Received: 23 October 2020 | Revised: 25 August 2021 | Accepted: 5 September 2021

DOI: 10.1111/jzs.12540

JOURNAL OF ZOOLOGICAL SYSTEMATICS AND EVOLUTIONARY RESEARCH — WILEY

ORIGINAL ARTICLE

Evidence of cryptic diversity in *Podarcis peloponnesiacus* and re-evaluation of its current taxonomy; insights from genetic, morphological, and ecological data

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Abstract

The Peloponnese wall lizard, *Podarcis peloponnesiacus*, is endemic to the Peloponnese. Although the phylogeny and species diversity of the Balkan species of *Podarcis* have been extensively studied, the intraspecific relationships of *P. peloponnesiacus* are not yet well defined. The aim of this study was to investigate the intraspecific diversity in this species and clarify its taxonomic status by analyzing eight gene fragments (two mitochondrial and six nuclear) and several morphological traits, typically used for systematic inferences within the genus *Podarcis*. Together with ecological niche modeling, we provided an integrative evaluation of the differentiation between lineages. The combination of several phylogenetic, species delimitation, and chrono-phylogenetic analyses revealed the existence of two highly supported and divergent clades with a distinct geographical and parapatric distribution and high niche overlap. The differentiation of the two clades dates back to the Pleistocene and is probably correlated with the paleogeography of the Peloponnese. These clades also differed in several phenotypic traits, which, however, exhibit extensive overlap and are not fully diagnostic. The combination of the above results allowed us to identify the two revealed clades as distinct species.

KEYWORDS
mitochondrial DNA, nuclear DNA, phylogeny, phylogeography, southern Balkans, species delimitation, systematics

1 | INTRODUCTION

The Peloponnese (south continental Greece) is an important area for biodiversity, both in terms of landscape and species diversity (Valakos et al., 2008). It is considered one of the most important speciation centers in the Balkan Peninsula (Gkontas et al., 2016) with high levels of endemism for plants, invertebrates, and vertebrates (Jablonski et al., 2016). It is home to endemic reptile taxa, such as the species *Podarcis peloponnesiacus* (Bibron and Bory de Saint-Vincent, 1833) and *Anguis cephallonica* (Werner, 1894). There is even endemism there at the genus level, in the case of *Hellenolacerta graeca* (Bedriaga, 1886) (Valakos et al., 2008).

The high diversity of this region is related to the complex paleogeographical history of southern Greece, including the Peloponnese (submergence and re-emergence of landmasses, due to tectonic, volcanic, and eustatic events; Creutzburg, 1963), in combination with the climatic changes of the Tertiary and Quaternary (Zachos et al., 2001). Several tectonic faults, which became active in the Corinthian

Zoobank Link: urn:lsid:zoobank.org:act:62535A3B-A26A-4AEB-8D97-4610401B3076 **Online ISSN**: 1439-0469

Gulf during the Pliocene, caused the isolation of the Peloponnese from continental Greece (3–4 Mya; Collier & Dart, 1991; Creutzburg, 1963; Zelilidis et al., 1998), turning it into an island, smaller in size than today, and closer to the coast (Dermitzakis, 1990). This isolation has been assumed to be the main cause of allopatric differentiation and speciation for several reptile and amphibian species, including the lizards *P. peloponnesiacus* (Poulakakis et al., 2005), *L. trilineata* (Sagonas et al., 2014), *A. cephallonica* (Jablonski et al., 2016), and *Algyroides moreoticus* (Strachinis et al., 2021), the nose-horned viper *Vipera ammodytes* (Ursenbacher et al., 2008), the alpine newt *Ichthyosaura alpestris* (Recuero et al., 2014; Sotiropoulos et al., 2007), and the tortoise, *Testudo hermanni* (Fritz et al., 2006), as well as, for several invertebrates, such as the snails [*Codringtonia* (Kotsakiozi et al., 2012) and *Josephinella* (Psonis et al., 2015)], the isopods [*Trachelipus* (Parmakelis et al., 2008)] and the beetle, *Gnaptor boryi* (Gkontas et al., 2016), all of which have differentiated lineages in the Peloponnese.

Lacertid lizards of the genus *Podarcis* have been proven to be a good model for biodiversity studies, as they have undergone a remarkable radiation, exhibiting high levels of differentiation in southern Europe (Oliverio et al., 2000), which is characterized by a complex geological history, high biodiversity, and a high percentage of endemism (Lymberakis & Poulakakis, 2010). This genus currently comprises 25 recognized species (Uetz et al., 2020), forming the predominant and most taxonomically diverse reptile group in southern Europe (Harris & Sá-Sousa, 2002). The southern Balkans host 10 native *Podarcis* species, five of which are endemic to Greek islands and the Peloponnese: *P. cretensis* (Wettstein, 1952) on the island of Crete, *P. gaigeae* (Werner, 1930) on the Skyros island group, *P. levendis* Lymberakis et al., 2008, on Pori and Lagouvardos islets, *P. milensis* (Bedriaga, 1882) on the Milos island group and *P. peloponnesiacus* on the Peloponnese (Lymberakis & Poulakakis, 2010; Pafilis, 2010; Speybroeck et al., 2020; Uetz et al., 2020); additionally, two alien *Podarcis* species (*P. vaucheri* and *P. siculus*) have also been found in Greece (Adamopoulou, 2015; Spilani et al., 2018). The species found in the Balkans (excluding the alien ones) are divided into three groups (Psonis et al., 2021). These are the *P. tauricus* group, including *P. tauricus* (Pallas, 1814), *P. gaigeae*, *P. milensis*, *P. melisellensis* (Braun, 1877), and *P. ionicus* (Lehrs, 1902), the *P. erhardii* group, which encompasses *P. erhardii* (Bedriaga, 1882), *P. cretensis*, *P. levendis*, and *P. peloponnesiacus*, and finally, *P. muralis* (Laurenti, 1868), which is widely distributed across the whole northern part of the range of the genus and is hypothesized to belong to the Italian group (Harris & Arnold, 1999). However, its position within this group is disputed (Psonis et al., 2018; Salvi et al., 2021). Moreover, two very recent studies based on genome data supported the phylogenetic relationship of *P. muralis* with the Iberian group of species (Garcia-Porta et al., 2019; Yang et al., 2021). It is surprising that based on these studies, the temporal diversification of the *P. cretensis*, *P. levendis*, and *P. peloponnesiacus* group coincides with the end of the Messinian Salinity Crisis (Krijgsman et al., 1999), so their divergence can be attributed to vicariant events, in contrast to the speciation events between other lineages of the genus *Podarcis*, which are attributed to the interactions of regional topography, climate, and geological history events (Salvi et al., 2021). This is in full agreement with previously published studies on the *Podarcis* species of the Balkans (Poulakakis et al., 2005; Psonis et al., 2018, 2021; Spilani et al., 2019), identifying the isolation of Crete (*P. cretensis*) from the Peloponnese (*P. peloponnesiacus*) and Pori (*P. levendis)* as the major vicariant event that led to diversification of those species at the end of the Miocene.

The focal species of this study, *P. peloponnesiacus*, commonly known as the Peloponnese wall lizard, is the most common lizard species of the Peloponnese (Valakos et al., 2008). It exhibits a snout–vent length (SVL) of up to 8.5 cm and a tail that can be twice as long. Males are larger than females, and the dorsum has striped markings in both sexes. Females are brown in color with yellowish dorsolateral stripes from the neck until the tail base, while males are more colorful, with greenish-brown backs and blue spots between the flanks and the fore limbs. On each side of the head, there is one postnasal scale and 0–7 supraciliary granules (Valakos et al., 2008). To date, three subspecies of *P. peloponnesiacus* have been recognized: *P. p. peloponnesiacus* (Bibron and Bory de Saint-Vincent, 1833), distributed in the Provinces of Laconia, Messenia, and Arcadia (Southern Peloponnese); *P. p. lais* (Buchholz, 1960), in the Provinces of Achaia and perhaps parts of Ilis (Northern Peloponnese); and *P. p. thais* (Buchholz, 1960), in the Province of Argolis (Northeast Peloponnese) and the islands of the Argosaronic gulf (Bringsøe, 1986; Chondropoulos, 1986). Their respective type localities noted in figure 2 of Buchholz (1960) initially designated four subspecies based exclusively on color patterns and coloration differences. This approach has been reviewed by Bringsøe (1986 and references therein) who restricted the subspecies to the aforementioned three, sinking the fourth subspecies of Buchholz (1960), *P. p. phryne*, in synonymy with *P. p. lais*. The species has a continuous range in the Peloponnese, with the exception of the northwestern part, where it has not been found (Bringsøe, 1986). Recently, the first occurrence of *P. peloponnesiacus* outside the Peloponnese was reported (in a southeastern district of Sterea Ellada, see locality 15 in Figure 1; Hedman et al., 2017). However, it is not clear whether this individual was an autochthonous specimen or one that was introduced by humans. As for its conservation status, *P. peloponnesiacus* is classified as Least Concern according to the IUCN criteria (Böhme & Lymberakis, 2009). At present, there are no phylogenetic studies focusing on *P. peloponnesiacus*, but in the studies of Lymberakis et al. (2008), Spilani et al. (2019), and Psonis et al. (2021), which used molecular phylogenetic methods, there were preliminary indications that *P. peloponnesiacus* was splitting into two distinct lineages. However, these two lineages have not been investigated in detail to date.

These findings prompted us to investigate the phylogeny of *P. peloponnesiacus* further, in order to provide an accurate description of the intraspecific diversity of the species and to clarify the taxonomic status of different evolutionary units (see also Speybroeck et al., 2020). By performing several analyses, we aim to shed light on the taxonomic status of the species. We aim to test the hypothesis of the existence of two distinct species; and, if these two species exist, we aim to estimate the time of their divergence and investigate the biogeographic context that stimulated their differentiation.

