









Multilocus and morphological analysis of south-eastern Iberian Wall lizards (Squamata, *Podarcis*)

Marta Bassitta¹  | Joana M. Buades¹ | Ana Pérez-Cembranos²  |
 Valentín Pérez-Mellado²  | Barbara Terrasa¹  | Richard P. Brown³  |
 Pilar Navarro⁴ | Javier Lluch⁴ | Jesús Ortega⁵  | Jose A. Castro¹  |
 Antònia Picornell¹  | Cori Ramon¹

¹Laboratori de Genètica, Departament de Biologia, Universitat de les Illes Balears, Palma de Mallorca, Spain

²Departamento de Biología Animal, Universidad de Salamanca, Salamanca, Spain

³School of Biological & Environmental Sciences, Liverpool John Moores University, Liverpool, United Kingdom

⁴Departamento de Zoología, Facultad de Ciencias Biológicas, Universitat de València, Valencia, Spain

⁵Evolutionary Ecology Unit, Department of Biology, Lund University, Lund, Sweden

Correspondence

Marta Bassitta, Laboratori de Genètica, Departament de Biologia, Universitat de les Illes Balears, Crta. de Valldemossa, km 7.5, 07122, Palma de Mallorca, Illes Balears, Spain.
 Email: m.bassitta@uib.es

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Abstract

The phylogenetic relationships among the wall lizards of the *Podarcis hispanicus* complex that inhabit the south-east (SE) of the Iberian Peninsula and other lineages of the complex remain unclear. In this study, four mitochondrial and two nuclear markers were used to study genetic relationships within this complex. The phylogenetic analyses based on mtDNA gene trees constructed with ML and BI, and a species tree using *BEAST support three divergent clades in this region: the Valencia, Galera and Albacete/Murcia lineages. These three lineages were also corroborated in species delimitation analyses based on mtDNA using bPTP, mPTP, GMYC, ABGD and BAPS. Bayesian inference species delimitation method (BPP) based on both nuclear data and a combined data set (mtDNA + nuclear) showed high posterior probabilities for these three SE lineages (≥ 0.94) and another Bayesian analysis (STACEY) based on combined data set recovered the same three groups in this region. Divergence time dating of the species tree provided an estimated divergence of the Galera lineage from the other SE group (*Podarcis vaucheri*, (Albacete/Murcia, Valencia)) at 12.48 Ma. During this period, the Betic–Rifian arc was isolated, which could have caused the isolation of the Galera form distributed to the south of the Betic Corridor. Although lizards from the Albacete/Murcia and Galera lineage are morphologically similar, they clearly represent distinct genetic lineages. The noteworthy separation of the Galera lineage enables us to conclude that this lineage must be considered as a new full species.

KEYWORDS

Iberian Peninsula, mtDNA, nuclear genes, phylogenetics, *Podarcis*, South-eastern

1 | INTRODUCTION

Morphological and genetic studies spanning the last 25 years have attempted to clarify relationships both within and between lineages of the major lacertid lizard genus *Podarcis* from the Iberian Peninsula and North Africa. These studies reported that all *Podarcis* that inhabit this region, with the

exception of *Podarcis muralis* (Laurenti, 1768), form a monophyletic group (Arnold, Arribas, & Carranza, 2007; Carranza, Arnold, & Amat, 2004) that is generally referred to as the *Podarcis hispanicus* (Steindachner, 1870) species complex.

Currently, seven genetically distinct lineages within this complex have been raised to species level: *Podarcis guarramae*, *Podarcis virescens*, *Podarcis liolepis*, *Podarcis*

bocagei, *Podarcis carbonelli*, *Podarcis vaucheri* and the nominal taxon *P. hispanicus* (Caeiro-Dias et al., 2018). The description of some of these species has been given in different works, so Harris and Sa-Sousa (2002) found molecular differences between two morphotypes (named types 1 and 2) from Western Iberia that were later described as two full species (Geniez, Sa-Sousa, Guillaume, Cluchier, & Crochet, 2014): *Podarcis gadarramae* and *P. virescens*, respectively. Moreover, they also divided *P. gadarramae* into two subspecies: *Podarcis gadarramae gadarramae* and *Podarcis gadarramae lusitanicus*. Harris and Sa-Sousa (2002) also described a further morphotype (type 3) that was later elevated to species rank as *P. liolepis* (Renoult, Geniez, Bacquet, Guillaume, & Crochet, 2010). Previously, other Iberian *Podarcis* were raised to species level, including *P. bocagei* (Lopez-Seoane, 1885), *P. carbonelli* (Pérez-Mellado, 1981) and *P. vaucheri* (Busack, Lawson, & Arjo, 2005).

The nominal taxon within the species complex *P. hispanicus* (Steindachner, 1870) was described as from the south-east (SE) of the Iberian Peninsula (Geniez, Cluchier, Sá-Sousa, Guillaume, & Crochet, 2007). Within this group, Pinho, Ferrand, and Harris (2006) described a new mtDNA lineage from Galera locality, placed in the Baza Depression of SE Spain, represented by only a single specimen. This mtDNA lineage and that of *P. liolepis* clustered separately to other members of the species complex. Later work using allozyme markers (Pinho, Harris, & Ferrand, 2007) largely corroborated the existence of a differentiated group in Galera area. In these first studies, monophyly of the Galera specimens with respect to other members of the species complex could not be addressed, due to the inclusion of individuals from a single location. Later genealogies based on two nuclear introns (Pinho, Harris, & Ferrand, 2008) did not support *Podarcis* from Galera as a monophyletic group and pointed to nuclear gene flow between the Galera and other lineages identified by mtDNA analyses, where they are in sympatry, at least across parts of their distributions. Renoult, Geniez, Bacquet, Benoit, and Crochet (2009) identified three evolutionary lineages (*P. virescens*, *P. liolepis* and *P. hispanicus*) in the east of Iberian Peninsula from morphological characters and nuclear loci, while their analysis of mtDNA revealed four lineages (*P. virescens*, *P. liolepis*, *P. hispanicus* from Galera and *P. hispanicus* from Valencia) suggesting an ancient introgression. Other studies added to the Galera (Pinho et al., 2006) and Valencia (Renoult et al., 2009) forms, an additional mitochondrial lineage detected in SE Spain from the Albacete/Murcia area. The Valencia and Albacete/Murcia populations appear to comprise sister mitochondrial lineages, which together represent a sister group to all North African lineages, from which they diverged 6.99–9.44 Ma (Kaliontzopoulou, Pinho, Harris, & Carretero, 2011). In that study, a second individual from the Galera lineage was included and the results indicated that this mtDNA lineage

clustered with those from *P. liolepis*, in agreement with Pinho et al. (2006) and unlike the results found by Renoult et al. (2009) which established that *P. liolepis* and *P. hispanicus* from Galera were two clearly independent groups. The cluster composed by *P. liolepis* and the Galera lineage was estimated to have diverged from other members of the species complex 9.44–13.94 Ma (Kaliontzopoulou et al., 2011). All these studies trying to establish the phylogeny of *Podarcis* from the SE (Galera, Valencia and Albacete/Murcia lineages) are based on a very low number of individuals for each lineage. Consequently, the phylogenetic relationships between these groups remain unsolved and require a re-examination based on more extensive information.

For many years, the designation of mitochondrial lineages within the *P. hispanicus* complex has been identified solely by numbers and has coexisted with numerous systematic proposals for these lineages (Geniez et al., 2007, 2014). Despite the large number of studies carried out on this species complex, the phylogenetic relationships within the complex are not well established and there could be undiscovered independent lineages. The adequate assignment of potentially new species is a valuable instrument for conservation (Geniez et al., 2007, 2014; Renoult et al., 2010).

In this paper, we investigate the *P. hispanicus* complex with the main aim of establishing the phylogenetic relationships within the SE forms of *Podarcis* (Valencia, Galera and Albacete/Murcia lineage) and between them and other *Podarcis* from the Iberian Peninsula and North Africa. This is achieved through analyses of mtDNA, nuclear DNA and morphology. These analyses will contribute to knowledge of the evolutionary history and taxonomy of Iberian *Podarcis*, providing deeper biogeographical insights and important information for conservation bodies.

2 | MATERIAL AND METHODS

2.1 | Sampling

In total, 105 *Podarcis* individuals from the Iberian Peninsula and North African region were captured (Table S1 and Figure 1). Lizards were caught by careful noosing in their natural habitats with the specific permits delivered by the competent body in each locality. All morphological measurements were taken in situ, and 1 cm of the tail tip was removed and stored in 100% ethanol. All lizards were released at their capture site.

Because the distribution of different lineages of *P. hispanicus* complex is not well-known in the SE Iberian Peninsula (see, for example, Caeiro-Dias et al., 2018 and references therein), we sampled some of the localities used by previous authors, where particular lineages were detected and described. In addition, we tried to include new localities within

