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Hidden diversity in the *Podarcis tauricus* (Sauria, Lacertidae) species subgroup in the light of multilocus phylogeny and species delimitation

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ABSTRACT

The monophyletic species subgroup of *Podarcis tauricus* is distributed in the western and southern parts of the Balkans, and includes four species with unresolved and unstudied inter- and intra-specific phylogenetic relationships. Using sequence data from two mitochondrial and three nuclear genes and applying several phylogenetic methods and species delimitation approaches to an extensive dataset, we have reconstructed the phylogeny of the *Podarcis* wall lizards in the Balkans, and re-investigated the taxonomic status of the *P. tauricus* species subgroup. Multilocus analyses revealed that the aforementioned subgroup consists of five major clades, with *P. melisellensis* as its most basal taxon. Monophyly of *P. tauricus* sensu stricto is not supported, with one of the subspecies (*P. t. ionicus*) displaying great genetic diversity (hidden diversity or cryptic species). It comprises five, geographically distinct, subclades with genetic distances on the species level. Species delimitation approaches revealed nine species within the *P. tauricus* species subgroup (*P. melisellensis, P. gaigeae, P. milensis*, and six in the *P. tauricus* complex), underlining the necessity of taxonomic re-evaluation. We thus synonymize some previously recognized subspecies in this subgroup, elevate *P. t. tauricus* and *P. g. gaigeae* to the species level and suggest a distinct Albanian-Greek clade, provisionally named as the *P. ionicus* species complex. The latter clade comprises five unconfirmed candidate species that call for comprehensive studies in the future.

1. Introduction

Wall lizards of the genus *Podarcis* Wagler, 1830 belong to the family of Lacertidae, currently including 23 species (Sindaco et al., 2013; Uetz and Hošek, 2016). The genus is western European in origin, its diversity being the result of several vicariance events mainly related to the fragmentation of the western microplates during the Miocene (Oliverio et al., 2000). It is now the predominant reptile group in southern Europe, distributed from northwestern Africa through the Iberian and the Italian peninsulas to the Balkans, northwestern Asia Minor and the Crimean peninsula (Arnold, 1973). Taxonomy within *Podarcis* is complicated and continuously subject to revision, due to the existence of substantial intra-specific variability (Arnold et al., 1978). The first molecular phylogenetic studies on the genus (Harris and Arnold, 1999; Oliverio et al., 2000) divided it into several species groups, with relationships mainly unresolved.

The focal taxa of this study form part of the Balkan species group, which is phylogenetically comprised of two distinct species subgroups: (a) the *P. erhardii* subgroup, including *P. cretensis*

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(Wettstein, 1952), *P. erhardii* (Bedriaga, 1882), *P. levendis* Lymberakis, Poulakakis, Kaliontzopoulou, Valakos, & Mylonas, 2008, and *P. peloponnesiacus* (Bibron & Bory, 1833); and (b) the *P. tauricus* species subgroup, consisting of *P. gaigeae* (Werner, 1930), *P. melisellensis* (Braun, 1877), *P. milensis* (Werner, 1930) and *P. tauricus* (Pallas, 1814). In the second subgroup, *P. gaigeae* and *P. milensis* are island endemics. Two morphological subspecies of the former are currently recognized: *P. g. gaigeae* (Werner, 1930) on the Skyros Archipelago, and *P. g. weigandi* (Gruber & Schultze-Westrum, 1971) on the islet of Piperi. On the other hand, *P. milensis* includes three morphological subspecies [*P. m. milensis* (Bedriaga, 1882), *P. m. adolfjordansi* (Buchholz, 1962), and *P. m. gerakuniae* (Müller, 1938)], distributed on the Milos Archipelago. *Podarcis tauricus* is the species with the widest distribution within the subgroup, ranging mainly in the southern Balkans and eastern Europe (Fig. 1C). It is subdivided into three currently recognized subspecies (Sindaco and Jeremcenko, 2008): (a) *P. t. tauricus* (Pallas, 1814); (b) *P. t. ionicus* (Lehrs, 1902); and (c) *P. t. thasopulae* (Kattinger, 1942), of which the first two are geographically isolated by the Pindos mountain range (Fig. 1C), and the third is a stenoendemic subspecies inhabiting the islet of Thasopoula (north Aegean). Finally, *Podarcis melisellensis* is distributed along the Dalmatian coast and on many of its islands, currently represented by two subspecies: *P. m. melisellensis* (Braun, 1877), *P. m. fiumana* (Werner, 1891), and one undescribed lineage (Podnar et al., 2004; Sindaco and Jeremcenko, 2008).

