. nigropunctatus</i>	Albania: Syri i Kalter	39,92603	20,19191	MW373234	MW373144	MW373325	MW373409
46	NHMC80.3.118.14	<i>A. n. nigropunctatus</i>	Greece: Zagori, Ioannina	39,865529	20,761152	MW373235	MW373145	MW373326	-

47	NHMC80.3.118.15	<i>A. n. nigropunctatus</i>	Greece: Antinioti sea lake, Corfu	39,800000	19,800000	MW373236	MW373146	MW373327	MW373410
48	NHMC80.3.118.16	<i>A. n. nigropunctatus</i>	Greece: Desi, Trikala	39,560400	21,371900	MW373237	MW373147	MW373328	MW373411
49	NHMC80.3.118.17	<i>A. n. nigropunctatus</i>	Greece: Desi, Trikala	39,560400	21,371900	MW373238	MW373148	MW373329	-
50	NHMC80.3.118.18	<i>A. n. nigropunctatus</i>	Greece: Mystras, Ioannina	39,570700	21,102000	MW373239	MW373149	MW373330	-
51	NHMC80.3.118.24	<i>A. n. nigropunctatus</i>	Greece: Gezovitisas lake, Corfu	39,770000	19,940000	MW373240	MW373150	MW373331	MW373412
52	NHMC80.3.118.25	<i>A. n. nigropunctatus</i>	Greece: Gezovitisas lake, Corfu	39,770000	19,940000	MW373241	MW373151	MW373332	MW373413
53	NHMC80.3.118.27	<i>A. n. nigropunctatus</i>	Greece: Gaidarana lake Corfu	39,650000	19,800000	MW373242	MW373152	MW373333	MW373414
54	NHMC80.3.118.28	<i>A. n. nigropunctatus</i>	Greece: Tebloni, Corfu	39,640000	19,800000	MW373243	MW373153	MW373334	MW373415
55	NHMC80.3.118.29	<i>A. n. nigropunctatus</i>	Greece: Pogoniskos, Ioannina	40,060100	20,523000	MW373244	MW373154	MW373335	-
56	NHMC80.3.118.30	<i>A. n. nigropunctatus</i>	Greece: Pogoniskos, Ioannina	40,060100	20,523000	MW373245	MW373155	MW373336	MW373416
57	NHMC80.3.118.31	<i>A. n. nigropunctatus</i>	Greece: Drymades, Ioannina	40,061400	20,439800	MW373246	MW373156	MW373337	MW373417
58	NHMC80.3.118.32	<i>A. n. nigropunctatus</i>	Greece: Molyvoskepastis monastery	40,061300	20,593700	MW373247	MW373157	MW373338	-
59	NHMC80.3.118.33	<i>A. n. nigropunctatus</i>	Greece: Ioannina	39,813300	20,940700	MW373248	MW373158	MW373339	MW373418
60	NHMC80.3.118.34	<i>A. n. nigropunctatus</i>	Greece: Drymades, Ioannina	40,061400	20,439800	MW373249	MW373159	MW373340	MW373419
61	NHMC80.3.118.36	<i>A. n. nigropunctatus</i>	Greece: Drymades Ioannina	40,061400	20,439800	MW373250	MW373160	MW373341	MW373420
62	NHMC80.3.118.37	<i>A. n. nigropunctatus</i>	Greece: Grevena	39,978900	21,178500	MW373251	MW373161	MW373342	MW373421
63	NHMC80.3.118.41	<i>A. n. nigropunctatus</i>	Greece: Voio mt., Grevena	40,181800	21,338300	MW373252	MW373162	MW373343	MW373422
64	NHMC80.3.118.42	<i>A. n. nigropunctatus</i>	Greece: Voio mt., Grevena	40,181800	21,338300	MW373253	MW373163	MW373344	MW373423
65	NHMC80.3.118.44	<i>A. n. nigropunctatus</i>	Greece: Grammos mt., Ioannina	40,235800	20,921900	MW373254	-	MW373345	-
66	NHMC80.3.118.65	<i>A. n. nigropunctatus</i>	Greece: Kastoria	40,651700	21,179100	MW373255	MW373164	MW373346	MW373424
67		<i>A. n. nigropunctatus</i>	Slovenia: Dragonja	45,448150	13,676490	MW373256	MW373165	MW373347	-
68		<i>A. n. nigropunctatus</i>	Slovenia: Dragonja	45,448150	13,676490	MW373257	MW373166	MW373348	MW373425
69		<i>A. n. nigropunctatus</i>	Albania: Cajupit pass	40.18512	20.18229	MW373258	MW373167	MW373349	MW373426
70		<i>A. n. nigropunctatus</i>	North Macedonia: Oteshevo	40.97784	20.91241	MW373259	MW373168	MW373350	MW373427