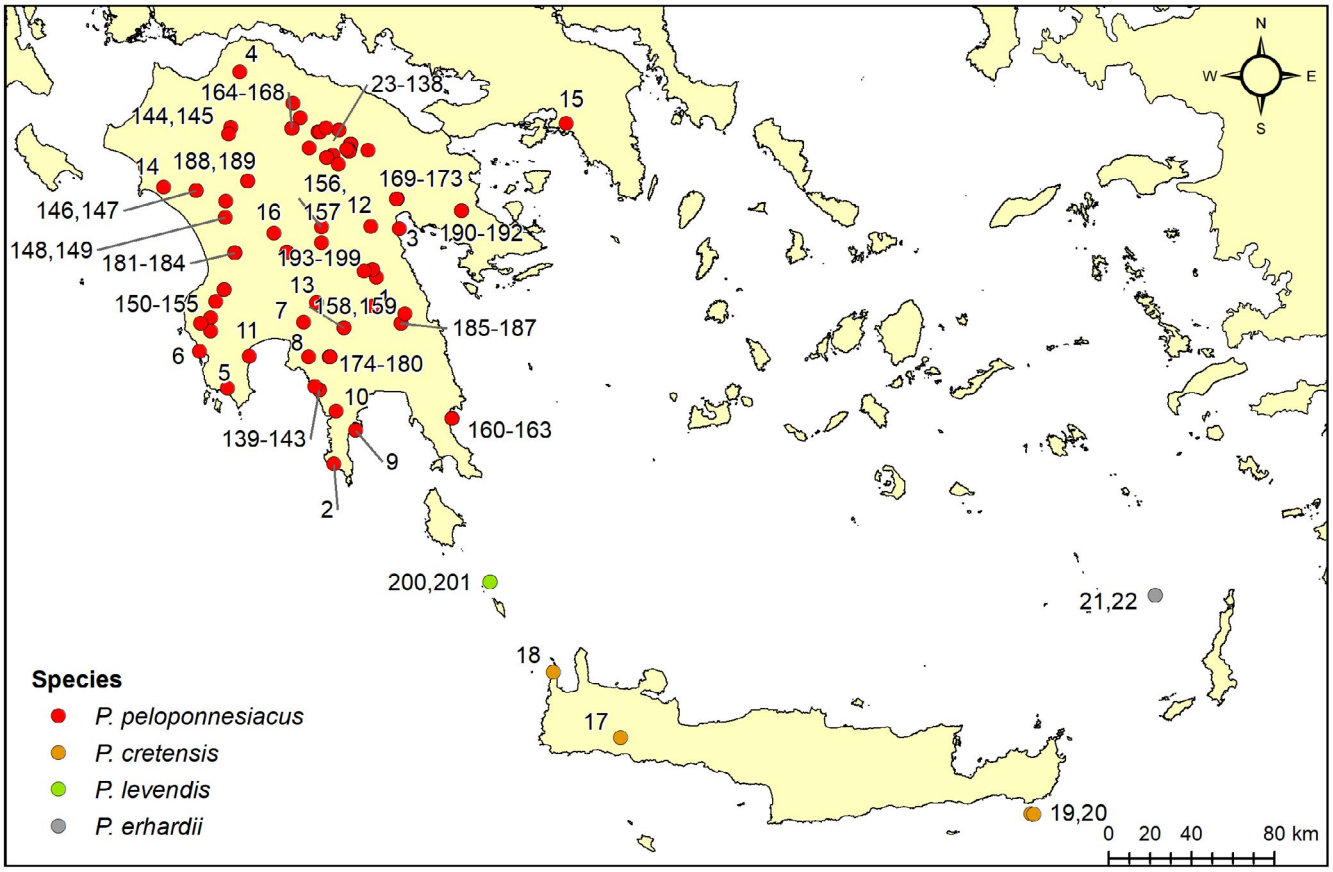

FIGURE 1 The geographical localities of all specimens used for the phylogenetic analyses. Numbers refer to the serial number of specimens (a/a) given in Table S1

2 | MATERIALS AND METHODS

2.1 | Specimens, DNA extraction, PCR amplification, and sequencing

For this study, a total of 193 specimens of *P. peloponnesiacus* were used, which were collected from 51 geographical localities. Additionally, two specimens of *P. levendis* and four specimens of *P. cretensis* were also included for comparative reasons and two *P. erhardii* specimens were used as the outgroup. All specimens had been deposited in the collections of the National History Museum of Crete—University of Crete, where they were conserved in 95% ethanol or were frozen (−86°C). For 142 specimens, we conducted total genomic DNA extraction from muscle tissue using a typical ammonium acetate protocol (Bruford et al., 1998), while the remaining 51 extractions were obtained from previous studies (Lymberakis et al., 2008; Spilani et al., 2019). Double-stranded PCR was used to amplify two mitochondrial gene fragments [large subunit of ribosomal RNA (*16S*) and cytochrome b (*cyt b*)] and six nuclear gene fragments [melanocortin 1 receptor (*MC1R*), recombination activating gene 1 (*Rag1*), natural killer tumor recognition receptor (*NKTR*), ubinuclein 1 protein (*UBN1*), and two anonymous loci *Pod55* and *Pod15b* (Pereira et al., 2013)]. The total number of samples used, their code, their location, and the genetic loci amplified are presented in Table S1 (Accession numbers MW832559 to MW832696, MW846641 to MW846854, MW880774 to MW880855, and MW880857 to MW880888). The geographical localities of the samples used are illustrated in Figure 1.

Primers used for each genetic locus and their PCR conditions are shown in Table S2. Each PCR reaction was performed in 20 μl volume, in which 1 μl template DNA was mixed with 2.5 units Taq polymerase (Dream Taq DNA Polymerase, Thermo Scientific), 1× PCR Buffer, 1.5–3 mM $MgCl_2$, 0.2 mM dNTPs, and 0.2 mM of each primer. The PCR cycles comprised a 10-min pro-incubation at 94°C, followed by 35 cycles of 1-min denaturation at 94°C, 1-min annealing temperature of each primer, and 1-min extension at 72°C, and then the cycling terminated with a 10-min extension at 72°C.

Double-stranded sequencing was conducted in an automated sequencer ABI3730XL (CeMIA Company; Larissa, Greece) using the Big-Dye Terminator v.3.1 Cycle Sequencing kit®, following the manufacturer's protocol and using the same primers as in the PCR reactions.

2.2 | Alignment, genetic divergence, and model selection

Sequences were edited in a Codon Code Aligner (v. 3.7.1; Codon Code Corporation®), and the homology of the targeted loci was

verified with the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Alignment was performed for each locus separately. For the mitochondrial gene fragments *cyt b* and *16S*, the alignment was performed using the ClustalW algorithm, which is embedded in the MEGA program (v. 7.0.26; Kumar et al., 2016). The nuclear loci were aligned with MAFFT (v. 7; Katoh & Standley, 2013) based on the FFT-NS-2 algorithm (Fast: Progressive method). Alignment gaps were inserted to resolve length differences between non-coding sequences (*16S*, *Pod15b*, *Pod55*). *Cytochrome b*, *MC1R*, *RAG1*, *NKTR*, and *UBN1* sequences were translated into amino acids prior to further analysis, in order to ensure the absence of stop codons. 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.

Genetic distances were calculated with MEGA (v. 7) based on the Tamura-Nei model of evolution.

For the phylogenetic, chronophylogenetic, and species delimitation analyses, three datasets were prepared. A mitochondrial dataset (mtDNA) comprised of the two mtDNA gene fragments for all *P. peloponnesiacus*, *P. levendis*, *P. cretensis*, and *P. erhardii* specimens. The second dataset (nuDNA) was created using the six nuclear gene markers (*Pod55*, *MC1R*, *Rag1*, *Pod15b*, *NKTR*, and *UBN1*). For this dataset, representatives from each one of the major evolutionary lineages that were identified from the phylogenetic tree produced from the initial mtDNA dataset were selected. The selection of the representatives was based on the results of the multi-rate Poisson Tree Processes (mPTP) analysis (v. 0.2.4; Kapli et al., 2017) on the phylogenetic tree produced from the mtDNA dataset. Based on this analysis (see Section 3), three groups were recognized within *P. peloponnesiacus*. Finally, a concatenated dataset was constructed, including all loci (mtDNA and nuDNA). Gene matrices are available on TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S27763?x-access-code=38e8a3d614ca6d44fc62c4e7b3e081e&format=html).

The nucleotide substitution model selection tests were carried out separately for the two types of genomes used in this study (mtDNA and nuDNA). The mtDNA alignment was subdivided into four pre-defined blocks; three of them corresponded to each codon position for cyt *b* and the fourth to *16S* (non-coding genetic locus). The nuclear loci were subdivided into 14 blocks corresponding to the 1st, 2nd, and 3rd codon positions for the genes *MC1R*, *Rag1*, *NKTR*, *UBN1*, and two blocks for *Pod55* and *Pod15b*. The alignments and the pre-defined blocks were then analyzed in PartitionFinder2 (PF) v.2.1.1 (Lanfear et al., 2017) to find the best-fit partitioning scheme and evolutionary models for each downstream analysis, evaluating the models that were implemented in each type of software (RAxML, MrBayes). Data blocks had linked branch lengths, and the model selection was based on the Bayesian information criterion, ignoring the evolutionary models that contained both gamma distribution and invariable sites (Yang, 2006). The optimal combination of block sequences was done with the "greedy" algorithm.

2.3 | Phylogenetic analyses

Phylogenetic analyses were performed separately for each dataset, based on maximum likelihood (ML) and Bayesian inference (BI). ML analysis was performed using the RAxML program (v. 8.1.21; Stamatakis, 2014). The best ML tree for each dataset was selected from 50 ML searches, and the statistical support of the branches was evaluated based on 1000 thorough bootstraps. BI analysis was performed in MrBayes (v. 3.2.6; Ronquist et al., 2012), based on the partition results and models revealed in PF2. Four runs were performed with eight independent sampling chains for each run. Each chain "ran" for 10^7 generations for each dataset, except the mitochondrial dataset, which "ran" for 1.5×10^7 generations, sampling every 5000 generations. Four MCMC diagnostics were analyzed in Tracer v.1.6. (Rambaud et al., 2014) to check for convergence and stabilization of the analysis, such as the average standard deviation of split frequencies, the plot of the generation versus the log probability of the data (the log likelihood values), the average potential scale production factor (PSRF), and the estimated sample size (ESS). The first 25% of 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 50% 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).

For nuclear loci, the relationships among haplotype sequences were determined using a haplotype network and a median joining model implemented in NETWORK 10.2 (Bandelt et al., 1999). The analysis was performed for each gene fragment separately.

2.4 | Species delimitation

The evaluation of the putative species' boundaries was performed using four different approaches: the mPTP, the Assemble Species by Automatic Partitioning (ASAP; Puillandre et al., 2021), the Bayesian Phylogenetics and Phylogeography—BPP v.4.3.8 (Flouri et al., 2018), and the Species Tree And Classification Estimation, Yarely—STACEY v.1.2.2 (Jones, 2017) implemented in BEAST2.

The ASAP builds species partitions from single-locus sequence alignments. For this reason, we performed this analysis based on the mtDNA alignment (*cyt b* and *16S*). The analysis was run using all of the three distance methods available (Jukes-Cantor, Kimura 2-parameter and simple p-distances) under default parameters (Split groups below 0.001 probability and highlighting results between 0.005 and 0.05 genetic distances).

In mPTP, the species were delimited using the mPTP ML approach. Given that mPTP analysis is restricted to a single locus, we used the ML phylogenetic tree produced by the mtDNA dataset.