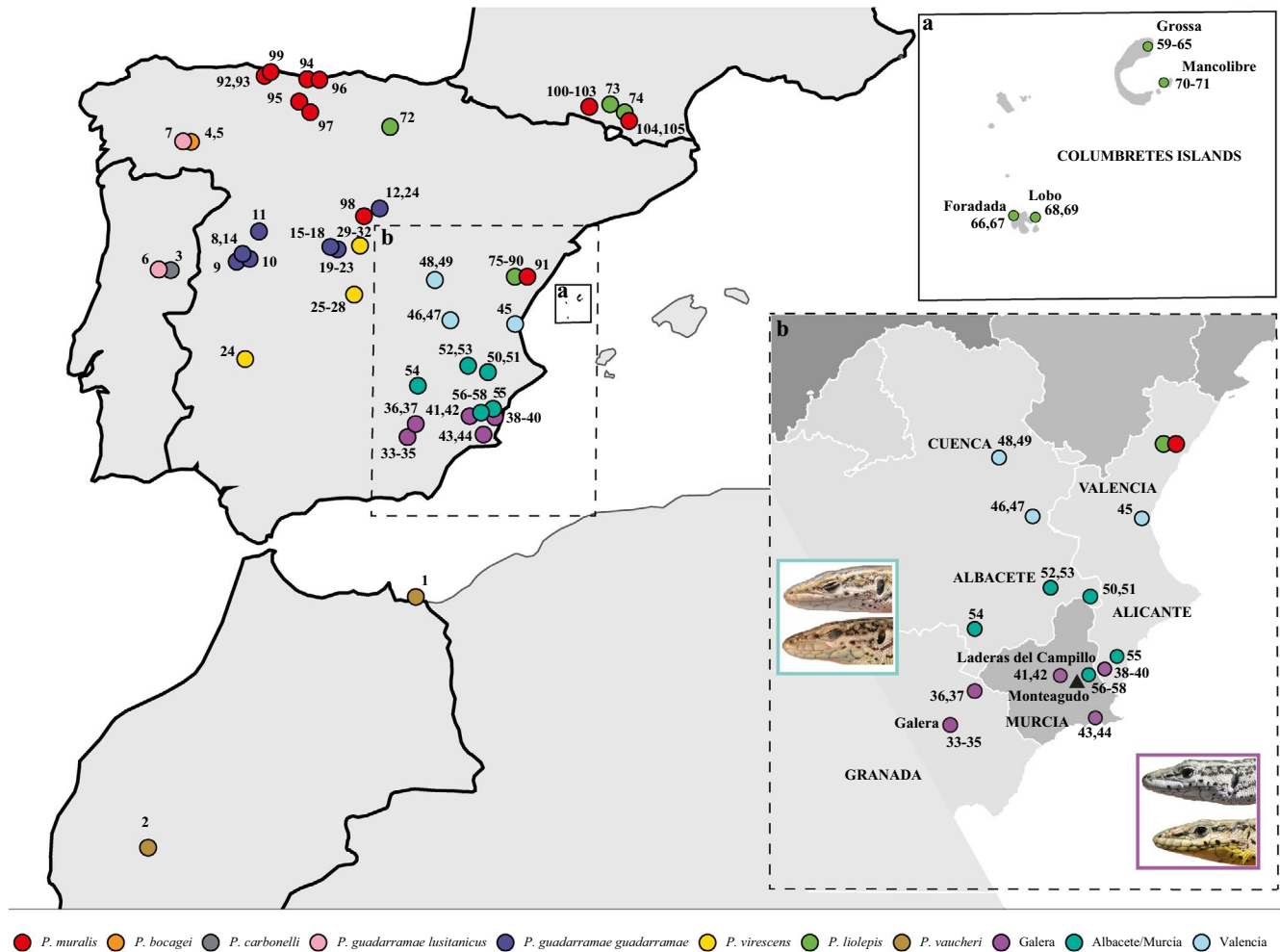


FIGURE 1 Locations of all *Podarcis* samples from the Iberian Peninsula and North Africa used in this study. The colours correspond to the different species indicated in the legend below and the numbers correspond to those in Table S1. The black triangle marks the locality type of *Podarcis hispanicus* sensu stricto (Monteagudo, Murcia)

the expected range of each lineage. Similar to previous authors (Geniez et al., 2007), we intensively searched the restricted type locality of *P. hispanicus* (Steindachner, 1870), Monteagudo, close to Murcia town, twice in 2019. Despite suitable climatic conditions, we did not find any *Podarcis* at the type locality but did detect a small population at Laderas del Campillo, 4.5 km north from Monteagudo (Figure 1). To our knowledge, this is the closest location to the type locality of *P. hispanicus* sensu stricto (Geniez et al., 2007). Lizards from Galera (Granada) were captured with a Permit of Scientific Capture from the Consellería de Agricultura, Ganadería, Pesca y Desarrollo Sostenible of Junta de Andalucía (permit No: 201999900530233 issued on 24/07/2019).

2.2 | DNA extraction, amplification and sequencing

A standard phenol–chloroform protocol was used for DNA extraction (González et al., 1996). The following

four non-overlapping mtDNA fragments were obtained for each specimen: (a) partial 12S rRNA (360 bp), (b) partial control region (CR) (476 bp), (c) two partial fragments of cytochrome *b* (CYTB) (306 and 483 bp, respectively) and (iv) two partial subunits of the NADH dehydrogenase gene and associated tRNAs (referred to as ND1 (48 bp), ND2 (415 bp), tRNA_{Ile}, tRNA_{Gln} and tRNA_{Met} (213 bp)). Two partial nuclear genes were amplified and sequenced: (a) melanocortin-1 receptor gene (MC1R) (663 bp) and (b) recombination-activating gene 1 (RAG1) (939 bp) (Table S1). Primers and amplification conditions are the same as those used in our previous studies of *Podarcis* (Buades et al., 2013; Rodríguez et al., 2013, 2014, 2017; Terrasa et al., 2009). Both strands of the PCR products were sequenced on an automated ABI 3130 sequencer (Applied Biosystems) using a BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems) and edited using CodonCode Aligner software (CodonCode Corporation). Nuclear data were phased using the PHASE algorithm (Stephens, Smith, & Donnelly, 2001) within DnaSP v.6 (Librado & Rozas, 2009). Eighty-five

sequences that had been published by previous studies were also used (Table S1).

2.3 | Phylogenetic analyses

We identified 88 unique haplotypes within the concatenated mitochondrial DNA data set (2,301 bp) from the *Podarcis* specimens with DnaSP v.6 (Librado & Rozas, 2009). Individuals from the Balearic Islands were included as out-groups (five *Podarcis lilfordi* and three *Podarcis pityusensis*). Sequences were aligned in the MAFFT v7.423 online server (Katoh & Toh, 2008) using the iterative refinement method (FFT-NS-i). Best-fit nucleotide substitution models and partitioning scheme were chosen simultaneously using PartitionFinder v2.1.1 (Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2016) under the Akaike information criterion (AIC). The partitioning schemes were defined manually (by gene and by codon), with branch lengths of alternative partitions ‘unlinked’ to search for the best-fit scheme. The proportion of invariable sites (I) parameter was discarded if the favoured model incorporated both the I and the Gamma site rate heterogeneity (G) parameters as simultaneous use of these parameters can have undesirable effects (Yang, 1993).

We performed phylogenetic analyses using maximum-likelihood (ML) and Bayesian inference (BI) methods. Maximum-likelihood analyses were performed using IQ-TREE version 1.6.10 (Nguyen, Schmidt, von Haeseler, & Minh, 2014). We applied the partitions and the best-fit substitution model and performed 10^6 bootstrap replicates based on the ultrafast bootstrap approximation (UFBoot) (Hoang, Chernomor, Von Haeseler, Minh, & Vinh, 2017; Minh, Nguyen, & von Haeseler, 2013) for statistical support.

Bayesian analyses were performed with MrBayes 3.2.6 (Ronquist et al., 2012). MCMC chain lengths were 10^7 generations with a sampling frequency of 10^3 generations. We used two simultaneous runs of three hot and one cold chain each. Convergence was confirmed by examining the stationarity of the log-likelihood (lnL) values of the sampled trees and the observation of average standard deviations of the split frequencies being <0.01 . Run characteristics such as effective sample sizes (ESS) were also assessed in Tracer v1.7 (Rambaut, Drummond, Xie, Baele, & Suchard, 2018). The fifty per cent majority-rule consensus tree was summarized using sumt command with the first 25% of each run discarded as burn-in. The resulting phylogenetic tree was visualized and edited using FigTree v1.4.2 (Rambaut, 2014).

Haplotype networks were constructed for each phased nuclear locus using the Population Analysis with Reticulate Trees (PopART, <http://popart.otago.ac.nz>) (Leigh & Bryant, 2015) with the TCS method (Clement, Posada, & Crandall, 2000).

2.4 | Species tree and divergence times estimates

A standard likelihood ratio test of the molecular clock was performed for both mtDNA and nuclear data sets in MEGA7 (Kumar, Stecher, & Tamura, 2016). This test reliably informs whether a strict or relaxed clock model is most suitable for divergence time dating (Brown & Yang, 2011).

The species tree approach that is implemented in *BEAST (Heled & Drummond, 2010) was used in an attempt to simultaneously infer the phylogenetic relationships and divergence times between the different lineages of the Iberian and North African *Podarcis* based on: (a) all mtDNA sequence data sets (108 individuals) and (b) both mtDNA and the phased nuclear loci (mtDNA + nuclear) (RAG1: 95 individuals and MC1R: 102 individuals). For mtDNA analyses, the species tree was calibrated using a mean split time of 5.3250 Ma with relatively little uncertainty around this estimate, replicating the calibration in Rodríguez et al. (2013). The calibration was specified from a log-normal distribution with mean 1.6724 and standard deviation 0.002 (the central 95% of this distribution ranges from 5.304 to 5.346, in real space). This calibration is based on knowledge of the timing of the end of the Messinian salinity crisis (5.33 Ma) and the very rapid refilling of the Mediterranean basin that would have separated the two Balearic island *Podarcis* (i.e. *P. lilfordi* and *P. pityusensis*; see Brown et al., 2008 and references therein). Three partitions were assigned as: (a) 12S rRNA, CR, all tRNAs (b) CYTB/ND1/ND2 1st + 2nd codon position and (c) CYTB/ND1/ND2 3rd codon position. Evolutionary models were the same as those used for MrBayes. The *BEAST MCMC sampler was run twice for 5×10^8 generations, with one step per 50,000 being sampled. A relaxed log-normal clock model was specified since the molecular clock likelihood ratio test indicated rate variation across the gene trees. A coalescent Yule speciation process was used for the tree prior. For mtDNA + nuclear analyses, we used the same calibration described for the mtDNA-only analyses. The same DNA substitution model was used for the three mtDNA partitions and the JC69 model was used for the two nuclear loci. Tracer v1.7 (Rambaut et al., 2018) was used to check for convergence. Posterior trees were combined to obtain the tree with the maximum sum of posterior clade probabilities using mean heights for node annotation.