71		<i>Algyroides moreoticus</i>	Greece: Ithaca	38,324050	20,740620	MW373260	MW373169	MW373351	MW373428
72		<i>Algyroides moreoticus</i>	Greece: Ithaca	38,324050	20,740620	MW373261	MW373170	MW373352	MW373429
73		<i>Algyroides moreoticus</i>	Greece: Chanandritsa, Achaia	38,119270	21,761980	MW373262	MW373171	MW373353	MW373430
74		<i>Algyroides moreoticus</i>	Greece: Kephallonia	38,191487	20,569350	MW373263	MW373172	MW373354	MW373431
75		<i>Algyroides moreoticus</i>	Greece: Elos Agvias	38,280550	21,755283	MW373264	MW373173	MW373355	MW373432
76	NHMC80.3.71.5	<i>Algyroides moreoticus</i>	Greece: Agaliani, Messinia	37,349226	21,728553	MW373265	MW373174	-	MW373433
77		<i>Algyroides moreoticus</i>	Greece: Chimara, Peloponnese	36.64580	22.46898	MW373266	MW373175	MW373356	MW373434
78		<i>Algyroides moreoticus</i>	Greece: Chimara, Peloponnese	36.64580	22.46898	MW373267	MW373176	MW373357	MW373435
79		<i>Algyroides moreoticus</i>	Greece: Avramianika	36.71307	22.41583	MW373268	MW373177	MW373358	MW373436
80		<i>Algyroides moreoticus</i>	Greece: Karea, Peloponnese	36.73171	22.41468	MW373269	MW373178	MW373359	MW373437
81		<i>Algyroides moreoticus</i>	Greece: Chora Getson	36.94844	22.25404	MW373270	MW373179	MW373360	MW373438
82		<i>Algyroides moreoticus</i>	Greece: Artemisia, Peloponnese	37.09895	22.22251	MW373271	MW373180	MW373361	MW373439
83		<i>Algyroides moreoticus</i>	Greece: Ag. Vasilios	37.07565	22.27064	MW373272	MW373181	MW373362	MW373440
84		<i>Algyroides moreoticus</i>	Greece: Doxas, Peloponnese	37.93098	22.28293	MW373273	MW373182	MW373363	MW373441
85		<i>Algyroides moreoticus</i>	Greece: Sikionas, Peloponnese	37.96655	22.69628	MW373274	MW373183	MW373364	MW373442
86		<i>Algyroides moreoticus</i>	Greece: Kalamata, Peloponnese	37.03158	22.13802	MW373275	MW373184	MW373365	MW373443
87		<i>Algyroides moreoticus</i>	Greece: Stamfani, Strofades	37,248800	21,008000	MW373276	MW373185	MW373366	MW373444
88		<i>Algyroides moreoticus</i>	Greece: Stamfani, Strofades	37,248800	21,008000	MW373277	MW373186	MW373367	MW373445
89		<i>Algyroides moreoticus</i>	Greece: Stamfani, Strofades	37,248800	21,008000	MW373278	MW373187	MW373368	MW373446
90		<i>Algyroides moreoticus</i>	Greece: Stamfani, Strofades	37,248800	21,008000	MW373279	MW373188	-	MW373447
91		<i>Algyroides moreoticus</i>	Greece: Stamfani, Strofades	37,248800	21,008000	MW373280	MW373189	-	MW373448
Amar1		<i>Algyroides marchi</i>	Spain, La Hueta	-	-	GQ142133	-	KX080715.1	-
Amar2		<i>Algyroides marchi</i>	Spain, El Toril	-	-	AF080307	-	KX080716	-
Af1		<i>Algyroides fitzingeri</i>	Italy: Mt. Albo, Sardinia	-	-	GQ142134	-	KX080713	-
Af2		<i>Algyroides fitzingeri</i>	Italy: Mt. Albo, Sardinia	-	-	GQ142134	-	KX080712	-
Dmos1		<i>D. mosorensis</i>	Montenegro: Mt. Maglic	-	-	KF373648	-	KX080722	-
Dmos2		<i>D. mosorensis</i>	Montenegro: Mt. Durmitor	-	-	KF373646	-	KX080723	-
Dmon1		<i>D. montenegrina</i>	Albania: Skhoder	-	-	GQ142141	-	KX080721	-
Dmon2		<i>D. montenegrina</i>	Albania: Theth	-	-	KF373644	-	KX080720	-
Lv1		<i>Lacerta viridis</i>	Greece: Thessalia, Larissa	-	-	KJ940327	KP697870	-	KJ940408
Lv2		<i>Lacerta viridis</i>	Greece: Thessalia, Larissa	-	-	KJ940328	KP697852	-	KJ940407