Bayesian species delimitation was conducted in BP&P to explore different species delimitation models (A11 analysis). For the priors

on population sizes (θ) and divergence times (τ), we used inverse-Gamma priors with $\alpha = 3$. The β parameter was adjusted according to the mean estimate of nucleotide diversity for θ ($\beta = 0.2$) and node height for τ ($\beta = 0.09$). We performed the analyses using the reversible-jump MCMC algorithm 0 ($\varepsilon = 2$). The analysis ran in duplicate to check for convergence. Analyses were run for 200,000 MCMC steps, with samples drawn every five steps and with the first 20% of samples discarded as burn-in. Assignment was based on the mtDNA phylogeny, and the maximum number of mtDNA potential species (seven species; see Section 3) revealed from the single-locus species delimitation analysis (mPTP). Following the suggestion of the authors of BP&P, our analysis was based on nuclear loci only, and not in combination with the mitochondrial ones (see manual of BP&P).

In STACEY, BEAUti v. 2.4.7 (which is also included in BEAST2 v. 2.4.7) (Bouckaert et al., 2014), was used to prepare the input files. All loci were included in the analysis (the two mtDNA genes were considered a single locus). Each individual was considered as a distinct species *a priori*. For the selection of the most appropriate substitution model, we used the bModelTest tool. Ploidy values were fit to 2.0 and 0.5 for nuclear and mitochondrial genes, respectively, and the species tree was estimated with a Yule model with collapsed height nodes of $\varepsilon = 1 \times 10^{-4}$, and birth diff rate of 100 *a priori*. The collapse weight parameter (ω), which affects the number of clusters, was given a flat prior bound from 0 to 1.0, and thus, any possible grouping scheme received the same initial probability. As for other priors, the strict clock model for describing the molecular clock was used. The analysis was run twice with 5×10^8 generations. Results from the STACEY runs were summarized with the species delimitation analysis tool provided along with the STACEY package (speciesDA.jar) using collapse height = 0.001 and sim cutoff = 1.0 (no cluster similarity binning; Jones et al., 2015).

2.5 | Species tree construction and divergence time estimation

All loci (mtDNA and nuDNA) were used to estimate a species tree and divergence times, with the StarBeast2 package included in BEAST2. The required import file (xml) was created using the BEAUti program. As in the case of STACEY, the two mtDNA gene fragments were considered a single locus (the two mtDNA genes were unlinked in site and clock models and linked in trees). Nucleotide substitution models were given *a priori* based on the PF analysis. Priors were used in advance: "Birth Death Model" for the species tree, "Linear with Constant Root Populations" for the population model, and "Uncorrelated Lognormal" for the molecular clock description. The separation between the Peloponnese and Crete and the island group of Pori (*P. levendis*), and therefore the divergence between *P. peloponnesiacus* from *P. cretensis* and *P. levendis*, was used as a calibration point and defined at 5.3 million years ago (Normal distribution with a mean value of 5.3 and a standard deviation of 0.15; see Dermitzakis, 1990 and Introduction above). The MCMC analysis ran for 10^9 generations, extracting results every 5000 generations and rejecting the first 25% of them (burn-in). The resulting log files were analyzed with Tracer (v. 1.6) to determine that convergence of the analysis had occurred and a satisfactory effective sample size (ESS) was recovered (>200). Next, Tree Annotator v. 2.4.7, included in BEAST2, was used to estimate the species tree, calculating the maximum clade credibility tree that best represented the posterior distribution.

2.6 | Morphological data

2.6.1 | Recorded traits

To investigate the morphological differentiation of the two divergent evolutionary clades of *P. peloponnesiacus* (see Section 3), we examined a total of 332 specimens, which included museum specimens from the herpetological collections of the Natural History Museum of Crete (NHMC), the Natural History Museum, London (NHMUK), and the Natural History Museum, Vienna (NMW), and specimens sampled in the field. Specimens were collected from 43 geographical localities. We examined both linear biometric and pholidotic traits. Biometric variables were only considered in adult specimens ($N = 305$) and were always recorded by AK to reduce sources of measurement error. The total number of samples used, their code, their location, and the morphological traits recorded for each one are presented in Table S3, while the geographical localities of the samples used are illustrated in Figure S1.

Each of the specimens examined for morphological analyses was assigned to one of the two phylogenetic clades (1 or 2) based on (1) the genetic analyses of samples from the same population, when available; or (2) the geographical location of the population of origin well within the inferred range of each clade, when such assignment could be done without doubt. Specimens for which their assignment to one of the clades was ambiguous were excluded altogether. Note that for specimens from Stymfalia Lake, where both clades are found in syntopy (Spilani et al., 2019; see also results below), we only included genetically identified individuals, which could be unambiguously assigned to one of the two clades.

For each specimen examined, we measured nine biometric characters to the closest 0.01 mm using electronic calipers: SVL, trunk length (TRL, defined as the closest distance between anterior and posterior limbs), head length (HL), pileus length (PL), head width (HW), head height (HH), mouth opening (MO) measured from the tip of the snout to the posterior border of the last supralabial scale, forelimb length (FLL), and hindlimb length.

In addition, we recorded several pholidotic traits typically used for systematic inferences in lacertids (Kaliontzopoulou et al., 2012; Lymberakis et al., 2008), including both meristic and presence/absence characters. Specifically, for each specimen, we recorded collar scales number (CSN), gular scales number (GSN), the number of transversal rows of ventral scales (VSN), femoral pores number (FPN), subdigital lamellae number under the 4th toe (SDLN), supratemporal scales number (STSN), supraciliary scales number (SCSN), supraciliary granules number (SCGN), the number of supralabial

scales (SLAB), and the number of supralabial scales before the subocular (SLAB_suboc). Furthermore, we recorded the presence/absence of the masseteric (MASS) and the tympanic scale (TYMP); the fragmentation of the tympanic scale (TYMPfr); the presence/absence of contact between the internasal and frontal scales (IN_F), between the occipital and interparietal scales (O_IP), and between the rostral and internasal scales (R_IN); and the presence/absence of a third scale between the aforementioned pairs of scales (3rdIN_F, 3rdO_IP, 3rdR_IN). All bilateral characters were recorded on the right side of the lizard body when possible.

2.6.2 | Statistical analyses

All biometric traits were log-transformed prior to data analyses to better approximate the normal distribution. In order to test for differences in linear biometry between both evolutionary clades considered, we used AN(C)OVA comparisons, where we first tested for differences in body size, as represented by SVL; and in continuation, we used SVL as the covariate to compare variation in the remaining traits while taking size variation into account. The significance of examined AN(C)OVA models was evaluated through 1000 permutations of randomized residuals as implemented in the R-package *RRPP* (Collyer & Adams, 2018, 2019). To further investigate the biometric differentiation of both clades and identify those traits that contributed the most to discriminating them, we performed linear discriminant analyses, with a leave-one-out cross-validation procedure. For this, we first used a linear regression on SVL to remove size effects from the remaining biometric traits. Due to the existence of marked sexual dimorphism in body size and shape in *Podarcis* wall lizards (Kaliontzopoulou et al., 2015; Kaliontzopoulou et al., 2006, 2010), and since sexual differentiation was not the focus of this study, we performed all aforementioned analyses in each sex separately.

The analysis of pholidotic traits focused on different character sets, depending on whether they could be treated as continuous, or should rather be considered as categorical, variables. This provided three sets of pholidotic variables: (1) continuous meristic traits, including CSN, GSN, VSN, FPN, and SDLN; (2) meristic traits that—due to their reduced variation range—were considered as ordinal categorical (STSN, SCSN, SCGN, SLAB, SLAB_suboc); and (3) binary traits (i.e., presence/absence ones, as described above). To test for differences in continuous pholidotic traits between both clades, we followed the same procedures as for linear biometric traits described above, including the evaluation of ANOVA models through randomized residuals on a permutation procedure and discriminant analyses, applied separately to members of each sex. To evaluate the differentiation of both clades in ordinal and binomial variables, we used logistic-regression models with a Poisson and a binomial error distribution respectively. For those traits found to be significantly different between clades, we then inspected the frequency of occurrence of each character state to gain insights with respect to traits that could be diagnostic.

2.7 | Species distribution modeling and niche similarity analyses

Species distribution modeling analyses test the impact of ecological factors on the current distribution of the lineages. Presence only datasets for the two clades of *P. peloponnesiacus* were analyzed. All the presence points used in the analyses were extracted from the NHMC collections database. In total, we used 193 points, 91, and 102 for each of the identified clades, resulting from the phylogenetic analyses. After the clean-up of the dataset for duplicate records and the spatial thinning of the occurrence data, the number of records was reduced to 41 for clade 1 and 17 for clade 2. The area of the modeling was based on the current species distribution (Böhme & Lymberakis, 2009) along with an extension in part of the central Greece and Attiki region due to a record in Athens (Hedman et al., 2017) (Figure S2).

Environmental data, at 1 × 1 km spatial resolution, from the CHELSA database were used (Bobrowski & Udo, 2017; Karger et al., 2017). The spThin R package (Aiello-Lammens et al., 2015) with an occurrence thinner radius of 2 km was used for the minimization of the effects of sampling bias (Boria et al., 2014) and the USDM R package (Naimi et al., 2014) for the calculation of variance inflation factor (VIF) for the set of selected predictors and 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 modeling, allowing the fine tune (Hao et al., 2020) of the MaxEnt algorithm using the ENMeval R package (Muscarella et al., 2014). The "random k-fold" partition scheme of ENMeval was selected for all the analyses (Muscarella et al., 2014) for training/validation. 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 reached 0, when they dropped 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 each clade. The final models selected to best approximate the niche of clades 1 & 2 were then used for the calculation of the niche overlap between clades. To this end, the relevant indices for niche overlap, *D* and *I*, based on Schoener's *D* and modified Hellinger *I* distances, respectively, as proposed by Warren et al. (2008) were calculated using the *dismo* R package (Hijmans et al., 2011). Values with ranges from 0 to 0.2 correspond to "no or limited" niche overlap,

0.2 to 0.4 correspond to "low" overlap, 0.4 to 0.6 correspond to "moderate" overlap, 0.6 to 0.8 correspond to "high" overlap, and 0.8 to 1.0 correspond to "very high" overlap (Rödder & Engler, 2011).

2.8 | Diagnostic characters based on molecular data

Regarding the use of molecular data as diagnostic characters, we adopt the position of Nygren and Pleijel (2011). To this end, that is, to recover diagnostic combinations of nucleotides (DNCs) for pre-defined groups of DNA sequences, we used the program MolD (Molecular Diagnoses) v. 1.3 (Fedosov et al., 2019). A pure composite nucleotide character—*that is*, a combination of nucleotides at specific positions in the DNA alignment that are shared by all members of a focus taxon, and by none of the non-focus taxa members, is termed a primary diagnostic nucleotide combination (pDNC), whereas a diagnostic nucleotide combination, which combines several pDNCs (or characters from several pDNCs) aiming for an increased robustness of a diagnosis, is termed a secondary DNC (sDNC). MolD is designed to retrieve pDNCs and sDNCs for the pre-defined assemblages of DNA sequences that correspond to taxa (which may range from species, subspecies, or even populations to any higher level). The most polymorphic gene fragment (*cyt b*) was used for this analysis, aiming to identify sDNCs for the two major clades of *P. peloponnesiacus* (see Section 3). The numbering was based on the complete cytochrome *b* sequence of *P. muralis*, which is available in GenBank (MT027220).