2.5 | Genetic structure and species delimitation analyses

Two tree-based (bPTP, mPTP and GMYC) and one distance-based (ABGD) species delimitation methods were performed on the concatenated mitochondrial haplotypes data set. bPTP (Zhang, Kapli, Pavlidis, & Stamatakis, 2013) analyses were

TABLE 1 Results from BPP analyses for each combination of priors based on phased nuclear loci only (first row) and on combined data set mtDNA + nuclear loci (second row)

Priors	PP of each species											PP for number of species								
	θ	α	[Bal]	[Gal]	[Lio]	[Alb]	[Mur]	[Gua]	[Val]	[Lus]	[Vir]	[Boc]	[Car]	[Vau]	[Boc-Car]	[Vir-Car]	P	P	P	P
G (3. 0.02)	G (3. 0.004)	Nuclear	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.95	0.97	0.92	1.00	0.03	0.04	0.91	0.09	0	0
G (3. 0.02)	G (3. 0.004)	mt + nuclear	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.96	0.95	1.00	0.04	0.00	0.95	0.05	0	0
G (3. 0.02)	G (3. 0.2)	Nuclear	1.00	1.00	1.00	1.00	1.00	1.00	0.99	0.90	0.94	0.83	1.00	0.06	0.10	0.82	0.18	0	0	0
G (3. 0.02)	G (3. 0.2)	mt + nuclear	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.89	0.89	1.00	0.11	0	0.89	0.11	0	0	0
G (3. 0.02)	G (3.0.02)	Nuclear	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.95	0.97	0.91	1.00	0.03	0.05	0.91	0.09	0	0	0
G (3. 0.02)	G (3.0.02)	mt + nuclear	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.95	0.95	1.00	0.05	0	0.95	0.05	0	0	0
G (3. 0.2)	G (3.0.02)	Nuclear	1.00	1.00	1.00	0.99	1.00	1.00	0.96	0.71	0.61	0.62	0.31	0.85	0.27	0.24	0.22	0.52	0.23	0.23
G (3. 0.2)	G (3.0.02)	mt + nuclear	1.00	1.00	1.00	1.00	1.00	1.00	0.99	0.87	0.77	0.59	0.38	0.92	0.37	0.18	0.34	0.57	0.09	0.09
G (3. 0.2)	G (3. 0.2)	Nuclear	1.00	1.00	1.00	0.98	1.00	1.00	0.94	0.54	0.45	0.60	0.20	0.69	0.22	0.26	0.11	0.41	0.41	0.41
G (3. 0.2)	G (3. 0.2)	mt + nuclear	1.00	1.00	1.00	1.00	1.00	1.00	0.98	0.76	0.70	0.44	0.23	0.88	0.48	0.18	0.18	0.62	0.20	0.20
G (3. 0.002)	G (3. 0.004)	Nuclear	1.00	1.00	1.00	1.00	1.00	1.00	0.97	1.00	0.93	0.93	0.86	1.00	0.07	0.05	0.85	0.15	0	0
G (3. 0.002)	G (3. 0.004)	mt + nuclear	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.99	0.96	0.95	1.00	0.04	0.01	0.95	0.05	0	0
G (3. 0.002)	G (3. 0.04)	Nuclear	1.00	1.00	1.00	1.00	1.00	1.00	0.98	0.99	0.92	0.92	0.84	1.00	0.08	0.09	0.82	0.17	0	0
G (3. 0.002)	G (3. 0.04)	mt + nuclear	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.99	0.99	0.94	0.93	0.99	0.06	0.01	0.93	0.07	0	0

Note: The posterior probability (PP) values are averages from three independent runs of module A11.

Abbreviations: Bal, Balearic outgroup; Car, *Podarcis carbonelli*; Gal, Galera lineage; Lio, *Podarcis liolepis* Alb, Albacete/Murcia lineage; Lus, *Podarcis guadarramae lusitanicus*; Mur, *Podarcis muralis* Gua, *Podarcis guadarramae guadarramae*; Val, Valencia lineage; Vau, *Podarcis vaucheri*Vir, *Podarcis virescens* Boc, *Podarcis bocagei*.

performed using the online server (<https://species.h-its.org/>) and the ML tree from IQ-TREE. We ran 500,000 generations with a thinning of 500 and a burn-in of 0.1, then assessed convergence visually using the MCMC iteration vs. log-likelihood plot automatically generated. Next, we applied the mPTP method (Kapli et al., 2017) using the ML tree from IQ-TREE. We performed two simultaneous Markov chain Monte Carlo (MCMC) runs of 100,000,000 steps, sampling every 10,000 steps. Convergence was confirmed with the likelihood plot of the combined runs and the average standard deviation of delimitation support values (0.000009), which indicate convergence on the same distribution as it approaches to zero. The -multi option was used to allow differences in rates of coalescence among species. Finally, we incorporated the single-threshold GMYC (Fujisawa & Barraclough, 2013; Pons et al., 2006) model. This model is based on an ultrametric phylogenetic tree and uses differences in branching rates to infer species delimitation. The input tree was generated in BEAST v2.6 (Bouckaert et al., 2019) under a relaxed clock and Yule model tree prior. The same partitioning model used for the ML and BI trees was used as estimated using AIC in PartitionFinder v2.1.1 (Lanfear et al., 2016). Analyses were run for 200 million generations, sampling every 2,000 generations. Convergence and mixing were monitored in Tracer v1.7 (Rambaut et al., 2018), and ESS values >200 indicated adequate sampling of the posterior. TreeAnnotator within BEAST was used to create a tree with the maximum sum of posterior clade probabilities using mean heights for node annotation and a 10% of burn-in. The GMYC analysis was conducted using the single-threshold method in the splits package on R v. 3.6.1.

Automatic Barcode Gap Discovery (ABGD; Puillandre, Lambert, Brouillet, & Achaz, 2012) uses pairwise genetic distances to determine the differences between intra- and interspecific divergence and establishes the number of different species based on those differences. ABGD was performed using the online tool (<http://www.wabi.snv.jussieu.fr/public/abgd/abgdweb>) with default values for the prior intraspecific divergences (Pmin 0.001, Pmax 0.1, steps 10), relative gap width (1.5) and distance distribution (20). Results were compared using both JC69 and K80 models.

Structural analysis was performed with the Bayesian model-based clustering algorithm implemented in BAPS 6.0 (Corander & Tang, 2007) using the mtDNA haplotypes alignment. The method was performed using clustering with linked loci and codon linkage model. We ran BAPS for values of k ranging from one to fifteen, performing three replicates for each value of k .

Bayesian Phylogenetics and Phylogeography (BPP v4.0) (Yang & Rannala, 2010) analysis was performed on two different data sets: (a) two-phased nuclear loci and (b) mtDNA + two-phased nuclear loci. We also included Balearic *Podarcis* in the analysis as the out-group. BPP is

a robust means of inferring species from recently diverged lineages using multilocus data (Leaché, Zhu, Rannala, & Yang, 2018; Rannala, 2015) under the multispecies coalescent model (MSC) (Rannala & Yang, 2003). This Bayesian method assumes no gene flow among species and no recombination among loci and explains gene tree discordance by incomplete lineage sorting (ILS). BPP implements a reversible jump Markov chain Monte Carlo (rjMCMC) search to estimate the sum of posterior probability (PP) of all species delimitation models considered. The speciation models PP can be affected by the prior distributions chosen for the ancestral population size (θ) and root age (τ). We used a range of different prior scenarios (Table 1), including small population size (θ) and deep divergence (τ), or large population size and shallow divergence that favours more conservative models containing fewer species (Yang & Rannala, 2010). We used the A11 model (speciesdelimitation = 1 and speciestree = 1), which jointly infers the species tree and the species delimitation (Rannala & Yang, 2017). The assignment of individuals to hypothesized species was based on the identified mtDNA lineages. The MCMC chain was run for 10^4 steps (following a burn-in of 2,000 steps), sampled every 25 steps. Each analysis was run three times to confirm that they converged on the same posterior.

Finally, we used STACEY v1.8.0 (Jones, 2017), a Bayesian multispecies coalescent method implemented in BEAST v2.5 (Bouckaert et al., 2019) that allows inference of species delimitation and species phylogeny. The author of this analysis prefers the term 'cluster' over 'species' (Jones, 2017), and so, we use both terms when discussing this analysis. STACEY provided a parallel analysis to that in BPP and so was particularly useful to ensure the robustness of the species delimitation results. STACEY was preferred over a similar approach within *BEAST (Grummer, Bryson, & Reeder, 2014; Heled & Drummond, 2010) because it provided better convergence of the MCMC chain. It differs from BPP in several ways, including the implementation of a birth–death–collapse model, which eliminates the need for a rjMCMC algorithm, and use of different population size parameters which are integrated out. As for BPP analyses, we used the three loci comprised mtDNA and the two-phased nuclear loci with the JC69 substitution model and a strict clock specified for each. Two independent runs of 450 million generations of the MCMC chains were performed sampling every 5,000 steps. Convergence was checked in Tracer 1.7 (Rambaut et al., 2018), and posterior trees from both runs were combined (the first 20% of trees from each run were removed as burn-in). The resulting 144,000 species trees were processed in SpeciesDelimitationAnalyser (Jones, 2017) with a collapse height of 0.0001 (the same value used in STACEY analysis) and default similarity cut-off (0.9). Posterior trees were also combined to obtain a maximum sum of clade credibilities tree.

2.6 | Morphological analyses

To avoid any confusion regarding their lineage assignment, our morphometric analyses were restricted to individuals that we personally generated sequence data for. Thus, we analysed a rather small sample of lizards (25 individuals from Galera lineage and 12 belonging to Albacete/Murcia lineage). Our aim was only to show general similarities and differences between the two lineages of Albacete/Murcia and Galera. A more thorough analysis will be subsequently carried out with a larger sample of individuals from the entire Iberian *Podarcis* species complex.

Six body dimensions, as well as body mass (Weight), were included in this study: snout–vent length (SVL), intact tail length (TL), pileus length (PL), head width (HW), head height (HH) and left hindlimb length (HLL). All measurements were made with a digital calliper to the nearest 0.01 mm, except for SVL and intact tail length, which was measured with a steel rule to the nearest 1 mm. Weight was obtained with a spring scale Pesola®. Six scalation characters were recorded: gularia, collaria, dorsalia, ventralia, femoralia and left fourth digit lamellae (see Pérez-Mellado & Gosá, 1988) for methodological details of body measurements and scalation counts). Not all characters could be recorded from all individuals. Due to sexual dimorphism, males and females were analysed separately. Raw or log-transformed data were checked for normality (Shapiro–Wilk test) and homogeneity of variances (Fligner test) prior to statistical comparisons (one-way ANOVA). If assumptions for the use of parametric techniques were not met, we employed non-parametric equivalents. All analyses were done within R (R Core Team, 2018). In addition, we studied colour and design variation in our samples, employing the same criteria as Geniez et al. (2007) in their diagnostic descriptions. Colour descriptions were completed with the assignment of colour codes to the following body parts: background dorsum, pileus, flanks, belly, gular region, blue ocelli in outer ventral scales, if present, dorsolateral stripes, dorsal side of the tail and ventral side of the tail. Colour codes were assigned according with the catalogue of Köhler (2012).

3 | RESULTS

3.1 | Phylogenetic analysis

PartitionFinder v2.1.1 (Lanfear et al., 2016) identified three partitions with the following substitution models: non-coding fragments [GTR+I+G], 1st and 2nd codon of coding regions [HKY+I+G] and 3rd codon of coding regions [GTR+I+G] (analyses were carried out with these models but without the I parameter, as discussed in the section 2). ML and BI

trees clustered the individuals into four major mitochondrial clades within the Iberian Peninsula and North African samples, corresponding to the following taxa/areas: group 1 (monophyletic) only includes *P. muralis*; the other groups are polyphyletic including group 2: *P. carbonelli*, *P. virescens*, *P. g. lusitanicus*, *P. bocagei* and *P. g. guadarramae*; group 3: *P. vaucheri*, Galera lineage, Valencia lineage and Albacete/Murcia lineage; and group 4: *P. liolepis* (Figure 2). The separation between groups 3 and 4 presents low support (posterior probability < 50; bootstrap support < 40). Divergence of the different SE mitochondrial lineages (Galera, Albacete/Murcia and Valencia) show high support (PP \geq 0.99; BS \geq 79). The results show Valencia and Albacete/Murcia as sister lineages to the North African form (*P. vaucheri*), while the entire group (*P. vaucheri*, (Albacete/Murcia, Valencia)) is a sister lineage to the Galera lineage.