Table S2. Primers and conditions used in PCR amplifications^a and in cycle sequencing reactions.

Gene	Primers	Sequence (5' – 3')	Size (bp)	Conditions	Reference
MC1R	MC1RF	GGCNGCCATYGTCAAGAACCGGAACC	~700	1.5mM MgCl, 94°C/1min, 59°C/1min, 72°C/1min x 35 cycles	Pinho et al., 2010
	MC1RR	CTCCGRAAGGCRATAATGATGGGGTCCAC			
NKTR	NKTRf19	GATGACATGGAGATYTGACTCC	~630	3mM MgCl, 94°C/1min, 47°C/1min, 72°C/1min x 35 cycles	Townsend et al., 2011
	NKTRr18	CTYCTDGAYCGACTTCTTGAGTGACT			
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	708	1.5mM MgCl, 94°C/1min, 47°C/1min, 72°C/1min x 35 cycles	Simon et al., 1994
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA			
	or		480		Folmer et al., 1994
	CIJ1718	GGAGGATTTGGAAATTGATTAGTTCC			
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA			
Cyt b	GLUDG	TGACTTGAARAACCAAYCGTTG	~425	1.5mM MgCl, 94°C/1min, 47°C/1min, 72°C/1min x 35 cycles	Palumbi, 1996
	CB2	CCCTCAGAATGATATTTGTCCTCA			

^a using single *Taq*DNA polymerase (KAPABIOSYSTEMS®)

Table S3. Remaining predictors for each of the focal species after VIF analysis.

	<i>Algyroides nigropunctatus</i>	<i>Algyroides moreoticus</i>
Isothermality (bio_3)	X	X
Temperature Seasonality (bio_4)	X	
Maximum temperature of the warmest month (bio_5)	X	
Mean Temperature of Wettest Quarter (bio_8)	X	
Mean Temperature of Driest Quarter (bio_9)	X	X
Precipitation Seasonality (bio_15)	X	X
Precipitation of Warmest Quarter (bio_18)		X
Precipitation of Coldest Quarter (bio_19)	X	X