3 | RESULTS

3.1 | Phylogenetic reconstruction

For the mtDNA dataset, a total of 982 base pairs (bp) were aligned (426 bp for *cyt b* and 556 bp for *16S*), 190 (19.3%) of which were variable, 160 (16.3%) when the outgroup was excluded. For the nuDNA dataset, a total of 3874 bp were aligned (426 bp for *Pod55*, 670 bp for *MC1R*, 983 bp for *Rag1*, 541 bp for *Pod15b*, 590 bp for *NKTR*, and 664 bp for *UBN1)*, 130 sites (3.4%) of which were variable, 115 sites (2.9%) when the outgroup was excluded. Finally, for the concatenated dataset, a total of 4799 bp were aligned (417 bp for *cyt b*, 508 bp for *16S*, 426 bp for *Pod55*, 670 bp for *MC1R*, 983 bp for *Rag1*, 541 bp for *Pod15b*, 590 bp for *NKTR*, and 664 bp for *UBN1*).

The best-fit partitioning scheme for each downstream analysis and the selected nucleotide substitution models are given in Table S4.

Both phylogenetic analyses (ML and BI) on the mtDNA dataset produced trees with fairly similar topologies. The ML analysis resulted in a topology with lnL = −2979.33, while the BI analysis resulted in a topology with mean lnL = −3166.40. Phylogenetic analyses recovered two main very well-supported clades within *P. peloponnesiacus*, the sister group relationship of which has low statistical support (pp = 0.75, bs = 49; Figure 2). On the other hand, the monophyly of *P. cretensis* and *P. levendis* was well supported. The phylogenetic analyses of the nuDNA dataset produced trees with similar topologies (lnL = −6464.78 for ML and lnL = −6487.52 for BI), in which *P. peloponnesiacus* was divided into two main clades, with higher statistical support (pp = 1, bs = 62) compared to the mtDNA dataset. Of these two clades, clade 1 is distributed in western and central Peloponnese (hereinafter *Podarcis peloponnesiacus* West or 1_ and clade 2 in eastern Peloponnese (hereinafter *Podarcis peloponnesiacus* East or 2_ (Figure S3), showing sympatry in the area of lakes Stymfalia and Doxa, where individuals from both clades were found. More specifically, we analyzed 97 individuals from Lake Stymfalia, 24 of which belonged to clade 1 and 73 to clade 2. For the concatenated dataset, both phylogenetic analyses produced a resolved phylogenetic tree (Figure 3; lnL = −9039.30 for ML and lnL = −9086.09 for BI), with two major clades within *P. peloponnesiacus*.

The results of haplotype network analysis for each nuclear gene separately suggested the distinction of the two clades of *P. peloponnesiacus* identified in the gene trees (Figure S4).

3.2 | Genetic distances

Pairwise genetic distances (Tamura-Nei model) between individuals varied up to 7.8% for *16S* (up to 6.4% without the outgroup and up to 5.6% within *P. peloponnesiacus*) and up to 18.4% for *cyt b* (up to 12.2% without the outgroup and up to 11.6% within *P. peloponnesiacus*). Sequence divergence ranged up to 1.4% for *Pod55*, up to 1.8% for *MC1R*, up to 4.9% for *Rag1*, up to 1.5% for *Pod15b*, up to 1.7% for *NKTR*, and up to 2.6% for *UBN1*. Genetic distances among the major phylogenetic lineages are presented in Table 1.

3.3 | Species delimitation, species tree, and divergence times estimations

The mPTP analysis revealed seven clusters (potential species) in the phylogenetic tree of the mtDNA dataset with the best score for multi coalescent rate of 408.29. Three of them correspond to *P. peloponnesiacus* (one to the west clade, and two to the east clade; see Figure 2), two to *P. cretensis*, and the last two to each one of the remaining species (*P. levendis* and *P. erhardii*).

In all cases of ASAP analyses (using Jukes-Cantor, Kimura 2-parameter, and *p* distance), the best partition, which means the partition with the lowest ASAP-score, was that with six potential species (*P. erhardii*, *P. levendis*, two in *P. cretensis*, and two in *P. peloponnesiacus* (east and west clades).

In STACEY analysis, the SpeciesDelimitationAnalyser found classification with five clusters (species) as the one with the highest posterior probability (0.86); *P. cretensis*, *P. levendis*, *P. erhardii*, and the two clades of *P. peloponnesiacus* (east and west) as distinct species. The species tree obtained as part of species delimitation is given in Figure S5, indicating the presence of two potential species within *P. peloponnesiacus* with very high posterior probabilities (1.00).

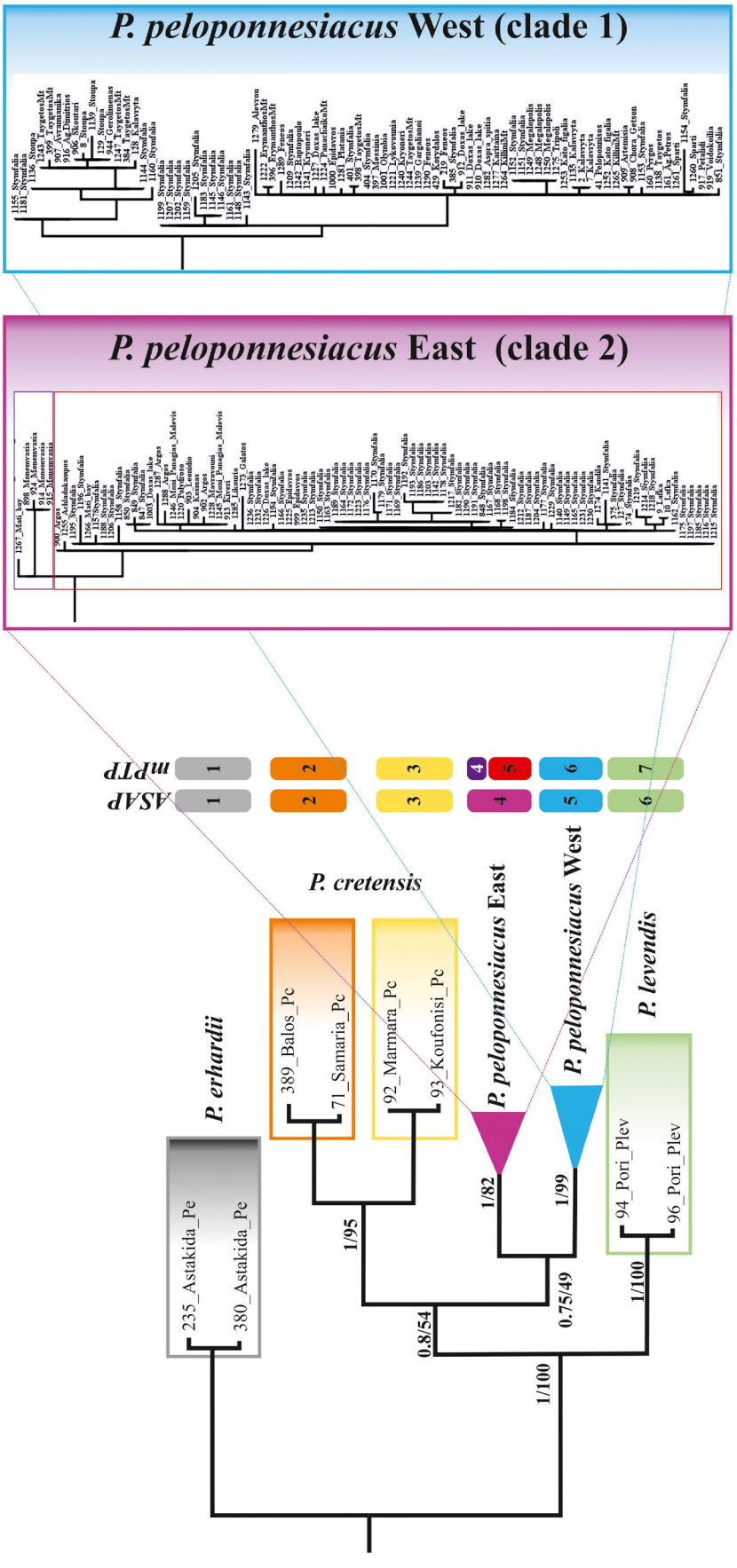

FIGURE 2 The ML mitochondrial gene tree (*cyt b* & 16S rRNA). The posterior probabilities and bootstrap supports (BI/ML) are presented on the branches. To clades, the vertical bars represent the number of distinct entities (potential species) based on mPTP (seven in total) and ASAP (six in total) analyses. On the right side, the two main clades of *P. peloponnesiacus* are shown in detail

The model estimated with BP&P (PP = 0.77) supported the presence of five species as in the case of STACEY, in which the posterior probabilities for the potential species in *P. peloponnesiacus* were 1.00 for the west clade and 0.97 for the east clade.

The species tree (Figure 4) resulting from StarBEAST2 showed high posterior ESS values (>342) for all parameters Topologically speaking, this species tree obtained from StarBEAST2 and STACEY and the gene trees from ML and BI analyses on the complete dataset led to similar conclusions regarding the relationships among the major clades (species) with the distinction of *P. peloponnesiacus* into two major clades (east and west) with high posterior probability [pp = 0.99 in StarBEAST2 (Figure 3) and 0.96 in STACEY (Figure S5)].

According to the chronophylogenetic analysis (Figure 4), the time of divergence between the two clades of *P. peloponnesiacus* was estimated at 1.72 million years ago (the beginning of the Pleistocene), with the range from 0.92 to 2.57 million years ago (95% HPD). The differentiation between *P. levendis* and *P. peloponnesiacus* dates back to 3.55 MYA, with a range from 1.65 to 3.8 million years.