Networks based on phased nuclear loci (Figure 3) show less structured interrelations between different *Podarcis* populations. *Podarcis muralis* and *P. vaucheri* are the only groups that share no haplotypes with any other *Podarcis* populations. The Galera lineage presents two shared haplotypes and nine species-specific haplotypes in the RAG1 network, and one shared haplotype and five unique haplotypes in the MC1R network. These haplotypes were shared with geographically close groups (*P. virescens*, *P. liolepis*, Valencia and Albacete/Murcia lineages), except for *P. g. lusitanicus*, indicating a common haplotype among these populations with posterior differentiation. The striking number of different haplotypes found in *P. liolepis* demonstrates the high genetic diversity present in this group and could be an indicator that its distribution range has not been fully studied genetically.

3.2 | Species tree and divergence time estimates

The mtDNA species tree (Figure 4) provided a posterior mean for the divergence of the Iberian and North African group with respect to the monophyletic group *P. muralis* at 15.60 Ma (95% highest posterior density, HPD: 22.31–10.71 Ma) and that for the divergence of Galera from the clade containing (*P. vaucheri* (Albacete/Murcia, Valencia)) at 12.48 Ma (95% HPD: 18.27–7.93 Ma). The Valencia and Albacete/Murcia lineage divergence appeared to be more recent at 7.11 Ma (95% HPD: 11.75–1.87 Ma). The split between *P. liolepis* and the remaining lineages from west Iberia would have taken place during the middle Miocene, in the late Serravallian (12.58 Ma, 95% HPD: 18.53–7.40 Ma), as also observed for the divergence of the Galera lineage. Despite the use of simpler models with fewer parameters, the mtDNA + nuclear analyses did not show repeatable convergence on the same posterior, and thus, the results are not presented here.

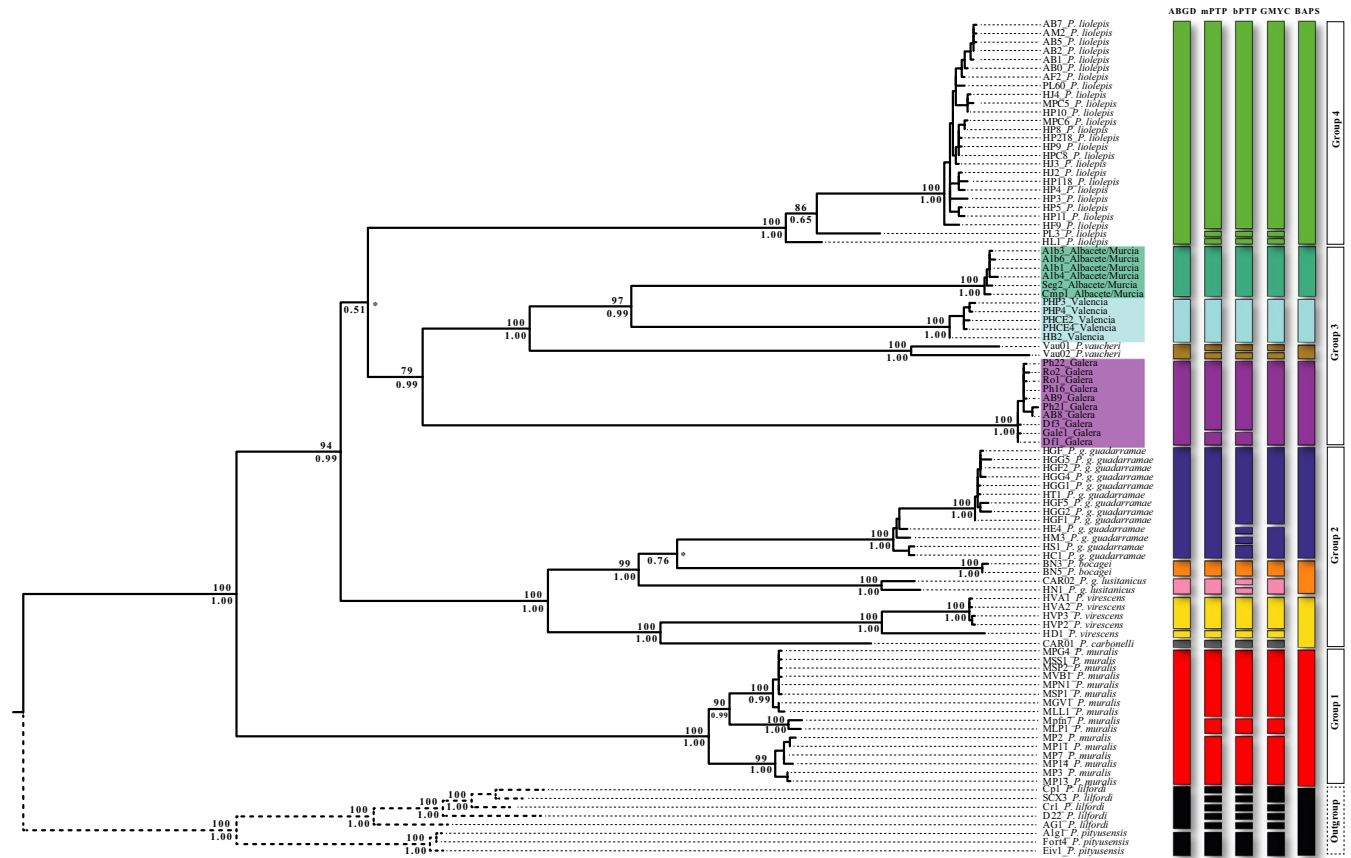


FIGURE 2 Gene tree based on maximum likelihood for mitochondrial data showing different scenarios of species delimitation between Iberian and North African *Podarcis* populations, using the Balearic clade as an out-group. The numbers above branches correspond to bootstrap support and numbers below branches are posterior probabilities from Bayesian analysis. Support values for nodes with $\leq 50\%$ support are not included. Asterisks indicate branches where different analyses produced different topology. Group 1: *Podarcis muralis*; Group 2: *Podarcis bocagei*, *Podarcis carbonelli*, *Podarcis guadarramae guadarramae*, *Podarcis guadarramae lusitanicus*, *Podarcis virescens*; Group 3: *Podarcis vaucheri*, Galera lineage, Albacete/Murcia lineage, Valencia lineage; and Group 4: *Podarcis liolepis*

3.3 | Genetic structure and species delimitation analysis

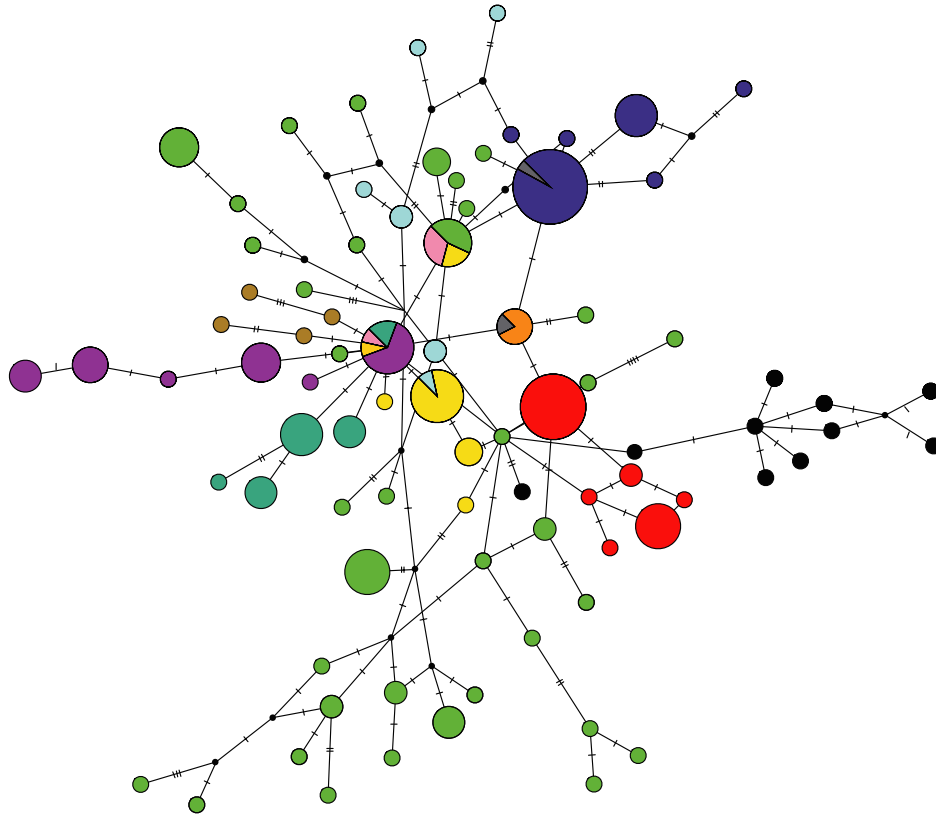
The different species delimitation methods (bPTP, mPTP, GMYC, ABGD and BAPS), based on mtDNA, returned different partition numbers, ranging from 9 to 22. All the methods corroborated the separation of the SE lineages (Valencia, Albacete/Murcia and Galera; Figure 2).