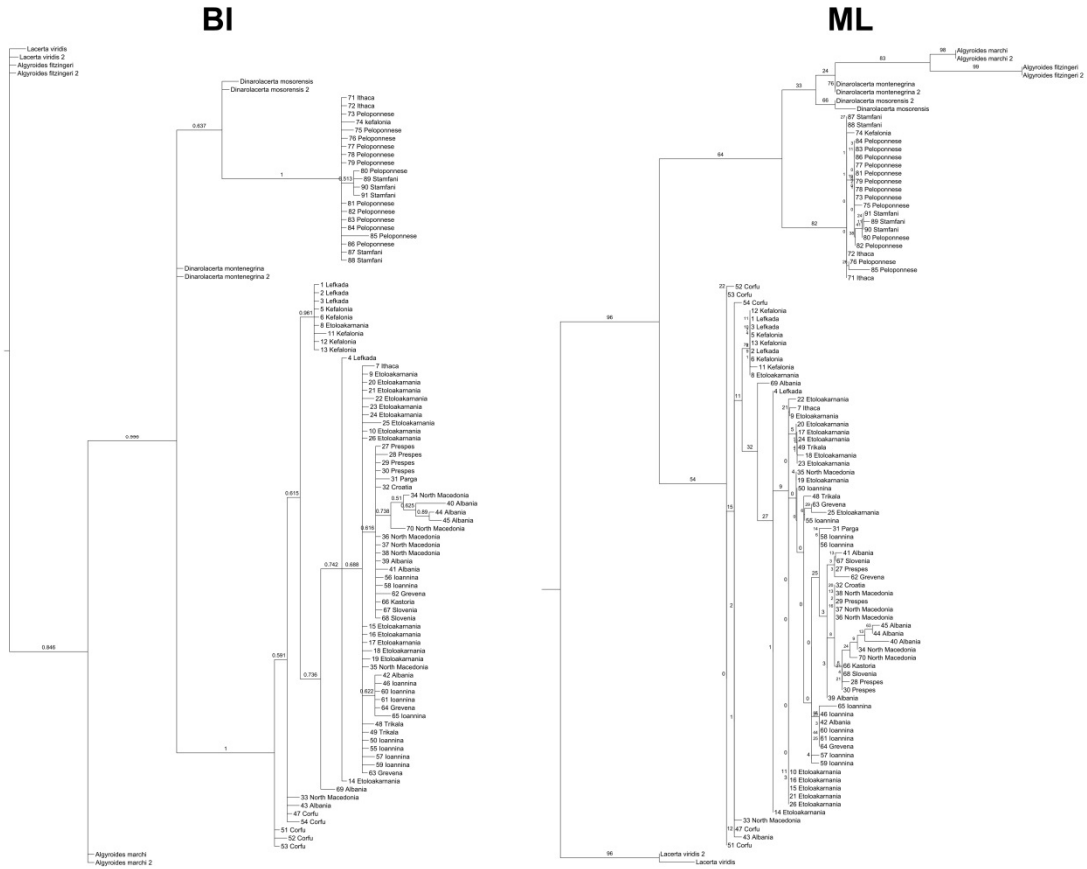


Figure S1. Concatenated gene trees produced by nuclear data only (NKTR and MC1R fragments), with Bayesian Inference on the left (BI) and Maximum Likelihood on the right (ML). Posterior probabilities and bootstrap values are given above the branches.