The phylogenetic relationships and phylogeography of the *P. tauricus* subgroup has previously been investigated on the basis of mitochondrial DNA (mtDNA) loci (Podnar et al., 2014, 2004; Poulakakis et al., 2005a,b). The analysis of a dataset including all the species of the *P. tauricus* subgroup, but with limited number of specimens solely from Greece, supported monophyly of the *P. tauricus* species subgroup with two major clades. The first included *P. tauricus* sensu stricto, and the second comprised *P. gaigeae*, *P. milensis*, and *P. melisellensis* (moderate statistical support). In addition, the existence of two major lineages within *P. tauricus* sensu stricto with substantial high genetic diversity was detected. Moreover, mtDNA data for *P. melisellensis* revealed the presence of three major subclades (*melisellensis, fiumana,* and *Lastovo* subclades) with distinct geographic structure that is in discordance with its current subspecies taxonomy (Podnar et al., 2014, 2004).

To date, there has been no comprehensive study of the *P. tauricus* species subgroup with extensive sampling coverage and genetic information from both mitochondrial and nuclear DNA. Such a study will contribute to (a) the discovery of hidden diversity, (b) comparison of phylogenetic assessment among nuclear and mitochondrial markers, (c) comparison between gene trees and species trees, (d) evaluation of the phylogenetic relationships among the focal taxa in the light of new findings, and (e) estimation of the number of species included in the *P. tauricus* species subgroup. To that end, two mitochondrial and three nuclear markers were analysed using an extensive dataset. This included samples from the entire distribution area of the focal taxa and involved several multilocus phylogenetic analyses and coalescence-based approaches.

2. Material and methods

2.1. Specimens, DNA extraction, amplification and sequencing

Total genomic DNA was isolated using a standard ammonium acetate extraction protocol from muscle, liver or blood of specimens preserved in absolute ethanol. The sampling localities are shown in Fig. 1 and the tissue samples are listed in Appendix A. All the samples were deposited in the Natural History Museum of Crete, University of Crete (NHMC), but see Appendix A. The identification of species was based on external morphological characters sensu Arnold and Ovenden (2002). In total, 317 individuals constituting the ingroup were used (298 belonging to the focal *P. tauricus* species subgroup), including 13 species from more than 200 localities.

Double-stranded PCR was performed to amplify partial sequences of two mitochondrial gene (mtDNA) fragments [the large subunit of ribosomal RNA (16S rRNA) and the cytochrome *b* (cyt *b*)], and three nuclear gene (nDNA) fragments [the melanocortin receptor 1 (MC1R) and two anonymous nDNA markers (Pod15b and Pod55)]. These two anonymous markers have been recently added to the genomic resources and used for phylogenetic, species delimitation, population genetics and phylogeographic studies in *Podarcis* spp. (Pereira et al., 2013). Primers and conditions used in PCR amplifications and in cycle sequencing reactions are given in Table 1.

Single stranded sequencing of the PCR product was performed using the Big-Dye Terminator (v3.1) Cycle Sequencing kit ® on an ABI3730 automated sequencer following the manufacturer's protocol and using the same primers as in PCR.

Sequences were viewed and edited using CodonCode Aligner v. 3.7.1 (CodonCode Corporation ®). The authenticity of the sequences and the homology to the targeted genes were evaluated with a BLAST search in the NCBI genetic database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All newly determined sequences have been deposited in GenBank (Appendix A). Sequences of *Podarcis* were retrieved from GenBank (131 in total) and included in the phylogenetic analyses. Moreover, sequences of *Atlantolacerta andreanskyi* (Werner, 1929), *Lacerta agilis* Linnaeus, 1758, and *Teira dugesii* (Milne-Edwards, 1829) were also retrieved and used as outgroups. Information for the downloaded sequences (gene sampling, corresponding accession numbers, and studies generating the sequences) are presented in Appendix A.

To ensure that nuclear copies of mtDNA (pseudo-genes) were not present in the dataset several precautions and observations were conducted: (a) the general agreement in the topology, between the two mtDNA markers, (b) the absence of indels in the cyt *b* since it is a protein-coding gene, and (c) the absence of double peaks in the sequence chromatographs.