Under five out of seven prior specifications for θ and τ , BPP analyses for both data sets nuclear and combined mtDNA + nuclear loci provided the greatest support for the delimitation of 12 species (Table 1). The analyses in which the parameters favoured a moderate populations size (θ : InvG (3, 0.02)) and divergence time (τ : InvG (3, 0.02)) provided the highest posterior probability (0.91 and 0.95, respectively) for 12 species. This contrasts with analyses that favoured a larger population size (θ : InvG (3, 0.2))

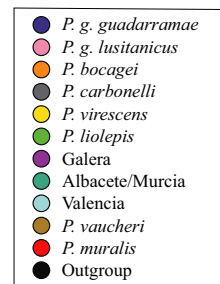
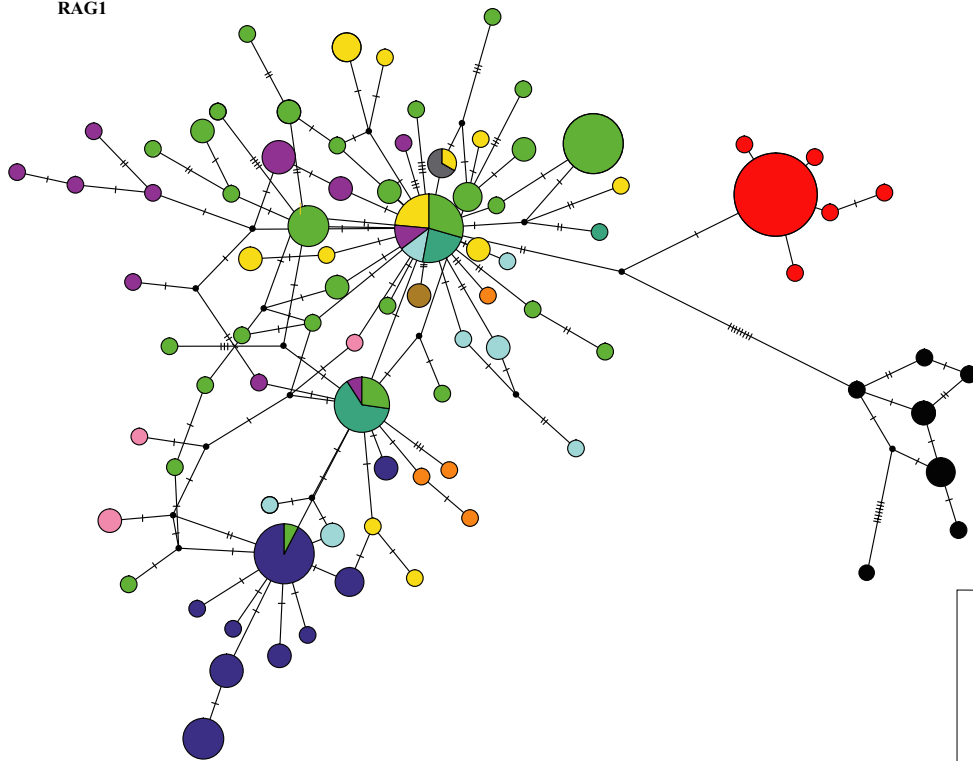
and a deep/moderate divergence time (τ : InvG (3, 0.2)) or (τ : InvG (3, 0.02)), which provided the greatest support for 11 species (0.520 and 0.62, respectively). *Podarcis bocagei* and *P. carbonelli* are the two populations that are not clearly defined as separate species (Table 1 and Figure 2). In terms of species delimitation, all prior combinations showed strong individual species support (≥ 0.94) for the Galera, Albacete/Murcia and Valencia lineages (Table 1). Nonetheless, the inferred species tree topologies tended to vary among models and even (to a lesser extent) showed some minor differences between runs for a given prior combination. Posterior support for nodes within the BPP species trees based on both nuclear data and mtDNA + nuclear combined data was generally low, except for *P. muralis* and *P. vaucheri*, and the topology differed from that described by phylogenetic trees based on the mtDNA concatenated data set.

FIGURE 3 TCS haplotype networks of Iberian and North African *Podarcis* nuclear loci for melanocortin receptor 1 (MC1R) and recombination-activating gene 1 (RAG1). Hatch marks between black dots represent one mutational step, haplotypes circle area is proportional to the number of individuals and the colour identifies the species

MC1R



RAG1



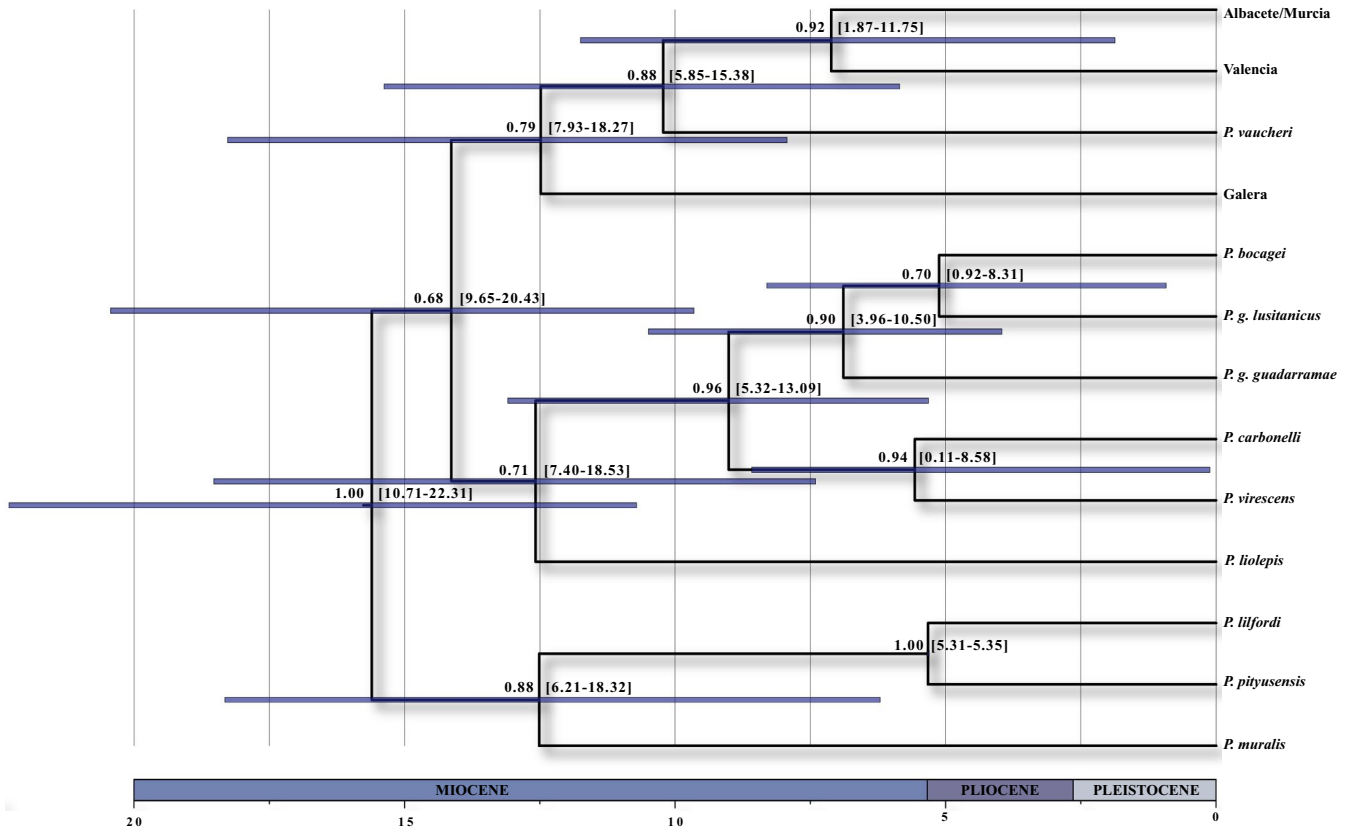


FIGURE 4 Species tree with estimated divergence time based on mitochondrial data (2,301 bp of 12S, CR, CYTB and NADH fragments) resolved from *BEAST analyses. The divergence times were estimated using the separation of the Balearic *Podarcis* clade as a calibration. Horizontal bars and numbers at the right of nodes (Ma) indicate a 95% credible interval of divergence and posterior probability values are shown to the left

Species delimitation analysis performed with STACEY provided similar results to those obtained using BPP and attributed highest posterior probabilities to 13 clusters (0.96). In this analysis, an extra group was found corresponding to the separation of the out-group into the two recognized species from the Balearic islands (*P. lilfordi* and *P. pityusensis*). Nonetheless, the three SE lineages from the Iberian Peninsula are confirmed to represent independently evolving lineages. Also similar to BPP analyses, support values were low for individual internal nodes within the species tree, except for the divergence of *P. vaucheri* from the rest of species complex.

3.4 | Morphological analysis

In the SE Iberian Peninsula, both groups of lizards from the Albacete/Murcia and Galera lineages are characterized by a very small body size (SVL and Weight) in comparison with the remaining recognized species of Iberian *Podarcis* (Kaliontzopoulou, Carretero, & Llorente, 2012). In both lineages, we found an SVL under 53 mm (Table S2). Body size is statistically similar in the Albacete/Murcia and Galera lizards (Table S2, adult males: one-way ANOVA,

$F_{1,22} = 1.816$, $p = .192$, adult females: $F_{1,11} = 0.173$, $p = .686$) as is body mass (males: $F_{1,19} = 0.0128$, $p = .991$, females: $F_{1,10} = 3.703$, $p = .083$). The number of lizards with an intact tail precluded any comparison between lineages (Table S2). For head measurements, only head width showed significant differences between males of Albacete/Murcia and Galera ($F_{1,22} = 9.541$, $p = .0054$) but not between females ($F_{1,11} = 2.041$, $p = .181$). The remaining head measurements were similar in both lineages (pileus length, males: $F_{1,22} = 1.658$, $p = .211$, females: $F_{1,10} = 0.06$, $p = .811$; head height, males: $F_{1,22} = 1.832$, $p = .19$, females: $F_{1,11} = 1.53$, $p = .242$). Hindlimb length was also similar in both lineages (males: $F_{1,22} = 3.1$, $p = .0922$, females: $W = 6$, $p = .106$).

With regard to scalation traits, the number of subdigital lamellae under the 4th toe is similar in both lineages (males: $F_{1,21} = 0.43$, $p = .519$, females: $F_{1,7} = 1.485$, $p = .262$). There is a greater number of femoral pores in males from the Albacete/Murcia lineage (Table S2, $F_{1,22} = 16.48$, $p = .00052$) but not in females ($F_{1,10} = 0.561$, $p = .471$). Also, the number of gular scales is greater in Albacete/Murcia males (Table S2, $F_{1,22} = 27.02$, $p = 3.26 \times 10^{-5}$) but not in females ($F_{1,10} = 0.944$, $p = .354$). We did not find differences in the number of dorsal scales (males: $F_{1,21} = 0.19$, $p = .668$,

females: $F_{1,9} = 2.065$, $p = .185$) nor in collaria (males: $F_{1,22} = 0.132$, $p = .72$, females: $F_{1,9} = 1.205$, $p = .301$). Finally, the number of ventral scales is significantly higher in Galera lizards (Table S2, males: Mann–Whitney test, $W = 23.5$, $p = .0123$, females: $F_{1,9} = 7.333$, $p = .024$).

Lizards from both lineages of Albacete/Murcia and Galera are small dorsally brownish or greyish lizards. The general appearance of the lizards from both lineages is very similar (Figures S1 and S2). In the Galera lineage, we observed dorsal colours from Drab (code 19 of Köhler, 2012) to Light Drab (269) and, especially, Olive-Brown (278) and Brownish Olive (276) (Table S4). The dorsal background of lizards from the Albacete/Murcia lineage is mainly Drab (19), Ground Cinnamon (270) or Light Drab (269). Thus, in the Albacete/Murcia lineage we can observe olive nuances that, apparently, are absent in the Galera lineage. A similar pattern was observed in pileus colour (Table S4). In the Galera lineage, bellies are mainly Smoky White (261) or Light Buff (2), while in the Albacete/Murcia lineage bellies are Pale Buff (1) or Light Buff (2) (Table S4).