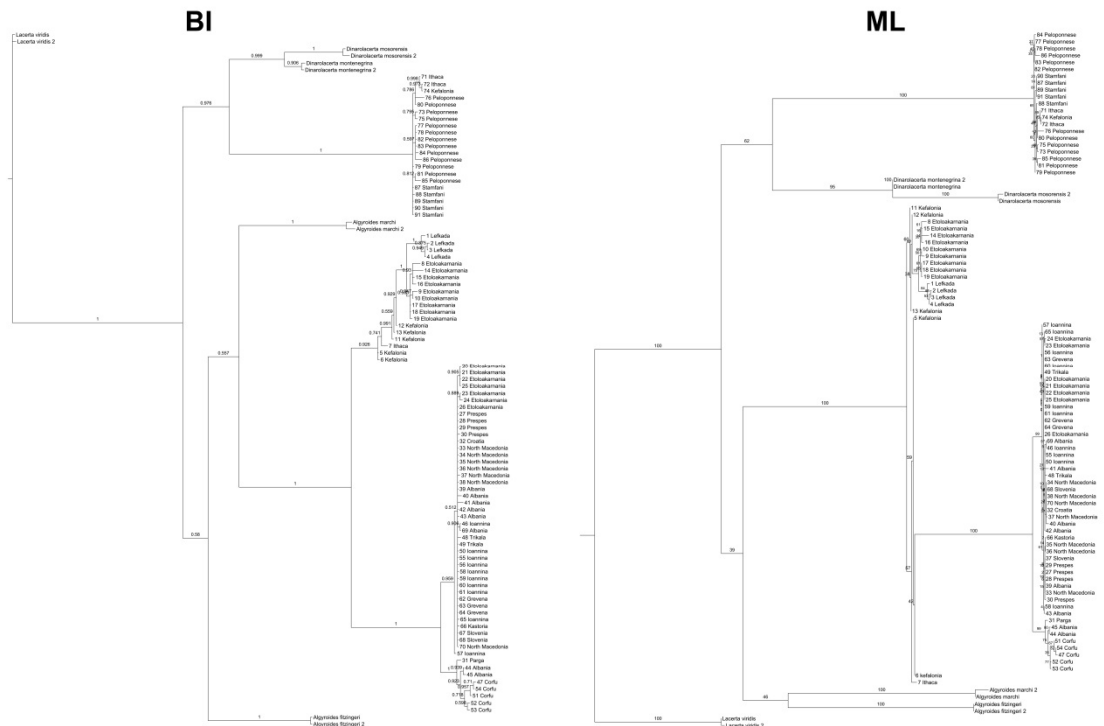


Figure S2. Concatenated gene trees produced by mtDNA data only (Cytb and COI fragments), with Bayesian Inference on the left (BI) and Maximum Likelihood on the right (ML). Posterior probabilities and bootstrap values are given above the branches.