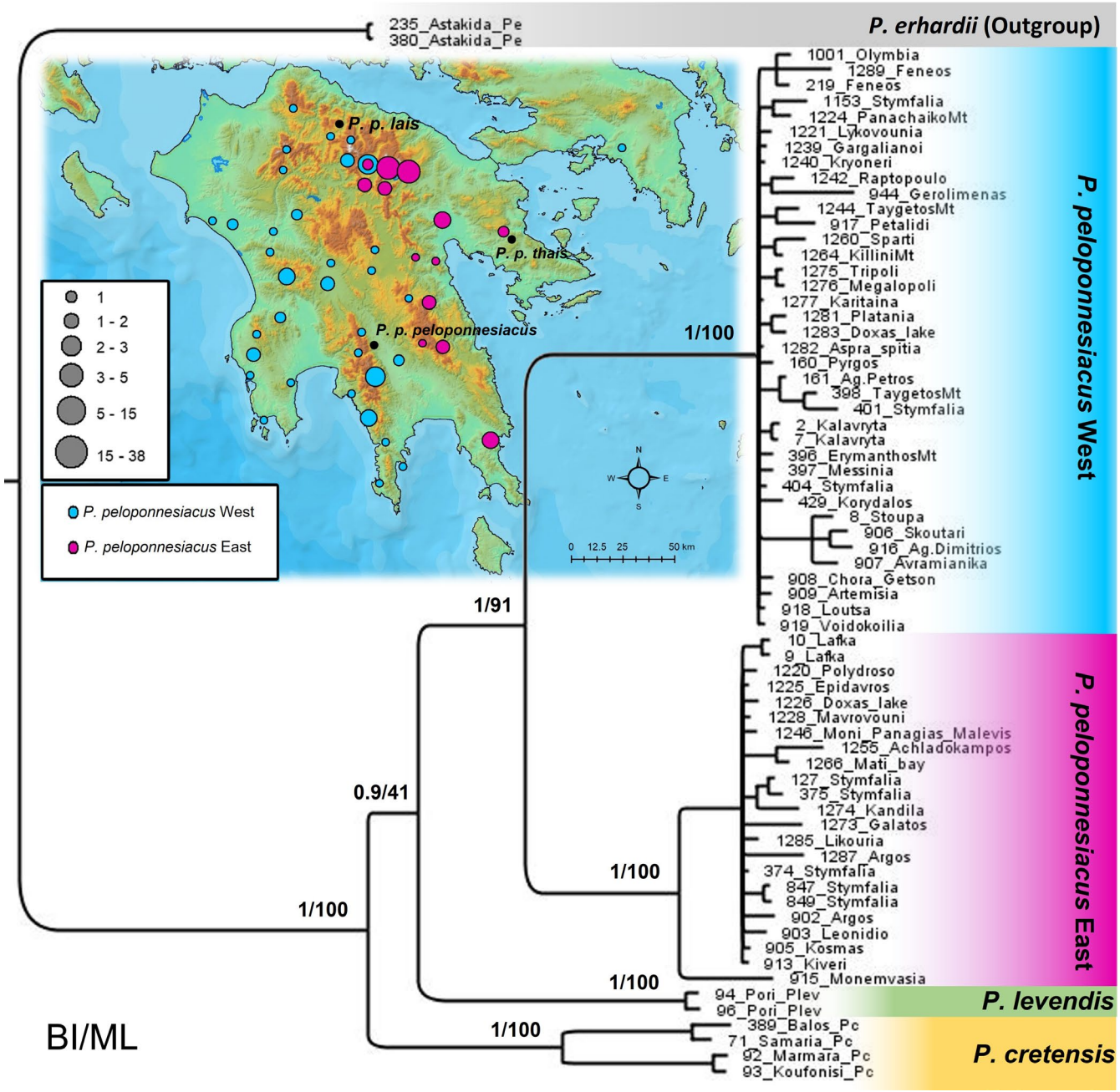

FIGURE 3 Bayesian inference tree based on the concatenated (mtDNA & nuDNA) dataset. The posterior probabilities (>0.95) and bootstrap support (>50%) of both phylogenetic methods are given on top of the branches. The map within the tree shows the geographical distribution of specimens of the two clades of *P. peloponnesiacus*. The size of the circles indicates the number of specimens per 100 km^2. Black dots show the type localities of previously defined subspecies

TABLE 1 Genetic distances using the Tamura-Nei model among major phylogenetic lineages: Cyt b/16S rRNA below diagonal-left, Pod55/MC1R/Rag1/Pod15b/NKTR/UBN1 above diagonal-right

	P. peloponnesiacus west	*P. peloponnesiacus* east	*P. levendis*	*P. cretensis*	*P. erhardii*
P. peloponnesiacus west		1/0.9/0.4/0.8/0.9/0.6	0.9/0.9/0.7/1.4/0.5/0.8	0.8/1/0.3/1.7/0.4/0.9	1.3/0.8/0.4/1.5/1.2/1.9
P. peloponnesiacus east	6.3/3.4		0.4/0.3/0.6/1/0.9/0.6	0.3/0.4/0.3/1.4/0.8/0.6	0.8/0.8/0.4/1/1.6/1.6
P. levendis	7.9/3.4	9.0/4.1		0.1/0.4/0.5/1.2/0.2/0.7	0.6/0.8/0.5/0.8/1/1.7
P. cretensis	8.0/3.9	8.6/4.5	9.4/5.1		0.5/0.9/0.1/1.3/0.9/1.4
P. erhardii	15.0/4.8	14.6/5.8	14.9/5.4	15.8/5.1	

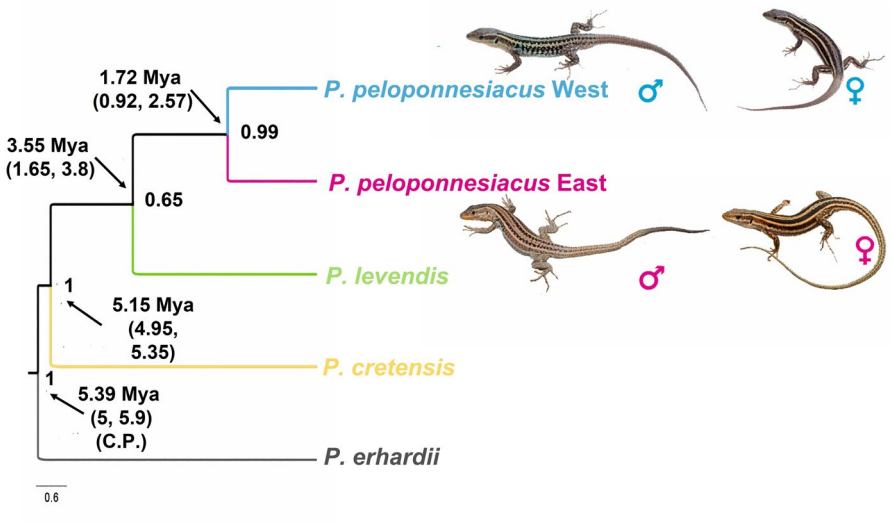

FIGURE 4 The tree produced by the chronophylogenetic analysis. For the species tree, the posterior probabilities are given near the branches. Values >0.95 indicate statistical significance. For the divergence times, numbers showing arrows represent the divergence time in millions of years, while the parentheses indicate the uncertainty (95% HPD) of the time estimation. C.P. refers to the calibration point

3.4 | Morphological analyses

The morphological comparisons performed between individuals of the two clades observed within *P. peloponnesiacus* indicated the existence of significant differences in some—but not all—biometric and pholidotic traits, which were more prominent in males than in females. Individuals of the two clades did not differ in body size in either sex, but there were several body parts that did differ in females, namely relative TRL, PL, and MO; and all body dimensions relative to SVL in males, except for HH and FLL (Table 2). Similarly, we found significant differences between clades in the number of collar scales and femoral pores in both sexes, in addition to the number of gular and ventral scales only in males (Table 3). Indeed, individuals of clade 2 (eastern lineage) exhibited a lower number of collar scales (CSN), but a higher number of femoral pores (FPN) in both sexes, while males of clade 2 also exhibited a higher number of gular (GSN) and a higher number of ventral (VSN) scales (Figure 5). Despite these differences, however, the ranges of variation overlapped widely between both clades, not allowing the retrieval of unique diagnostic traits (Table S5).

Discriminant analyses performed to (size-corrected) biometric and continuous pholidotic traits reinforced the notion that, despite statistically significant morphological differentiation in both sexes, the morphological properties of the two clades are quite similar, yielding a relatively low capacity of their correct discrimination. Indeed, leave-one-out cross-validation of the discriminant functions constructed based on biometric variables resulted in moderate percentages of correct classification, which were lower for clade 2 (females: 63.04%; males: 42.37%) than for clade 1 (females: 86.36%; males: 86.60%). Examination of discriminant vector composition revealed that relative TRL and relative MO were the most relevant traits for the discrimination of eastern and western female individuals, while relative MO and elative FLL contributed the most for discriminating males (Table S6). Similarly, discriminant functions constructed based on continuous pholidotic traits resulted in relatively low percentages of correct classifications, with clade 2 being classified correctly at a lower percentage (females: 55.77%; males: 59.15%) than clade 1 (females: 88.30%; males: 81.74%). The number of collar scales (CSN) and femoral pores (FPN) was the most relevant variables

TABLE 2 Results of AN(C)OVA comparisons of linear biometric traits between the two clades of *P. peloponnesiacus* for each sex separately

		Females ($N = 133$)				Males ($N = 179$)		
		SS	Z	*p*		SS	Z	*p*
SVL	clade	0.004	0.269	0.478	Clade	0.001	−0.377	0.751
TRL	SVL	2.223	3.490	**0.001**	SVL	1.496	3.414	**0.001**
	clade	0.061	2.118	**0.001**	Clade	0.030	1.586	**0.002**
	SVL: clade	0.001	0.245	0.498	SVL: clade	0.000	−0.759	0.833
HL	SVL	0.391	2.796	**0.001**	SVL	1.022	3.263	**0.001**
	Clade	0.002	0.535	0.340	Clade	0.037	1.779	**0.001**
	SVL: clade	0.009	1.105	0.072	SVL: clade	0.016	1.474	**0.011**
PL	SVL	0.532	3.309	**0.001**	SVL	0.902	3.081	**0.001**
	Clade	0.028	2.043	**0.001**	Clade	0.066	1.997	**0.001**
	SVL: clade	0.001	0.732	0.227	SVL: clade	0.009	1.139	0.082
HW	SVL	0.383	2.769	**0.001**	SVL	0.970	3.192	**0.001**
	Clade	0.012	1.212	0.051	Clade	0.045	1.805	**0.001**
	SVL: clade	0.000	−2.078	0.961	SVL: clade	0.000	−0.528	0.775
HH	SVL	0.595	2.944	**0.001**	SVL	0.944	2.881	**0.001**
	Clade	0.001	−0.129	0.644	Clade	0.016	1.038	0.113
	SVL: clade	0.000	−0.695	0.804	SVL: clade	0.001	−0.516	0.766
MO	SVL	0.582	3.462	**0.001**	SVL	1.065	3.320	**0.001**
	Clade	0.044	2.126	**0.001**	Clade	0.061	2.203	**0.001**
	SVL: clade	0.002	0.865	0.165	SVL: clade	0.000	−1.009	0.859
FLL	SVL	0.241	2.543	**0.001**	SVL	0.639	3.085	**0.001**
	Clade	0.008	0.927	0.116	clade	0.001	−0.041	0.621
	SVL: clade	0.007	0.953	0.125	SVL:clade	0.000	−2.398	0.971
HLL	SVL	0.275	3.055	**0.001**	SVL	0.596	3.162	**0.001**
	Clade	0.007	1.173	v	Clade	0.020	1.715	**0.001**
	SVL: clade	0.000	0.056	0.806	SVL: clade	0.001	0.177	0.521

Abbreviations: SS: sum of squares, Z: effect sizes, *p*: corresponding *p*-value based on 1000 residual randomizations. Significant effects are in bold text (see Section 2 for variable acronyms).