Taking into account the same diagnostic characters employed by Geniez et al. (2007) to define the nominotypical taxon, *P. hispanicus*, we can see that almost all diagnostic features of colour and design can be found in both lineages. However, all these traits are better represented by the Albacete/Murcia lineage (Table S3 and Figure S2). A wider sampling of its geographic region is required to provide an in-depth morphological description of the Valencia lineage.

3.5 | Taxonomic results

Geniez et al. (2007) carried out a morphological analysis of several lizards from the SE Iberian Peninsula, before the discovery of two new lineages: Albacete/Murcia and Valencia (Caeiro-Dias et al., 2018 and references therein). Consequently, it is possible that their sample contained lizards from different lineages. We do not know whether this was the case, but from our results, lizards from the Albacete/Murcia lineage are closer to the nominotypical taxon, as defined by Geniez et al. (2007) (see Table S3). In addition, at the closest locality to Monteagudo, Laderas del Campillo, we found lizards assigned to the Albacete/Murcia lineage. Thus, we propose that the Albacete/Murcia lineage could be considered the present-day representation of the nominotypical taxon, *P. hispanicus* sensu stricto. In fact, the phylogenetic tree (Figure 2) shows that there is a monophyletic group formed by the Valencia lineage and *P. hispanicus* sensu stricto (Albacete/Murcia lineage). This clade is the sister group to *P. vaucheri*. The Galera lineage is a monophyletic group, clearly separated from the other Eastern lineages of Iberian *Podarcis*. In line with this result and the

arrangement of different lineages in the phylogeny of Iberian *Podarcis* (Table 1 and Figures 2 and 4), we can conclude that the Galera lineage represents a new full species: *Podarcis galerae* sp. nov. The taxonomic arrangement of this lineage within the Iberian *Podarcis* complex and the description of the new species in comparison with the nominal taxon *P. hispanicus* is proposed below.

4 | TAXONOMY

Family LACERTIDAE

Genus *Podarcis* Wagler, 1830

4.1 | *Podarcis hispanicus* Steindachner, 1870

Lacerta oxycephala var. *hispanica* Steindachner, 1870. *Sitzungsber. Akad. Wiss. Wien, Math. Naturwiss. Kl.*, Abt.1, 62(8): 350.

Lacerta muralis steindachneri Bedriaga, 1886. *Abh. Senckenberg. Naturforsch. Ges.*, 14(2): 256.

Podarcis [*hispanicus*] *hispanicus* Steindachner, 1870, Geniez et al., 2007. *Herpet. J.*, 17:74.

4.1.1 | Type locality

Monteagudo (not Monte Agudo), province of Murcia. *Terra typica restricta* Geniez et al. (2007).

Geniez et al. (2007) designed as the lectotype the specimen NMW 16088:1 (Naturhistorisches Museum Wien, Austria).

4.1.2 | Diagnosis

Due to the coincidence of several morphometric traits of the lineages from Galera and Albacete/Murcia, the diagnosis of Geniez et al. (2007) is, in general, valid for both lineages, here recognized as separated species. A small lizard with a very flattened head (Table S2). Generally, it lacks the maseteric plate. Dorsum brown. In most lizards, there is a continuation of light dorsolateral stripes and dark stripes along parietal scales of the head. Normally (more than 83% of individuals), vertebral line is present and in the majority of cases, bi or even trifurcated (Table S3 and Figure S2). Belly with white colour, even if more than 30% of individuals can show a yellowish colour, in some cases covering the base of the tail (Figure S2). In 25% of individuals, blue ocelli are present in outer ventral scales. From 56 to 73 dorsal scales (Geniez et al., 2007, mention lizards with a minimum of 44 dorsal scales).

4.2 | *Podarcis galerae* sp.nov

4.2.1 | Holotype

Herpetological collection (Colección Herpetológica de la Universidad de Salamanca, CHUS) of the Department of Animal Biology (University of Salamanca, Salamanca, Spain), CHUS01140319, holotype by present designation; adult male captured by Ana Pérez-Cembranos and Valentín Pérez-Mellado on 14 September 2019 in the village of Galera (Granada province, Spain).

4.2.2 | Type locality

Galera, province of Granada (Spain).

4.2.3 | Description of the holotype

Adult male (Figure 1) with 48.5 mm of snout–vent length and 115 mm of its intact tail length, 11.67 mm of pileus length, 5.62 mm of pileus width, 5.29 mm of head height, 15.85 mm of front leg length, 26.42 mm of hindleg length and 11.67 mm of foot length. Scalation: 56 longitudinal rows of dorsal scales at mid-body (dorsalia), 25 gular scales (gularia), 10 collar scales (collaria), 21 transversal rows of ventral scales from the collar to the anal plate (ventralia), 14 femoral pores on left hindleg (femoralia), 15 femoral pores on right hindleg, 27 subdigital lamellae under the fourth toe, six submaxillary scales on each side, eight supralabial scales on each side, six supraocular scales on each side and 10 supraciliary granules on each side. Coloration on the live animal: iris light brown. Pileus uniformly light brown. Dorsum brown, finely dotted. The individual lacks a well-formed vertebral line, although there is an alignment of disconnected blackish points. Light brown on dorsolateral lines, well-marked and with sharp edges, with dark supra-dorsolateral bands formed by unconnected black spots. Dorsolateral stripes continue over the pileus. Tail greenish, clearly contrasted with the brown dorsum. Upper half of flanks dark brown, speckled with black. Lower half of flanks with light brown background, stained with dark brown. Ventral whitish-greyish. Gular area only with some black point in the lateral zones. Outer ventral scales with faded grey spots. Blue ocelli in all outer ventral scales. Submaxillary scales not pigmented (Figure S1).

4.2.4 | Etymology

The epithet *galerae* refers to the type locality: Galera, a village from north-eastern Granada province (Spain).

4.2.5 | Diagnosis

A small wall lizard with <53 mm of SVL (see Table S2 for averages, maximum and minimum values of morphometric and scalations characters). Dorsal pattern and colour very variable among localities and even within a given population. Dorsum always light or dark brown, greyish or even reddish, never green. Light dorsolateral stripes that can continue or not over parietal plates of the head. Supradorsolateral stripes dark brown or black, present in almost all individuals. As in other Iberian *Podarcis*, light dorsolateral stripes are better defined in adult females. Upper half of flanks with a light brown spotted with small dark brown or black dashes, profusely reticulated with black spots or uniformly black, particularly in adult females. Lower half of flanks with a light brown sparsely spotted with brown or black small spots. Between upper and lower half of flanks, frequently there is a light brown stripe, again better defined in adult females. Vertebral line is present in only a half of individuals (Table S2). When present, vertebral line is frequently bifurcated, especially on the upper half of dorsum. In a majority of adult individuals (Table S2), belly is white or grey. However, a minor proportion of lizards shows a yellowish or even an orange belly (Table S3 and Figure S1). More than 40% of adult males have blue ocelli on outer ventral scales. Masseteric plate is absent. Juveniles, many adult females and even some adult males, can have a blue or greenish tail, sharply contrasted with brown dorsum.

4.2.6 | Comparison with other species

Table S3 shows the main differences between *P. hispanicus* and *P. galerae* sp. nov., comparing coloration and design traits of both species with descriptions by Geniez et al. (2007). Body size and general aspect of *P. hispanicus* and *P. galerae* sp. nov. are quite similar. Males of *P. galerae* sp. nov. show a wider head width and a lower number of femoral pores than *P. hispanicus*. Both sexes of *P. galerae* sp. nov. show a higher ventralia. In addition, in *P. galerae* sp. nov. we observe a more pointed snout and a more flattened head (Figure S1). There are several individuals that lack a vertebral line. When present, vertebral line is bifurcated in less than a half of adult lizards (Table S3 and see above the diagnosis of *P. galerae* sp. nov.). In *P. galerae* sp. nov., belly is normally white as in *P. hispanicus*, but there are individuals with yellowish or even orange bellies. In addition, in several adult males it is possible to observe blue ocelli on outer ventral scales, a trait only observed in a minority of *P. hispanicus* males.

4.2.7 | Distribution and ecology

The geographical distribution of *P. hispanicus* and *P. galerae* sp. nov. is poorly known. According to our present-day data, *P. hispanicus* is approximately distributed from the latitude of Murcia town to the north. Apparently, *P. hispanicus* is today absent from its *T. t. restricta*, Monteagudo (Murcia province, Geniez et al., 2007 and pers. Obs.). We ignore the northern limit of this distribution, especially in relation to *P. liolepis*. Similarly, the western limit remains to be clarified in relation to the geographical area occupied by *P. virescens*. Our westernmost locality was Cañada del Provencio in Sierra de Alcaraz (Albacete province). From our survey, the easternmost locality of *P. hispanicus* would be Callosa de Segura (Alicante province). Unfortunately, we cannot resolve if lizards from Elche (Alicante) can be assigned to *P. hispanicus* (Renoult et al., 2009). *Podarcis galerae* sp. nov. is mainly present to the south of Murcia town, apparently reaching coastal areas of Almería province (between Dalías and Berja, Caeiro-Dias et al., 2018, from data of Renoult et al., 2009). To the east, *P. galerae* sp. nov. arrives to Embalse de la Pedrera, in Orihuela (Alicante province). To the west, it would be present 2 km to the north of Tíscar (Jaén province, Caeiro-Dias et al., 2018). Caravaca de la Cruz (Murcia province) would be the northernmost known locality of *P. galerae* sp. nov. (Kaliontzopoulou et al., 2011). It is clear that we would need a deeper survey of geographical ranges of both species. Particularly interesting will be to study overlap areas of both lizard species. According with known localities, the altitudinal range of both species seems to be very similar (from 90 to 1,180 m.a.s.l. in *P. hispanicus* and 120 to 1,164 m in *P. galerae* sp. nov.). Both species are clearly saxicolous. As other Iberian species (i.e. *P. guadarramae* (Geniez et al., 2014 and references therein)), *P. hispanicus* and *P. galerae* sp. nov. are mainly adapted to rupicolous habitats. *P. hispanicus* is found in arid landscapes close to Murcia town (Laderas del Campillo), occupying ruins and artificial walls with crevices. It is also present in rocky outcrops inside pine forests (Cañada del Provencio). We also found lizards isolated in small accumulations of stacked stones that were removed from cultivated areas (Montealegre del Castillo). *Podarcis galerae* sp. nov. occupies a range of arid Mediterranean landscapes characterized by a strong human impact and a poor xerophytic and thermophilous vegetation. A vast proportion of the distribution range is a high plateau area characterized by a remarkable aridity and semi-desertic conditions, where lizards are located in rocky outcrops on vacant lands (Puebla de Don Fadrique), in ruins of old buildings, artificial rock fences or in breakwaters of reservoirs (Embalse de los Rodeos and Embalse de la Pedrera).