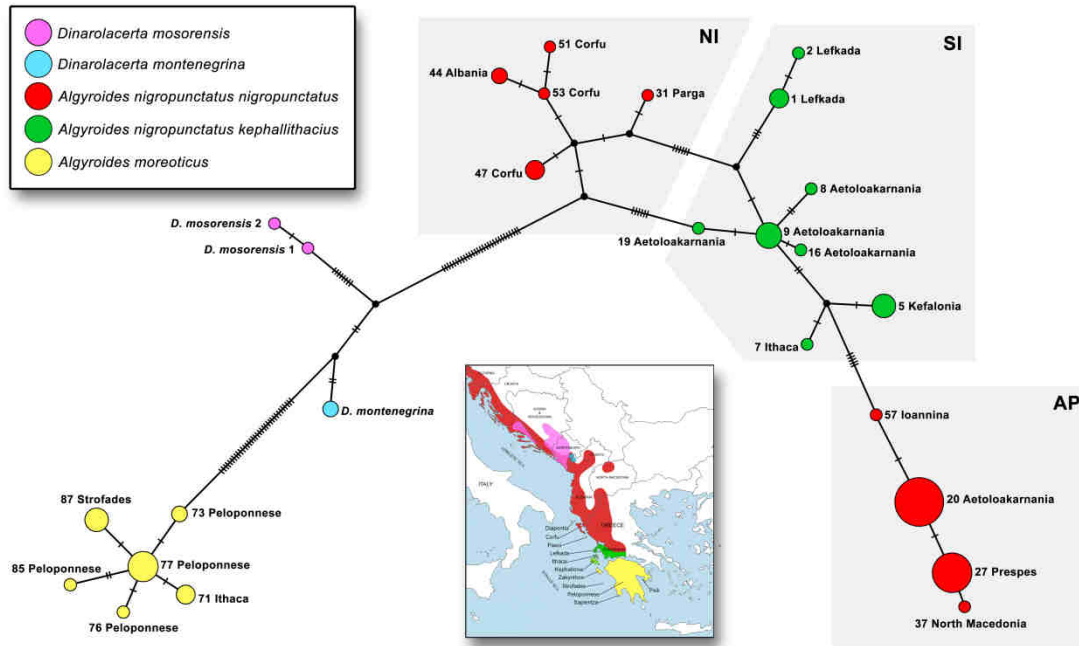


Figure S3. TCS haplotype network for *Algyroides* and *Dinarolacerta* species in southern Balkans. The three subclades of *Algyroides nigropunctatus* are framed with light grey color: NI) Northern Ionian (Corfu-Parga suclade), SI) Southern Ionian, and AP) Adria-Pindos, as described in Podnar and Mayer (2006).

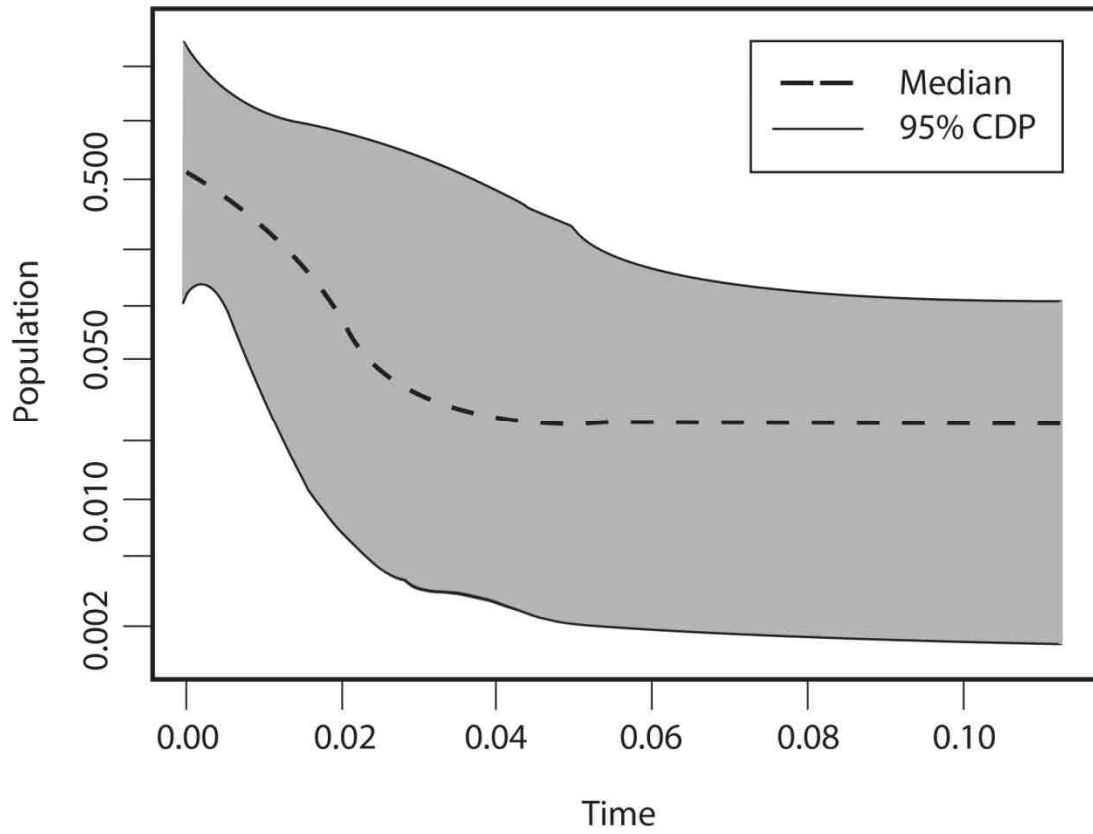


Figure S4. Extended Bayesian skyline plot for effective population sizes (N_e) through time (in millions of years) in sampled geographical areas for *A. moreoticus*. Lines represent median values and grey areas their 95% central posterior density (CPD) intervals.

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