TABLE 3 Results of ANOVA comparisons of continuous pholidotic traits between the two clades of *P. peloponnesiacus* for each sex separately

	FEMALES ($N = 145$)			MALES ($N = 185$)		
	SS	Z	Pr(>F)	SS	Z	Pr(>F)
CSN	0.320	2.143	**0.001**	0.242	1.868	**0.001**
SDLNright	0.000	−0.919	0.844	0.001	0.045	0.581
GSN	0.018	1.160	0.071	0.061	1.635	**0.001**
VSN	0.000	−0.942	0.848	0.018	1.936	**0.001**
FPN	0.171	2.129	**0.001**	0.179	2.120	**0.001**

Abbreviations: SS: sum of squares, Z: effect sizes, *p*: corresponding *p*-value based on 1000 residual randomizations. Significant effects are in bold text (see Materials & Methods for variable acronyms).

contributing to clade discrimination, in addition to the number of gular scales (GSN) in males (Table S7).

Finally, GLMs comparing the frequency distributions for categorical pholidotic traits revealed differences between clades in the number of supraciliary granules (SCGN), the probability of the presence of the masseteric scale (MASS), and the occurrence of contact between the rostral and internasal scales (R_IN; Table 4). Despite statistically identifiable differences between clades, however, none of these traits were strictly diagnostic, as all character states occurred in both clades (Figure 6).

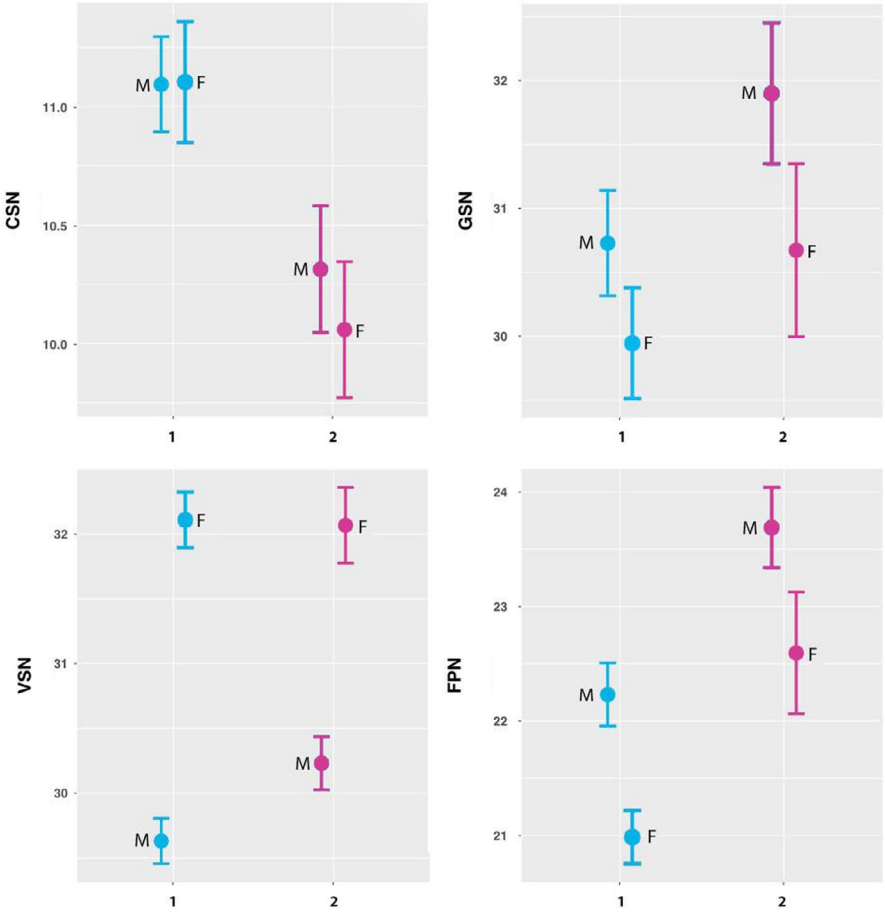

FIGURE 5 Means and confidence intervals for males (M) and females (F) of each clade (1 or 2) for continuous pholidotic traits (Numbers of: collar scales (CSN), gular scales (GSN), ventral scales (VSN), and femoral pores (FPN) for which significant differences between clades were identified)

TABLE 4 Results of GLMs applied to ordinal and binary pholidotic traits to test for differences between the two clades of *P. peloponnesiacus*

	Coef	SE	Z	*p*
Ordinal				
STSN	−0.087	0.057	−1.515	0.130
SCSN	−0.069	0.049	−1.391	0.164
SCGN	−0.809	0.112	−7.235	**$4.67*10^{-13}$**
SLAB	−0.029	0.039	−0.748	0.455
SLAB_suboc	0.000	0.054	−0.009	0.993
Binary				
MASS	−1.545	0.363	−4.252	**$2.11*10^{-05}$**
TYMP	1.644	1.160	1.417	0.157
TYMPfr	−0.946	0.920	−1.028	0.304
O_IP	−0.346	0.245	−1.409	0.159
X3rdO_IP	−0.048	0.582	−0.082	0.935
IN_F	−0.015	0.739	−0.020	0.984
X3rdIN_F	−0.043	0.464	−0.092	0.927
R_IN	1.447	0.550	2.631	**$8.50*10^{-03}$**
X3rdR_IN	0.872	1.124	0.776	0.438

Abbreviations: Coef, estimated regression coefficient; SE, standard error; Z, effect sizes; *p*, corresponding *p*-value. Significant effects are in bold text (see Materials & Methods for variable acronyms).

3.5 | Species distribution modeling and niche similarity analyses

Based on the VIF analysis, six out of 19 initial predictors were retained in the models, including temperature seasonality (BIO4), the maximum temperature of the warmest month (BIO5), the mean temperature of the wettest quarter (BIO8), precipitation seasonality (BIO15), precipitation of the warmest quarter (BIO18), and precipitation of the coldest quarter (BIO19). Four and two random folds for calibration/validation were created for clades 1 and 2 respectively. The difference was due to the final dataset (41 points for clade 1 and 17 for clade 2), for which 4100 and 1700 pseudoabsence points were randomly created following the recommendations of Barbet-Massin et al. (2012). Forty-five models have been run consecutively for each clade, and the model with the lowest delta corrected Akaike information criterion (*delta.AICc*) has been selected (Tables S8–S11). For clade 1, the selected model used the L, Q, and H features with a beta value of 1.5 (hereafter LQH_1.5) (Table S8) and clade 2 used the L, Q, and a beta value of 1 (LQ_1) (Table S9; Figure 7). Clade 1 had a training AUC of 0.8 and avg.test.AUC of 0.74, whereas clade 2 a train.AUC of 0.87 and avg.test.AUC of 0.6. For clade 1 (Table S10), the mean temperature of the wettest quarter (BIO8), the precipitation seasonality (BIO15), and the precipitation of the warmest quarter (BIO18) predictors seemed to contribute the

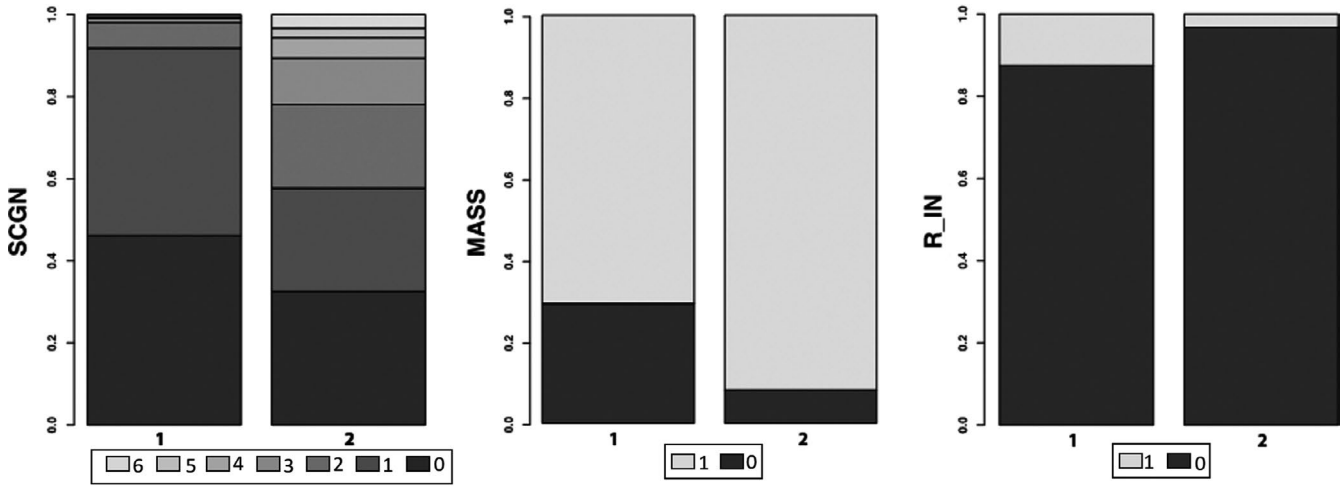

FIGURE 6 Relative frequencies of distribution observed in each clade (1 or 2) for the number of supraciliary granules (SCGN), the presence of a masseteric scale (MASS), and the presence of contact between the rostral and internasal scales (R_IN)