5 | Discussion

The mtDNA gene trees obtained show a clearer phylogeographical pattern than in previous attempts to unravel the history of the species complex (see, for example, Kaliontzopoulou et al., 2011). The most basal node in the tree gives rise to four groups: a monophyletic group including *P. muralis*, a western group (*P. carbonelli*, *P. virescens*, *P. guadarramae* and *P. bocagei*), a south-eastern group (*P. vaucheri*, Valencia, *P. hispanicus* sensu stricto (Albacete/Murcia) and *P. galerae* sp. nov. (Galera)) and a low supported north-eastern group represented by *P. liolepis* (Figure 2). This low support could be due to the lack of genetic data from the entire distribution range of *P. liolepis*, as corroborated by the high genetic diversity showed on TCS networks for nuclear genes. An interesting result was that *P. liolepis* was not found to be the sister group of *P. galerae* sp. nov., in contrast to what has been indicated in other studies, which suggests that *P. liolepis* is a sister lineage to all remaining lineages of Iberian *Podarcis* (Kaliontzopoulou et al., 2011; Pinho et al., 2006). The species delimitation and clustering analysis based on mtDNA identified three groups in the SE region (Valencia lineage, *P. hispanicus* sensu stricto and *P. galerae* sp. nov.). Even though species delimitation analysis based on nuclear loci and combined data set (mtDNA + nuclear) provided support for treatment of the Valencia lineage, *P. hispanicus* sensu stricto and *P. galerae* sp. nov. as different species, the species tree output in BPP and STACEY analyses could only provide significant support for the early divergence of *P. vaucheri*, unlike the findings based on mtDNA. All the species delimitation analysis based on both mtDNA and nuclear loci reported the same number of independent evolutionary units; therefore, no gene flow or introgression was detected, contrary to the different number of clusters found using only mtDNA data, or morphological and nuclear data between *P. hispanicus* lineages in Renoult et al. (2009). Networks based on nuclear genes indicated the uniqueness of *P. muralis* and *P. vaucheri* but showed a common haplotype between geographically close groups such as *P. virescens*, *P. liolepis*, *P. galerae* sp. nov., *P. hispanicus* sensu stricto or the Valencia lineage. A posterior differentiation of the defined groups is also represented with the presence of species-specific haplotypes.

The SE region of the Iberian Peninsula presents a complex geological history, highlighting the connection between the Mediterranean Sea and the Atlantic Ocean through the Betic corridor during the middle Miocene (Serravallian/Tortonian; 12.8–7.2 Ma) and the definitive closure during the Messinian (7.2–5.33 Ma) triggering the start of the Messinian Salinity Crisis (5.97–5.33 Ma) connecting Africa and the Iberian Peninsula (Krijgsman et al., 1996, 2000, 2018). The geological instability present during these periods is thought to have caused the origin of many groups of organisms (Busack et al., 2005; Carranza et al., 2004; Kaliontzopoulou et al., 2011;

Pinho et al., 2006). We found that *P. galerae* sp. nov. split from the SE Iberian *Podarcis* group (Valencia lineage, *P. hispanicus* sensu stricto and *P. vaucheri*) at 12.48 Ma. During this period, the Betic corridor was a connection between the Mediterranean Sea and the Atlantic Ocean dividing the Iberian Peninsula at the Betic cordillera. This scenario could have caused the isolation of *P. galerae* sp. nov. distributed in the south of this region. The separation between *P. liolepis* (distributed in the north-east) and the large clade of western lineages, including *P. carbonelli*, *P. virescens*, *P. guadarramae* and *P. bocagei*, also occurred during this period (12.58 Ma). The divergence between the Valencia lineage and the nominotypical taxon *P. hispanicus* sensu stricto occurred at 7.11 Ma. This period corresponds with the end of the Tortonian, when the Betic Corridor become colonizable by land with the definitive closure of the Betic Strait, causing the connection between the north and south regions of the Iberian Peninsula and Africa.

According to our genetic results, lizards from Laderas del Campillo (Murcia province) belong to the Albacete/Murcia lineage. This is the closest point to the restricted type locality of Monteagudo (Geniez et al., 2007) where we found *Podarcis* lizards. Even though *P. galerae* sp. nov. samples present morphologically similar traits, samples from the Albacete/Murcia lineage were identified as the nominal form of the complex, as this lineage shares almost all morphological features proposed by Geniez et al. (2007) for the name-bearing type specimens.

In addition, our findings support a clear delimitation of *P. galerae* sp. nov., on one hand, and the group that encompasses the lineages from Valencia, *P. hispanicus* sensu stricto and North Africa, on the other. This separation is noteworthy and supports full species status for the Galera lineage. The systematic status of the Valencia lineage remains unclear with additional genetic information on the lineage, wider sampling of its geographical range and a full morphological comparison with *P. hispanicus* sensu stricto and *P. galerae* sp. nov. required before full species status can be assigned.

It is difficult to establish the sympatry or non-sympatry of different lineages/species of *Podarcis* on the Iberian Peninsula. Previous studies evidenced a parapatric distribution in the eastern Iberian Peninsula; in contrast, in the west, species like *P. carbonelli* and *P. guadarramae* are largely sympatric in their distribution ranges, as well as *P. bocagei* and *P. guadarramae*. Further strict boundaries were proposed to separate eastern and southern species, such as *P. vaucheri*, *P. liolepis*, *P. virescens* and *P. hispanicus*. The limits among these species of the complex are not yet well defined due to samples being required from the entire distribution area. It is likely that the distribution areas will overlap, as it is the norm in Western Iberia and in other areas of the Mediterranean basin. The only way to take into account the extraordinary diversity of mitochondrial lineages of Iberian *Podarcis* is to document their morphological characteristics and make taxonomic decisions. We agree with the important argument that only formal

naming of different lineages will allow these new species to be considered in conservation policies (Geniez et al., 2007). We can always expect new discoveries or deeper analyses to change the general picture in the near future and, perhaps, certain taxa will change status, such as the Valencia lineage.

Despite both phylogenetic and species delimitation analyses corroborated with multiple methodological approaches and using different data sets (mtDNA, nuclear loci or combined data) showed high supports for the different SE *Podarcis* groups, genome-wide approaches would help to confirm these phylogenetic relationships and address the issues of introgression. Furthermore, to solve the puzzle of lineages of this species complex would be necessary to obtain a better picture of the distribution of *Podarcis* genus in the Iberian Peninsula. It could be particularly important to study the *P. liolepis* and Valencia lineages in a deeper way, including a greater number of individuals and locations. In addition, a global morphological study of *P. hispanicus* complex would be of interest and would help to clarify the wide diversity presents in this species complex.

In conclusion, this study provides a large genetic data set of mtDNA and nuclear sequences of the *P. hispanicus* complex based on a greater number of individuals and locations than in previous studies. These results allow a deeper phylogenetic analysis on SE *Podarcis* lineages that contributes to elucidating the controversy regarding this region. Our phylogenetic tree shows the group including the Valencia lineage and *P. hispanicus* sensu stricto, as sister group of the North African form (*P. vaucheri*). *Podarcis galerae* sp. nov. form a monophyletic group, separated (~12 Ma) from the rest of the SE and North African forms, in contrast to previous studies based on a very low number of individuals that showed the closest phylogenetic relationship between *P. galerae* sp. nov. and *P. liolepis* (Kaliontzopoulou et al., 2011; Pinho et al., 2006). Our results support the elevation of *P. galerae* sp. nov. to full species rank.