2.2. Alignment, genetic distances and model selection

The alignment of the sequences was performed separately for each gene with MAFFT (v.7; Katoh and Standley, 2013) with default parameters and the following alignment strategies of iterative refinement method: L-INS-i for cyt *b*, MC1R, and Pod-55, Q-INS-i for 16S rRNA, and E-INS-i for Pod15b. Alignment gaps were inserted to resolve length differences between sequences. Cytochrome *b* and MC1R sequences were translated into amino acids prior to analysis, and did not show any stop codons. Sequence divergences (*p*-distances) were estimated in MEGA (v.6.06; Tamura et al., 2013). The alignment used is available on request.

The alignment was partitioned into nine blocks, including 6 blocks for the 1st, 2nd, and 3rd codon positions for each one of the two protein-coding genes (cyt *b*, MC1R) and three blocks for each one of the other gene fragments (16S rRNA, Pod55, and Pod15b). This initial partition scheme was loaded in PartitionFinder (v.1.1.0; Lanfear et al., 2012) to calculate and select the best-fit partitioning scheme and models of molecular evolution for each downstream analysis (the model list was different for each analysis). The nine blocks were considered to have linked branch lengths and the model selection was based on the Bayesian Information Criterion (BIC; Schwarz, 1978), ignoring the models that include both gamma distribution and invariable sites (Yang, 2006). Finally, the greedy option was selected to search for the best-fit solutions.