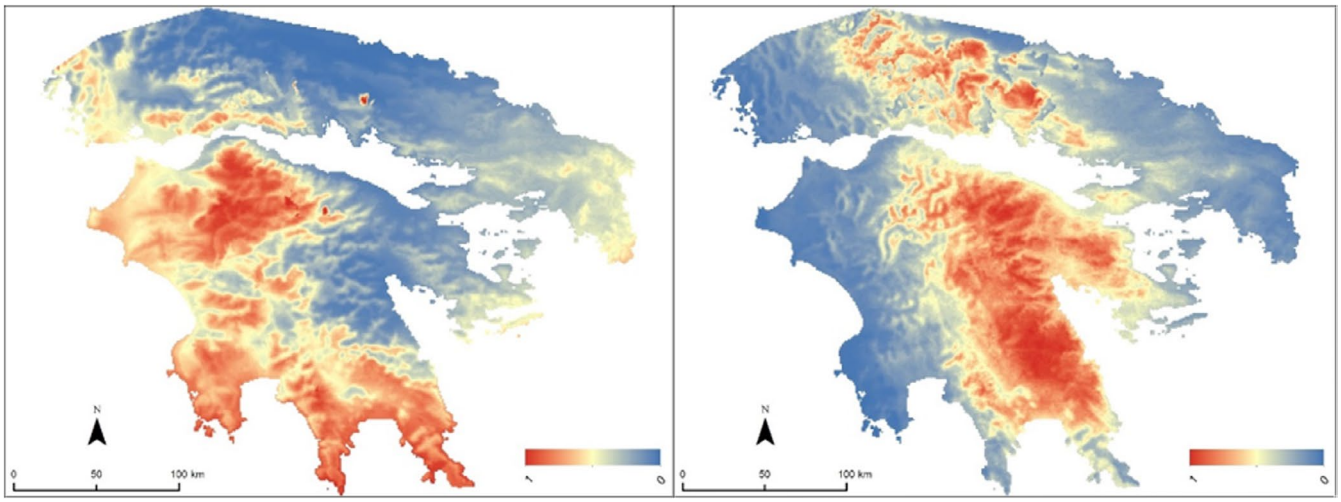

FIGURE 7 Model output for the western clade (clade 1) on the left and the eastern clade (clade 2) on the right of *P. peloponnesiacus*

most to the model, with 95% (cumulatively) of permutation importance. For clade 2 (Table S11), the mean temperature of the wettest quarter (BIO8), the precipitation seasonality (BIO15), the precipitation of the warmest quarter (BIO18), and the precipitation of the coldest quarter (BIO19) contributed with 92% of permutation importance.

Pairwise evaluation of niche similarity between the two clades indicated "moderate" to "high" niche overlap according to both indices (D = 0.6, I = 0.86).

3.6 | Diagnostic characters based on cyt *b*

For the two major clades of *P. peloponnesiacus* revealed in phylogenetic analyses (east and west), which were recognized as potential species by species delimitation analyses (STACEY, ASAP, and BP&P), there exist several diagnostic nucleotide sites in cyt *b*. For the western clade, these are: position 165, nucleotide 'C', 247: 'T', 261: 'G', 288: 'C', 294: 'T', 345: 'T', and for the eastern clade these are: 171: 'T', 192: 'T', 318: 'T'. As we mentioned before, this numbering is based on the complete cytochrome *b* sequence of *P. muralis* retrieved from GenBank (MT027220).

4 | DISCUSSION

This study constitutes the first integrative research on *P. peloponnesiacus*, analyzing genetic, phenotypic, and environmental data. The main question addressed through this integration of different evidence is whether the existence of two differentiated lineages identified within *P. peloponnesiacus* in previous studies (Lymberakis et al., 2008; Spilani et al., 2019, 2020) is confirmed with the inclusion of a larger number of samples and a more comprehensive representation of the geographical distribution of the

species. To this end, we analyzed a great number of genetic loci and carried out an assessment of morphological variation. We performed phylogenetic and species delimitation analyses together with species distribution modeling, and morphological (biometric and pholidotic) analyses. Ultimately, our main objective was to evaluate whether this subspecific divergence merits taxonomic distinction as different species, and whether these are morphologically and ecologically differentiated.

4.1 | Phylogenetic relationships within Podarcis peloponnesiacus

Phylogenetic analyses of the mtDNA dataset alone recovered two main clades within the species, with limited statistical support (p.p. = 0.75, b.s. = 49). However, analyses of the nuDNA dataset showed the same topology, with higher support values (p.p. = 1, b.s. = 62), which were further enhanced when using the concatenated (mtDNA and nDNA) dataset (p.p. = 1, b.s. = 92), which produced a completely resolved phylogeny. The inferred species tree, based on the combined dataset, showed the same topology (p.p. = 0.99) with that produced based on the concatenated dataset. In agreement with all of the above, the three different species delimitation approaches that were used to evaluate species' boundaries clearly supported the distinction of two differentiated groups of lineages within *P. peloponnesiacus*, with high posterior probabilities, while the two clades of *P. peloponnesiacus* are distinct at the nuclear level, as it is shown on the haplotype networks, but the discrimination is based on a limited number of samples compared to mitochondrial data. Equally important is the observation that the two groups of lineages are geographically separated, consisting of individuals from the western/central (clade 1) and the eastern (clade 2) Peloponnese (Figure 3). Similar distribution patterns in the area of the Peloponnese, that divide populations between the western and the eastern part of the peninsula, have also been found in other lizards of the family Lacertidae, such as *Hellenolacerta graeca* (unpublished data), in other groups of organisms such as spiders (Kornilios et al., 2016) and in isopods (Kamilari et al., 2014). Of particular interest is the fact that Lakes Stymfalia and Doxa form the intersection point of these clades, where individuals of both clades live in sympatry. Sympatric occurrence has also been confirmed for the same areas for the slow worms *A. graeca* and *A. cephallonica* (Jablonski et al., 2016).

Here, we should highlight that in the study of Spilani et al. (2019), where the population structure of *P. peloponnesiacus* was examined using microsatellite data, all the examined individuals of *P. peloponnesiacus* (they analyzed a total of 52 individuals, all of which are included in our study) were unequivocally assigned to two geographically distinct clusters, West and East Peloponnese. These findings, together with the evidence provided here, indicate that there is no genetic flow between individuals of the two clades and, probably, there are no hybrids. In the same study, phylogenetic analysis recovered two distinct subclades within *P. peloponnesiacus*, whose monophyly was well supported, and species delimitation analysis also revealed the existence of two species within *P. peloponnesiacus*. Another very recent paper (Psonis et al., 2021), using genomic data (SNPs data through ddRADseq), revealed the splitting of *P. peloponnesiacus* into two clades that coincide with the east and west clade of our study. The same study also uncovered the relationships between *P. peloponnesiacus*, *P. cretensis*, and *P. levendis*, where *P. levendis* is more closely related to *P. peloponnesiacus* than to *P. cretensis*, a fact that is also affirmed from our phylogenetic tree revealed from the concatenated dataset.

Genetic distances between the two clades of *P. peloponnesiacus* (6.3% for *cyt b* and 3.4% for *16S*) are of a similar order of magnitude to those found between species of the same genus, such as between *P. peloponnesiacus*, *P. levendis*, and *P. cretensis* (present study), or among members of the *P. tauricus* species group (Psonis et al., 2017, 2018) while they are very close to those of the species of the genus *Mesalina* (Kapli et al., 2008). Moreover, they are equivalent to those found between other species of the genus *Podarcis* outside Balkans, such as between *P. siculus* and *P. waglerianus* (6.6% for *cyt b* and 2.2% for *16S*; Psonis et al., 2017).

4.2 | Species divergence and biogeography

The "moderate" to "high" niche overlap between the two clades, in combination with the high overlap of the bioclimatic variables that most contribute to each model (BIO8, BIO15, and BIO18 for clade 1 and BIO8, BIO15, BIO18, and BIO19 for clade 2), suggest that the distinct distribution of the two clades is probably not related to ecological differentiation. Instead, it may be due to biogeographical events. Given that individuals of the two clades, in the areas where they are found in sympatry, retain their identities, we could assume that they are currently excluding each other, although our data are not enough to support it. The strong geographical cohesiveness of both the nuclear and mitochondrial genetic signal within *P. peloponnesiacus* suggests a history of parapatric divergence of the two clades. The chronophylogenetic analysis estimated the time of divergence between the two clades of *P. peloponnesiacus* to be about 1.72 Mya, a time that corresponds to the lower Pleistocene period, which is very close to the estimation of previously published studies (1.86 Mya) (Spilani et al., 2020). This divergence is also consistent with the most intraspecific divergences in the genus *Podarcis*, that have occurred within the Pleistocene, and imply a major role for climatic and sea-level changes during this epoch (Salvi et al., 2021).

From a biogeographical point of view, the Peloponnese's mountain massifs constitute an extension of the Pindos Mountain range and consequently of the External Hellenides [the western and southernmost part of the Hellenic Orogenic belt, that run from western continental Greece to the Peloponnese and Crete (Dornsiepen et al., 2001)], which expanded during the Pliocene (van Andel et al., 1993; Papanikolaou, 2010), while, by the same period, extensive lowland areas throughout the Peloponnese were inundated by the sea (Dermitzakis, 1990). Moreover, during the Pliocene, there was a series of tectonic rearrangements, which resulted in an important size reduction, with the present

mountainous areas becoming the only non-submerged land surfaces (Creutzburg, 1963). In the Upper Pliocene and Pleistocene, in the area around Megalopolis, there was a large deep freshwater lake (Falniowski & Szarowska, 2016), which may have been the initial geographical barrier that gradually isolated populations. Sea intervening between mountains also acted as a biogeographic barrier that prevented gene flow among populations (Psonis et al., 2018). In the Pleistocene, the Peloponnese was reconnected to mainland Greece, with paleogeographic illustrations showing wide land bridges between them (Ferentinos et al., 2012; Perissoratis & Conispoliatis, 2003) due to a major drop of sea level during the last glacial maximum (van Andel & Shackleton, 1982). In the middle Pleistocene, there were two sea introgressions throughout the Peloponnese (Siavalas et al., 2009), with populations probably surviving in higher altitudes. The high mountains of the Peloponnese (e.g., Helmos, Killini and Taygetos) had already attained their current altitude during the Pleistocene (Dimopoulos, 1993), providing suitable environments, for the survival of *P. peloponnesiacus* during ice ages. As for the contact zones of the two clades, lakes Stymfalia and Doxa, they formed at the end of the Pleistocene, with the separation of Killini from Mainalo and of Helmos from Killini respectively (Dimopoulos, 1993).

Based on the above, we could assume that *P. peloponnesiacus* was originally a single evolutionary unit, distributed throughout the Peloponnese, when it was still a continuous land mass. During the Pliocene, the species may have started to subdivide into diverging populations due to biogeographical barriers. Populations would then have been isolated in separate glacial refugia, resulting in reproductive isolation, leading to genetic differentiation due to drift (Abellán & Svenning, 2014). The Peloponnese could have served as an area with multiple subrefugia (Jablonski et al., 2016; Jesse et al., 2010). More specifically, the northern mountain ranges of Killini, Oligyrtos, Trachi, and Lyrkio, as well as Parnonas mountain in the southern part of the Peninsula, form a high-elevation line. This separates the western and eastern Peloponnese and could represent a geographical barrier (For a geographical map showing the mountain ranges of the Peloponnese, see Figure S6). At the end of the Pleistocene, when climatic conditions became favorable, western and eastern lineages could subsequently come into secondary contact.