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ORCID

Marta Bassitta  <https://orcid.org/0000-0001-6663-0820>

Ana Pérez-Cembranos  <https://orcid.org/0000-0002-8296-2283>

Valentín Pérez-Mellado  <https://orcid.org/0000-0002-7137-4945>

Barbara Terrasa  <https://orcid.org/0000-0003-2607-4633>

Richard P. Brown  <https://orcid.org/0000-0003-2401-6077>

Jesús Ortega  <https://orcid.org/0000-0001-6599-1675>

Jose A. Castro  <https://orcid.org/0000-0001-8262-560X>

Antònia Picornell  <https://orcid.org/0000-0003-4454-5304>

Cori Ramon  <https://orcid.org/0000-0003-1234-3337>

REFERENCES

- Arnold, E. N., Arribas, O., & Carranza, S. (2007). Systematics of the Palaearctic and Oriental lizard tribe Lacertini (Squamata: Lacertidae: Lacertinae), with descriptions of eight new genera. *Zootaxa*, 1430(1), 1–86.
- Bouckaert, R., Vaughan, T. G., Barido-Sottani, J., Duchêne, S., Fourment, M., Gavryushkina, A., & Matschiner, M. (2019). BEAST 2.5: An advanced software platform for Bayesian evolutionary analysis. *PLoS Computational Biology*, 15(4), e1006650.
- Brown, R. P., Terrasa, B., Pérez-Mellado, V., Castro, J. A., Hoskisson, P. A., Picornell, A., & Ramon, M. M. (2008). Bayesian estimation of post-Messinian divergence times in Balearic Island lizards. *Molecular Phylogenetics and Evolution*, 48(1), 350–358.
- Brown, R. P., & Yang, Z. (2011). Rate variation and estimation of divergence times using strict and relaxed clocks. *BMC Evolutionary Biology*, 11(1), 271.
- Buades, J. M., Rodríguez, V., Terrasa, B., Pérez-Mellado, V., Brown, R. P., Castro, J. A., ... Ramon, M. M. (2013). Variability of the mc1r gene in melanic and non-melanic *Podarcis lilfordi* and *Podarcis pityusensis* from the Balearic Archipelago. *PLoS One*, 8(1), e53088.
- Busack, S. D., Lawson, R., & Arjo, W. M. (2005). Mitochondrial DNA, allozymes, morphology and historical biogeography in the *Podarcis vaucheri* (Lacertidae) species complex. *Amphibia-Reptilia*, 26, 239–256.
- Caeiro-Dias, G., Luís, C., Pinho, C., Crochet, P. A., Sillero, N., & Kaliontzopoulou, A. (2018). Lack of congruence of genetic and niche divergence in *Podarcis hispanicus* complex. *Journal of Zoological Systematics and Evolutionary Research*, 56(4), 479–492.
- Carranza, S., Arnold, E. N., & Amat, F. (2004). DNA phylogeny of *Iberolacerta* and other lacertine lizards (Reptilia: Lacertidae): did competition cause long-term mountain restriction? *Systematics and Biodiversity*, 2(1), 57–77.
- Clement, M., Posada, D., & Crandall, K. A. (2000). TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, 9, 1657–1659.
- Corander, J., & Tang, J. (2007). Bayesian analysis of population structure based on linked molecular information. *Mathematical Biosciences*, 205, 19–31.
- Fujisawa, T., & Barraclough, T. G. (2013). Delimiting species using single-locus data and the Generalized Mixed Yule Coalescent approach: a revised method and evaluation on simulated data sets. *Systematic Biology*, 62(5), 707–724.
- Geniez, P., Cluchier, A., Sá-Sousa, P., Guillaume, C. P., & Crochet, P. A. (2007). Systematics of the *Podarcis hispanicus*-complex (Sauria, Lacertidae) I: Redefinition, morphology and distribution of the nominotypical taxon. *The Herpetological Journal*, 17(2), 69–80.
- Geniez, P., Sa-Sousa, P., Guillaume, C. P., Cluchier, A., & Crochet, P. A. (2014). Systematics of the *Podarcis hispanicus* complex (Sauria, Lacertidae) III: valid nomina of the western and central Iberian forms. *Zootaxa*, 3794(1), 1–51.
- González, P., Pinto, F., Nogales, M., Jiménez-Asensio, J., Hernández, M., & Cabrera, V. M. (1996). Phylogenetic relationships of the canary islands endemic lizard Genus *Gallotia* (Sauria: Lacertidae), inferred from mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution*, 6(1), 63–71.
- Grummer, J. A., Bryson, R. W. Jr, & Reeder, T. W. (2014). Species delimitation using Bayes factors: simulations and application to the *Sceloporus scalaris* species group (Squamata: Phrynosomatidae). *Systematic Biology*, 63(2), 119–133.
- Harris, D. J., & Sa-Sousa, P. (2002). Molecular phylogenetics of Iberian wall lizards (*Podarcis*): is *Podarcis hispanica* a species complex? *Molecular Phylogenetics and Evolution*, 23(1), 75–81.
- Heled, J., & Drummond, A. J. (2010). Bayesian inference of species trees from multilocus data. *Molecular Biology and Evolution*, 27, 570–580.
- Hoang, D. T., Chernomor, O., Von Haeseler, A., Minh, B. Q., & Vinh, L. S. (2017). UFBoot2: improving the ultrafast bootstrap approximation. *Molecular Biology and Evolution*, 35(2), 518–522.
- Jones, G. (2017). Algorithmic improvements to species delimitation and phylogeny estimation under the multispecies coalescent. *Journal of Mathematical Biology*, 74, 447–467.
- Kaliontzopoulou, A., Carretero, M. A., & Llorente, G. A. (2012). Morphology of the *Podarcis* wall lizards (Squamata: Lacertidae) from the Iberian Peninsula and North Africa: patterns of variation in a putative cryptic species complex. *Zoological Journal of the Linnean Society*, 164(1), 173–193.
- Kaliontzopoulou, A., Pinho, C., Harris, D. J., & Carretero, M. A. (2011). When cryptic diversity blurs the picture: a cautionary tale from Iberian and North African *Podarcis* wall lizards. *Biological Journal of the Linnean Society*, 103(4), 779–800.
- Kapli, P., Lutteropp, S., Zhang, J., Kobert, K., Pavlidis, P., Stamatakis, A., & Flouri, T. (2017). Multi-rate poisson tree processes for single-locus species delimitation under maximum likelihood and Markov chain Monte Carlo. *Bioinformatics*, 33, 1630–1638.
- Katoh, K., & Toh, H. (2008). Recent developments in the MAFFT multiple sequence alignment program. *Briefings in Bioinformatics*, 9(4), 286–298.
- Köhler, G. (2012). *Color catalogue for field biologists*. Offenbach, Germany: Herpeton Verlag.
- Krijgsman, W., Capella, W., Simon, D., Hilgen, F. J., Kouwenhoven, T. J., Meijer, P. T., ... Flecker, R. (2018). The Gibraltar corridor: Watergate of the Messinian salinity crisis. *Marine Geology*, 403, 238–246.
- Krijgsman, W., Garcés, M., Agustí, J., Raffi, I., Taberner, C., & Zachariasse, W. J. (2000). The ‘Tortonian salinity crisis’ of the eastern Betics (Spain). *Earth and Planetary Science Letters*, 181(4), 497–511.
- Krijgsman, W., Garcés, M., Langereis, C. G., Daams, R., Van Dam, J., Van der Meulen, A. J., ... Cabrera, L. (1996). A new chronology for the middle to late Miocene continental record in Spain. *Earth and Planetary Science Letters*, 142(3–4), 367–380.
- Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, 33(7), 1870–1874.
- Lanfear, R., Frandsen, P. B., Wright, A. M., Senfeld, T., & Calcott, B. (2016). PartitionFinder 2: new methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Molecular Biology and Evolution*, 34(3), 772–773.
- Leaché, A. D., Zhu, T., Rannala, B., & Yang, Z. (2018). The spectre of too many species. *Systematic Biology*, 68(1), 168–181.
- Leigh, J. W., & Bryant, D. (2015). PopART: Full-feature software for haplotype network construction. *Methods in Ecology and Evolution*, 6, 1110–1116.
- Librado, P., & Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, 25(11), 1451–1452.

- Minh, B. Q., Nguyen, M. A. T., & von Haeseler, A. (2013). Ultrafast approximation for phylogenetic bootstrap. *Molecular Biology and Evolution*, *30*(5), 1188–1195.
- Nguyen, L. T., Schmidt, H. A., von Haeseler, A., & Minh, B. Q. (2014). IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Molecular Biology and Evolution*, *32*(1), 268–274.
- Pérez-Mellado, V., & Gosá, A. (1988). Biometría y Folidosis en Lacertidae (Sauria, Reptilia). Algunos aspectos metodológicos. *Revista Española De Herpetología*, *3*(1), 97–104.
- Pinho, C., Ferrand, N., & Harris, D. J. (2006). Reexamination of the Iberian and North African *Podarcis* (Squamata: Lacertidae) phylogeny based on increased mitochondrial DNA sequencing. *Molecular Phylogenetics and Evolution*, *38*(1), 266–273.
- Pinho, C., Harris, D. J., & Ferrand, N. (2007). Comparing patterns of nuclear and mitochondrial divergence in a cryptic species complex: the case of Iberian and North African wall lizards (*Podarcis*, Lacertidae). *Biological Journal of the Linnean Society*, *91*(1), 121–133.
- Pinho, C., Harris, D. J., & Ferrand, N. (2008). Non-equilibrium estimates of gene flow inferred from nuclear genealogies suggest that Iberian and North African wall lizards (*Podarcis* spp.) are an assemblage of incipient species. *BMC Evolutionary Biology*, *8*(1), 63.
- Pons, J., Barraclough, T. G., Gomez-Zurita, J., Cardoso, A., Duran, D. P., Hazell, S., ... Vogler, A. P. (2006). Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Systematic Biology*, *55*(4), 595–609.
- Puillandre, N., Lambert, A., Brouillet, S., & Achaz, G. (2012). ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular Ecology*, *21*(8), 1864–1877.
- R Core Team (2018). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from <https://www.R-project.org/>
- Rambaut, A. (2014). *FigTree 1.4.2 software*, Institute of Evolutionary Biology, Edinburgh, UK: University of Edinburgh.
- Rambaut, A., Drummond, A. J., Xie, D., Baele, G., & Suchard, M. A. (2018). Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Systematic Biology*, *67*(5), 901–904.
- Rannala, B. (2015). The art and science of species delimitation. *Current Zoology*, *61*(5), 846–853.
- Rannala, B., & Yang, Z. (2003). Bayes estimation of species divergence times and ancestral population sizes using DNA sequences from multiple loci. *Genetics*, *164*(4), 1645–1656.
- Rannala, B., & Yang, Z. (2017). Efficient Bayesian species tree inference under the multispecies coalescent. *Systematic Biology*, *66*(5), 823–842.
- Renoult, J. P., Geniez, P., Bacquet, P., Benoit, L., & Crochet, P. A. (2009). Morphology and nuclear markers reveal extensive mitochondrial introgressions in the Iberian Wall Lizard species complex. *Molecular Ecology*, *18*(20), 4298–4315.
- Renoult, J. P., Geniez, P., Bacquet, P., Guillaume, C. P., & Crochet, P. A. (2010). Systematics of the *Podarcis hispanicus*-complex (Sauria, Lacertidae) II: the valid name of the north-eastern Spanish form. *Zootaxa*, *2500*(1), 58–68.
- Rodríguez, V., Brown, R. P., Terrasa, B., Pérez-Mellado, V., Castro, J. A., Picornell, A., & Ramon, M. M. (2013). Multilocus genetic diversity and historical biogeography of the endemic wall lizard from Ibiza and Formentera, *Podarcis pityusensis* (Squamata: Lacertidae). *Molecular Ecology*, *22*(19), 4829–4841.
- Rodríguez, V., Brown, R. P., Terrasa, B., Pérez-Mellado, V., Picornell, A., Castro, J. A., & Ramon, C. (2014). Genetic diversity and historical biogeography of the Maltese wall lizard, *Podarcis filfolensis* (Squamata: Lacertidae). *Conservation Genetics*, *15*(2), 295–304.
- Rodríguez, V., Buades, J. M., Brown, R. P., Terrasa, B., Pérez-Mellado, V., Corti, C., ... Ramon, M. M. (2017). Evolutionary history of *Podarcis tiliguerta* on Corsica and Sardinia. *BMC Evolutionary Biology*, *17*(1), 27.
- Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D. L., Darling, A., Höhna, S., ... Huelsenbeck, J. P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, *61*(3), 539–542.
- Stephens, M., Smith, N. J., & Donnelly, P. (2001). A new statistical method for haplotype reconstruction from population data. *The American Journal of Human Genetics*, *68*(4), 978–989.
- Terrasa, B., Pérez-Mellado, V., Brown, R. P., Picornell, A., Castro, J. A., & Ramon, M. M. (2009). Foundations for conservation of intraspecific genetic diversity revealed by analysis of phylogeographical structure in the endangered endemic lizard *Podarcis lilfordi*. *Diversity and Distributions*, *15*(2), 207–221.
- Yang, Z. (1993). Maximum likelihood estimation of phylogeny from DNA sequences when substitution rates differ over sites. *Molecular Biology and Evolution*, *10*, 1396–1401.
- Yang, Z., & Rannala, B. (2010). Bayesian species delimitation using multilocus sequence data. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(20), 9264–9269.
- Zhang, J., Kapli, P., Pavlidis, P., & Stamatakis, A. (2013). A general species delimitation method with applications to phylogenetic placements. *Bioinformatics*, *29*(22), 2869–2876.

SUPPORTING INFORMATION

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

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