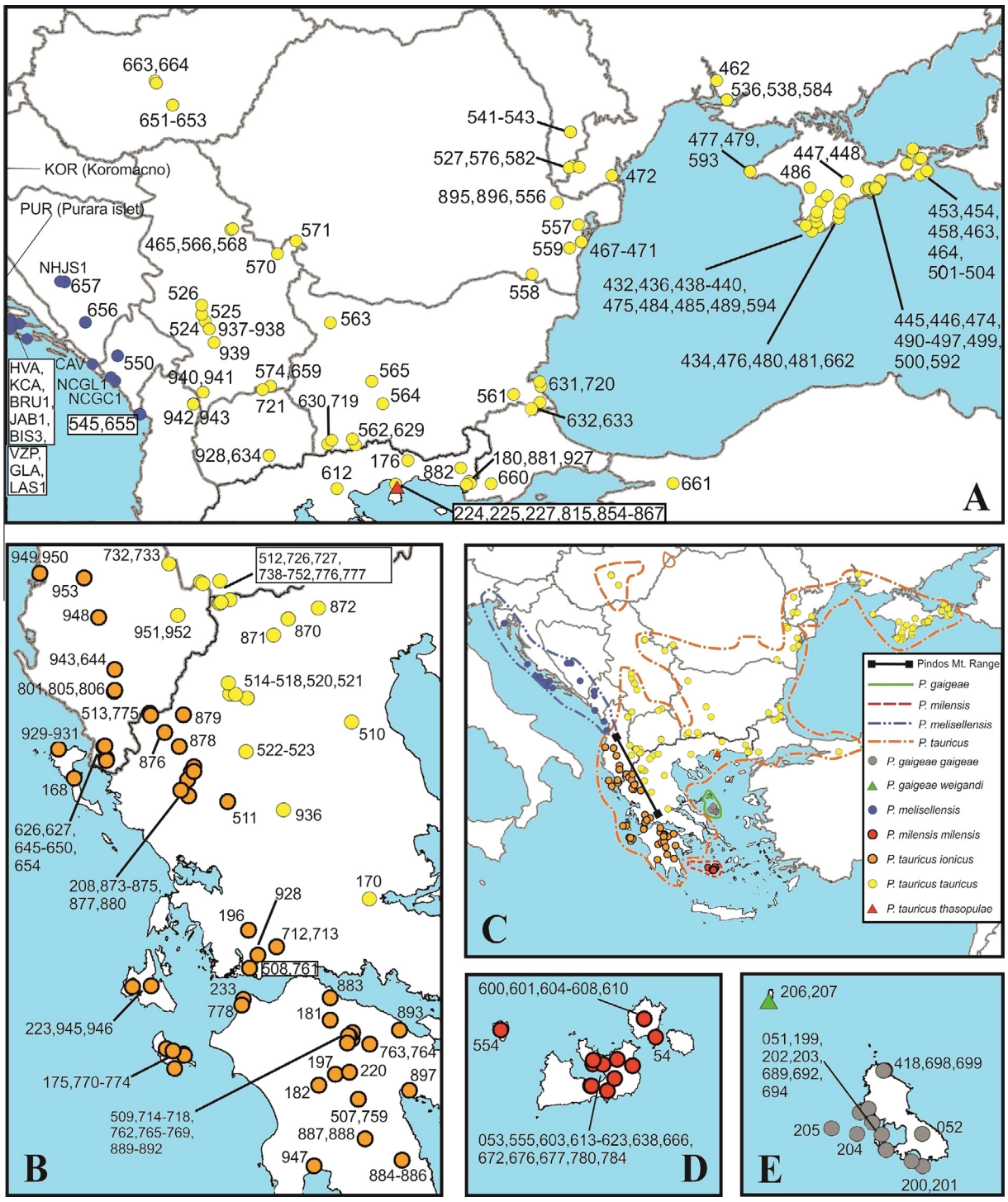

Fig. 1. Maps showing the localities of the samples and the distribution of the taxa belonging to the *P. tauricus* species subgroup: (A) northern parts of the entire range of *P. tauricus* species subgroup; (B) distribution pattern of *P. tauricus ionicus* and *P. t. tauricus* in the southwestern part of the Balkans; (C) sampled localities in the entire area of *P. tauricus* species subgroup; (D) Milos Island group and sampled localities of *P. milensis milensis*; (E) Skyros Island group and Piperi islet and sampled localities of *P. gaigeae*. The numbers/codes on the maps correspond to the sample codes of Appendix A.

To notice, in the species delimitation analysis and coalescent species tree inference the data were partitioned by loci due to requirement of ploidy provision.

2.3. Gene tree estimation on mtDNA, nuDNA and concatenated loci

Phylogenetic trees were constructed using Neighbor Joining (NJ) (Saitou and Nei, 1987), Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI). Neighbor Joining analysis was performed in MEGA using the p-distances. Bootstrapping with 1000 pseudo-replicates was used to examine the robustness of clades in the resulting tree (Felsenstein, 1985). Maximum Parsimony analysis was performed with PAUP* (v.4.0b10; Swofford, 2002) with heuristic searches using stepwise addition and performing tree-bisection-reconnection (TBR) branch swapping (Swofford et al., 1996). Confidence in the nodes was assessed by

Table 1
Primers and conditions used in PCR amplifications[a] and in cycle sequencing reactions.

Gene	Primers	Sequence (5′–3′)	Size (bp)	Conditions	Reference
16S rRNA	16SAr-l 16SBr-h	CGGCCGCCTGTTTATCAAAAACAT GGAGCTCCGGTTTGAACTCAGATC	~530	3 mM MgCl, 94 °C/1 min, 42–52.9 °C/1 min, 72 °C/1 min × 35 cycles	Palumbi (1996)
Cyt b	GLUDG CB2	TGACTTGAARAACCAYCGTTG CCCTCAGAATGATATTTGTCCTCA	~510	3 mM MgCl, 94 °C/1 min, 42–48.6 °C/1 min, 72 °C/1 min × 35 cycles	Palumbi (1996)
	L14841 CB2	AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA CCCTCAGAATGATATTTGTCCTCA	~350		Kocher et al. (1989) Palumbi (1996)
MC1R	MC1RF MC1RR	GGCNGCCATYGTCAAGAACCGGAACC CTCCGRAAGGCRTAAATGATGGGGTCCAC	~700	1.5 mM MgCl, 94 °C/1 min, 59 °C/1 min, 72 °C/1 min × 35 cycles	Pinho et al. (2010)
Pod55	pod55f pod55r	GGATCTTTATAGGAGAGTGCAGGCC TTCCAGATTGTGTTTATCCTGGTGG	~500		Pereira et al. (2013)
Pod15b	pod15bf pod15br	AATCCTGGCTAAATGCAAGCCTTGG GCCAGGAGAATAAGCTACTCCATCC	~550		Pereira et al. (2013)

[a] Using single *Taq* DNA polymerase (KAPA BIOSYSTEMS®).