4.3 | Morphological distinctiveness

In order to explore the phenotypic differentiation between the two clades of *P. peloponnesiacus*, we performed an extensive investigation of biometric and pholidotic traits. Unfortunately, none of these characters could be considered as diagnostic between clades, because even if there were some that had significant differences between two populations, their ranges of variation overlapped. However, it is not the first time that diagnostic characters have failed to be defined; the same has been observed in other species of the genus *Podarcis*, between closely related lineages that have experienced high genetic differentiation (Bruschi et al., 2006; Kaliontzopoulou et al., 2012; Rodríguez et al., 2017), where the range of scale counts among species largely overlap. It is well documented that *Podarcis* lizards, although exhibiting high phenotypic variation between populations and individuals of the same species (Carretero, 2008; Kaliontzopoulou et al., 2018), are characterized by a generally conserved morphology, leading to high levels of cryptic diversity (Kaliontzopoulou et al., 2011, 2012), and the study of these species has revealed that speciation is not always coupled with morphological change (Bickford et al., 2007).

4.4 | Taxonomy

Despite years of study, the taxonomy of the genus *Podarcis* is complex and continuously under reconsideration leading to repeated revisions and/or identifications of new species (Carretero, 2008; Harris et al., 2002; Kaliontzopoulou et al., 2011; Lima et al., 2010; Lymberakis et al., 2008; Pinho et al., 2007; Poulakakis et al., 2005; Psonis et al., 2017; Rodríguez et al., 2017; Salvi et al., 2017; Sá-Sousa & Harris, 2002; Senczuk et al., 2019). There are several cases of *Podarcis* species that were raised from subspecies to species rank (for a review see Senczuk, Harris, et al., 2019; Speybroeck et al., 2020) or that are considered species complexes due to high levels of cryptic genetic diversity (Bellati et al., 2011; Harris et al., 2005; Harris & Sá-Sousa, 2001; Pinho et al., 2006; Podnar, Mayer, & Tvrtković, 2004, 2005; Poulakakis et al., 2003, 2005; Psonis et al., 2017). Very recently, a new species (*Podarcis galerai*) was discovered in the *P. hispanicus* complex (Bassitta et al., 2020), based exclusively on molecular analyses, without any morphological differentiation from its closest lineage (*P. hispanicus*).

Although the monophyly of *P. peloponnesiacus* has been strongly supported by previous studies (Lymberakis et al., 2008; Poulakakis et al., 2005; Spilani et al., 2019), our data showed that a rearrangement of the taxonomic status of this monophyly is needed. Recapitulating essential relevant parts of the information presented above, there are three main issues to consider:

1. During recent years, there has been an accumulation of data from various sources indicating that the monophyletic species *P. peloponnesiacus*, consists of two distinct clades, which began diverging ~1.8 Mya. Specifically, presence data in numerous localities, combined with data of molecular markers (mtDNA, nuDNA, and microsatellites) in Spilani et al. (2019), phylogenomic data (ddRADseq) in Psonis et al. (2021), and molecular markers (mtDNA, nuDNA), together with morphological data in the present study, were statistically analyzed in a series of approaches including sequence relationships, chronophylogenetic analysis, species delimitation techniques, morphological comparisons, and niche similarity models. These produced overwhelmingly congruent data from distinct realms and are unequivocal in recognizing two distinct taxa, mostly parapatric, except for a single point of syntopic coexistence, nevertheless, with no evidence of genetic admixture whatsoever.

2. The data on which the designation of subspecies was initially based in Buchholz (1960) were rather weak in a taxonomic sense, as the author worked exclusively on coloration patterns and colors, for which he mentions overlap among the taxa recognized. This was combined with somewhat vague descriptions of the subspecies distributions. Bringsøe (1986) altered this view, sinking one of Buchholz's (1960) subspecies (*P. p. phryne*) in synonymy with *P. p. lais*. Nevertheless, distributions restricted in Bringsøe (1986) were still not based on defined limits among the taxa, but rather attributed to respective secondary divisions (Provinces) of the Greek administrative system (see Introduction).
3. The distributions of the two new taxa mentioned in the first point partially (inasmuch as their distribution areas were never strictly defined) coincide with the ones of previously designated subspecies as described in point 2. The relationships and distributions of the two groups of taxa (the three of Bringsøe (1986) and the two proposed in this study) are explicitly presented below in the description of the new taxa.

Following the reasoning above, we propose splitting the species *Podarcis peloponnesiacus* to two taxa, specifically:

Podarcis peloponnesiacus (Bibron and Bory de Saint-Vincent, 1833).

Synonymy: *Lacerta peloponnesiaca peloponnesiaca* Bibron and Bory de Saint-Vincent, 1833.

Podarcis peloponnesiacus peloponnesiacus (Bibron and Bory de Saint-Vincent, 1833).

Lacerta peloponnesiaca lais Bucholz, 1960 ("Kalavrita, Nordwest-Peloponnes").

Lacerta peloponnesiaca phryne Bucholz 1960 ("Berg Velia, südlich Kalavrita, oberhalb de Waldgürtels").

Type locality

"Mistras bei Sparti (Peloponnes)" restricted by Buchholz (1960). Holotype: MNHN-RA 2706 with the paratype series designated by the same author, including the nominotypical subspecies.

Distribution

The western part of the Peloponnese as presented in Figure 2.

Description

The description of the species follows Buchholz (1960) and is completed by the morphological data presented here (paragraph 3.4). However, there is no combination of morphological characters adequate to safely discriminate *P. peloponnesiacus* from the new taxon described at the species level below, *P. thais*.

Differential diagnosis

A differential diagnosis of the species regarding closely related congenerics is provided in Lymberakis et al. (2008). However, neither the latter nor the morphological characters examined here provide an unequivocal diagnosis with respect to *P. thais* (see below). Hence, we restrict our differential diagnosis exclusively to molecular characters: In our *Cyt b* alignment, nucleotide position 165 is occupied by base 'C'; nucleotide position 247 is occupied by base 'T'; nucleotide position 261 is occupied by base 'G'; nucleotide position 288 is occupied by base 'C'; nucleotide position 294 is occupied by base 'T'; and nucleotide position 345 is occupied by base 'T'.

Podarcis thais (Buchholz, 1960) **stat. nov**.

Synonymy: *Lacerta peloponnesiaca thais* Buchholz, 1960

Podarcis peloponnesiacus thais (Buchholz, 1960).

Type locality

"Aesculap-Heiligtum (400 m), Argolis".

Holotype

ZFMK-H 12261, and the type series designated by Buchholz (1960).

Distribution

The eastern part of the Peloponnese as presented in Figure 2. A discrepancy between the distribution proposed by Bringsøe (1986) and the one adopted here, regarding the specimens of the eastern slopes of Mt. Parnon and southwards from there (Figure 2). According to the present work, these populations fall unequivocally under the new taxon *P. thais*, which is elevated here.

Description

The description of the species follows Buchholz (1960) and is completed by the morphological data presented here (paragraph 3.4).

Differential diagnosis

In accordance with the reasoning of the differential diagnosis for *P. peloponnesiacus*, the differential diagnosis of *P. thais* is also exclusively based on molecular characters: In our *Cyt b* alignment, nucleotide position 171 is occupied by base 'T'; nucleotide position 192 is occupied by base 'T'; and nucleotide position 318 is occupied by base 'T'. The differential diagnosis with molecular characters supports differentiating the species from *P. peloponnesiacus*. For other closely related congenerics, the differential diagnosis in Lymberakis et al. (2008) of the former parent taxon (*P. peloponnesiacus*) is also valid for the new species.

4.5 | Conclusions

In this study, we showed there to be high intraspecific genetic variation within *P. peloponnesiacus*, highlighted the existence of a cryptic lineage with reproductive isolation [we combined our results with those of Spilani et al. (2019)] and a west-east geographical pattern. We also suggested, in accordance with many previous studies, that landscape and environmental events in the southern Balkan Peninsula (including the Peloponnese) were most probably major factors in species diversification processes. The revealed genetic structure of *P. peloponnesiacus* has considerable

implications for the taxonomy of the species and subsequently for its conservation. We proposed that the two clades within *P. peloponnesiacus* constitute two different taxa, and thus, we elevated *P. p. thais* to species level. Although *P. peloponnesiacus* has been suggested to be a species whose population and habitat are not threatened, it is now necessary to properly evaluate population sizes and revaluate possible threats to both these endemic taxa. Finally, our results clearly show that the general pattern of possible speciation events in *P. peloponnesiacus* is comparable to those of other taxa in the Peloponnese and the presence of divergent clades matches the historical biogeography of the Peloponnese peninsula.

ACKNOWLEDGMENTS

We thank Manolis Nikolakakis (NHMC, University of Crete) for helping in map drawing, Elias Tzoras for providing lizard photographs, and the reviewers and the editor, for providing comments that improved the first version of the manuscript. We would like also to thank Dr. Anna-Nicola Chapman (native speaker of English, Biology Department at the University of Athens) for manuscript proofreading. DJ was supported by the Slovak Research and Development Agency under contract no. APVV-19-0076.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the correspondig author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Figure S1. Geographic origin of the the specimens used in morphometrics.

Figure S2. Geographic origin of the reference points used in SDM analyses.

Figure S3. BI tree based on nuDNA.

Figure S4. Haplotype network for each nuclear locus separately for *P. peloponnesiacus*.

Figure S5. The species tree produced by STACEY analysis.

Figure S6. A geographical map of Peloponnese with the main mountains and Lake Stymfalia.

Table S1. List of specimens used in molecular analyses

Table S2. Primers used for the amplification of mtDNA and nuDNA loci.

Table S3. List of specimens used for morphological analyses.

Table S4. The selected models of nucleotide subsitutions.

Table S5. Descriptive statistics for males and females of the two clades of *P. peloponnesiacus*, for biometric and continuous pholidotic traits.

Tables S6&7. Discriminant vector composition (Table S6) and percentages of correct classification based on a leave-one-out cross-validation procedure (Table S7) for the discrimination of the two clades of *P. peloponnesiacus*.

Table S8. SDM for *Podarcis peloponnesiacus* west (Clade 1).

Tables S9. SDM for *Podarcis peloponnesiacus* east (Clade 2).

Table S10. The results of the variable importance test for *Podarcis peloponnesiacus* west (Clade 1).

Table S11. The results of the variable importance test for *Podarcis peloponnesiacus* east (Clade 2).

How to cite this article: Kiourtsoglou, A., Kaliontzopoulou, A., Poursanidis, D., Jablonski, D., Lymberakis, P., & Poulakakis, N. (2021). Evidence of cryptic diversity in *Podarcis peloponnesiacus* and re-evaluation of its current taxonomy; insights from genetic, morphological, and ecological data. *Journal of Zoological Systematics and Evolutionary Research*, 00, 1–21. https://doi.org/10.1111/jzs.12540