1000 bootstrap replicates, with the random addition of taxa. Maximum Likelihood analysis was performed with RAxML (v.8.1.21; Stamatakis, 2014). To ensure that the inferred ML tree was not a local optimum, 200 ML searches for each dataset were conducted. The confidence of the branches of the best ML tree was further assessed based on 1000 rapid bootstrap replicates (under the GTRCAT model). Bayesian Inference was performed in MrBayes (v.3.2.1; Ronquist et al., 2012), with four runs and eight chains for each run for 10^7 generations, and the current tree was saved to file every 1000 generations. In order to confirm that the chains had achieved stationarity, we evaluated "burn-in" by plotting log-likelihood scores and tree lengths against generation number using Tracer (v.1.6; Rambaut et al., 2013). The –lnL stabilized after approximately 10^6 generations and the first 25% of the trees were discarded by default, as a conservative measure to avoid the possibility of including random sub-optimal trees. A majority rule consensus tree ("Bayesian" 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, where probabilities $\geqslant$95% indicate significant support.

Mitochondrial genetic clusters that represent "independently evolving" entities were selected, considering only the ingroup, using the method of Zhang et al. (2013), which identifies genetic clusters using a Poisson Tree Processes (PTP) model. Identical sequences were omitted from this analysis. For the concatenated phylogenetic analyses, at least one exemplar representing each PTP group (genetic cluster) in the mtDNA analysis was selected for sequencing the three nuclear markers. Following the same procedure as in mtDNA data, all phylogenetic (NJ, MP, ML and BI) analyses were performed, additionally, on (a) a concatenated dataset containing the two mitochondrial (16S rRNA and cyt *b*) and the three nuclear genes (MC1R, Pod55, and Pod15b) and (b) a dataset including only the nDNA markers. These datasets included 60 entities representing 16 morphologically identified *Podarcis* species, for which all five genes were amplified. Sequences representing the above genes were also obtained from NCBI belonging to *Lacerta agilis* (used as outgroup), *P. bocagei* (Seoane, 1884), *P. filfolensis* (Bedriaga, 1876), *P. muralis*, and *P. tiliguerta* (Gmelin, 1789).

2.4. Coalescent species tree

The coalescent species tree analysis was performed using the BEAST 2 software package (v.2.4.0; Bouckaert et al., 2014). The input files (xml) were created using BEAUti v. 2.4.0, implemented also in the BEAST 2 package. The nucleotide substitution models were not given *a priori* but instead the BEAST Model Test option was selected. As for other priors the Yule Model was selected for speciation and the Uncorrelated Lognormal Model for describing the relaxed molecular clock. The MCMC analysis was run for 4×10^8 generations, saving the result every 5.000 generations. The obtained log files were analysed with Tracer v.1.6. (Rambaut et al., 2014) to verify that the convergence of the analysis had been achieved and that satisfactory effective sample sizes had been obtained (ESSs values >200). The value of $-\ln L$ was stabilized after 4×10^7 generations and the first 10% from the 20.000 saved ones were discarded. To display the species tree the softwares FigTree (v. 1.4.2; part of the BEAST 2 package) and DensiTree (v.2.2.4; Bouckaert, 2010) were used.

2.5. Species delimitation

Bayesian species delimitation was conducted using BP&P (v.3.1 as implemented in BPPx; Yang, 2015) with the dataset for the five molecular markers considered as four independently evolving loci (mtDNA, MC1R, Pod55, and Pod15b) and solely including the nine major clades and subclades of *P. tauricus* species subgroup as potential distinct species. The method uses the multispecies coalescent model to compare different models of species delimitation and species phylogeny in a Bayesian framework, accounting for incomplete lineage sorting due to ancestral polymorphism and gene tree-species tree conflicts (Rannala and Yang, 2013; Yang and Rannala, 2014, 2010). For the prior distributions the approach of Leaché and Fujita (2010) was followed, considering three different combinations of prior: $\theta \sim G(1, 10)$ and $\tau^0 \sim G(1, 10)$, both with a prior mean = 0.1 and variance = 0.01, (b) assuming relatively small ancestral population sizes and shallow divergences among species: $\theta \sim G(2, 2000)$ and $\tau^0 \sim G(2, 2000)$, both with a prior mean = 0.001 and variance = 5×10^{-7}, and (c) assuming large ancestral populations sizes: $\theta \sim G(1, 10)$ and relatively shallow divergences among species: $\tau^0 \sim G(2, 2000)$, which is a conservative combination of priors that should favour models containing fewer species. The rjMCMC analyses (algorithm 1) were performed for 100,000 generations (sampling interval of three) with a burn-in period of 2500 and each species delimitation model was assigned equal prior probability. Each analysis was run at least twice, initiated with different starting seeds, to confirm consistency between runs. The topology of the phylogenetic tree based on the concatenated dataset was given as starting tree.

For comparison reasons, species delimitation was also performed in STACEY (v. 1.2.1; Jones, 2015), which is implemented in the software package BEAST 2. The input files (xml) were created using BEAUti. The nucleotide substitution models were not given *a priori* but instead the BEAST Model Test option was selected. As for other priors the Fossilized Birth Death Model was selected for speciation and the Uncorrelated Lognormal Model for describing the relaxed molecular clock. The MCMC analysis was run for 10^8 generations, saving the result every 5000 generations. The obtained log files were analysed with Tracer to verify that the convergence

of the analysis had been achieved and that satisfactory effective sample sizes had been obtained. The value of −lnL was stabilized after 10^7 generations and the first 10% from the 20,000 saved ones were discarded. The analysis and the display of the results of the species delimitation and its statistical support were made by SpeciesDelimitationAnalyser (Jones et al., 2015).

3. Results

The best-fit partitioning schemes for each downstream analysis (BI and ML) and the selected nucleotide substitution models are summarized in the Supplementary Table S1. Different analyses and datasets resulted in different partitioning schemes and selected models.

In PTP analysis, 34 distinct evolutionary entities were identified (Suppl. Fig. S1). Up to 10 specimens from each evolutionary entity (Appendix A) were chosen and sequenced for the nuclear markers (MC1R, Pod55, and Pod15b), with the exception of the samples 947 (*P. tauricus* from Kalamata) and 132 (*P. muralis* from Kisavos Mt.), for which we failed to amplify the nDNA markers, as well as for the samples for which the mtDNA sequences were retrieved from GenBank (e.g. Lastovo subclade of *P. melisellensis*).

3.1. Concatenated gene trees

For the phylogenetic analyses at mtDNA level, a concatenated (cyt *b* and 16S rRNA) data set including 365 individuals (86 unique haplotypes) was used (sequences both generated here and downloaded), with 362 of them constituting the ingroup. A total of 1004 base pairs (cyt *b* 465 bp and 16S rRNA 539 bp) were aligned, with 294 (29.3%) alignment sites being variable and 266 (26.5%) parsimony informative (349 and 279, respectively, when outgroups were included in the analysis). For the nuDNA data, a total of 1739 base pairs (MC1R 696 bp, Pod55 474 bp, and Pod15b 569 bp) were aligned, with 164 (9.4%) alignment sites being variable and 103 (5.9%) parsimony informative (172 and 103, respectively, when outgroup was included in the analysis). The length of the sequences produced in the present study varied from 576 to 696 bp for the MC1R gene, from 408 to 474 bp for the Pod55 gene, and from 415 to 541 bp for the Pod15b gene. Finally, for the concatenated dataset (all five molecular markers) a total of 2718 base pairs were aligned, with 438 (16.1%) alignment sites being variable and 353 (13.0%) parsimony informative (468 and 355, respectively, when outgroup was included in the analysis).

Sequence divergence (p-distance) ranged from 0 to 23.7% for the cyt *b* gene and from 0 to 19.9% for the 16S rRNA gene. The mtDNA genetic distances among and within the major clades revealed by the phylogenetic analyses are shown in Tables 2 and 3, whereas the pairwise mtDNA genetic distances are shown in Supplementary Table S2. Sequence divergence ranged from 0 to 2.7% for the MC1R gene, from 0 to 3.3% for the Pod55 gene, and from 0 to 5.9% for the Pod15b gene. Table 4 shows the nDNA genetic distances among and within the major clades revealed by the phylogenetic analyses, whereas the pairwise nDNA genetic distances are shown in Supplementary Table S2.

Table 2
Genetic p-distances of the cyt *b* (below diagonal–left) and 16S rRNA genes (above diagonal–right) among the major clades. Values in diagonal (italics) are within clades sequence divergence (cyt b/16S rRNA), whereas dashes indicate the absence of the taxon from the 16S rRNA gene sampling, and n.a. indicate inability to compute inter–lineage divergence due to unitary representative of the clade.

Clade	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. *P. t. tauricus & P. t. thasopulae*	*0.4/0.1*	5.3	5.6	5.2	5.9	6.7	7.3	5.6	5.8	4.8	5.7	–	6.1	7.3	6.8	7.3	–	10.9	15.4	13.4
2. *P. t. ionicus*	10.6	*5.0/1.4*	5.0	4.4	4.7	6.6	6.5	5.2	5.6	5.0	5.8	–	6.2	6.7	5.44	6.3	–	9.	14.8	12.2
3. *P. gaigeae*	11.9	11.4	*0.3/0.2*	4.7	5.5	6.2	6.4	5.9	6.0	5.0	5.5	–	5.6	5.8	6.0	6.6	–	11.2	15.4	11.3
4. *P. milensis*	11.0	10.6	9.6	*1.0/0.4*	4.3	6.9	6.1	5.5	5.5	5.0	6.2	–	5.5	6.0	6.3	6.8	–	9.7	14.6	12.3
5. *P. melisellensis*	12.2	12.3	10.8	10.4	*3.7/1.3*	6.1	6.1	5.1	5.9	4.6	5.6	–	5.2	5.1	5.1	5.1	–	8.3	12.7	11.7
6. *P. cretensis*	13.4	13.3	14.6	13.4	15.2	*4.6/2.3*	3.7	3.4	5.7	5.1	6.4	–	6.9	7.3	6.4	5.6	–	8.9	14.1	12.8
7. *P. levendis*	12.4	14.1	15.2	13.3	14.4	7.9	*0.2/0.0*	2.9	5.0	5.3	6.4	–	6.8	7.2	6.0	5.7	–	9.6	15.3	12.8
8. *P. peloponnesiacus*	14.1	14.7	15.9	14.3	16.3	7.3	7.5	*0.3/0.0*	4.0	3.8	5.1	–	5.5	6.6	6.0	5.5	–	9.3	14.6	12.4
9. *P. erhardii*	13.8	13.5	15.1	13.0	13.5	13.6	12.4	13.2	*2.7/1.3*	4.9	5.5	–	5.5	6.5	6.4	6.0	–	9.9	15.0	12.6
10. *P. pityusensis*	18.2	16.2	17.7	15.4	15.4	15.9	18.6	17.6	16.9	*0.3/0.0*	1.5	–	4.9	5.3	5.0	5.7	–	8.3	13.7	11.4
11. *P. lilfordi*	16.4	14.5	15.2	15.1	13.8	13.8	124.7	14.2	16.2	10.4	*1.1/0.2*	–	4.7	5.4	6.0	6.4	–	9.9	14.3	11.5
12. *P. tiliguerta*	14.0	17.2	17.6	16.1	15.4	14.2	15.4	14.3	16.1	14.4	15.8	*3.3/–*	–	–	–	–	–	–	–	–
13. *P. siculus*	12.0	13.2	14.2	13.8	12.0	14.5	15.3	16.5	12.1	13.7	15.3	14.3	*1.0/0.0*	2.2	5.3	6.5	–	10.5	11.	12.5
14. *P. waglerianus*	13.3	13.6	13.8	13.4	11.5	16.1	14.6	16.4	13.3	12.4	13.4	13.2	6.6	*n.a./n.a.*	5.4	6.1	–	10.6	12.5	12.2
15. *P. muralis*	14.3	14.0	16.9	14.3	13.7	13.3	14.0	15.3	13.3	16.3	16.0	13.2	11.8	12.1	*3.9/1.2*	5.5	–	9.4	14.3	11.5
16. *P. bocagei*	14.4	14.9	14.7	15.8	14.2	15.4	15.5	16.0	16.2	17.2	15.2	13.2	15.2	12.7	12.1	*0.7/0.0*	–	8.8	13.4	11.4
17. *P. filfolensis*	16.6	171.0	16.1	16.8	16.0	17.6	14.8	17.3	16.3	17.1	15.0	16.3	15.4	14.3	16.0	15.4	*2.0/–*	–	–	–
18. *T. dugesii*	18.2	18.7	20.2	19.4	20.2	15.9	14.4	16.4	17.6	19.4	18.1	18.3	17.5	17.5	17.9	17.0	16.8	*n.a./n.a.*	12.6	10.8
19. *A. andreanskyi*	18.3	18.6	19.2	18.1	20.6	19.1	18.8	20.2	17.5	22.8	23.7	22.1	20.4	20.2	19.7	17.6	21.1	16.8	*n.a./n.a.*	16.0
20. *L. agilis*	17.8	19.0	20.0	17.2	17.8	15.9	15.0	18.5	16.6	17.6	15.7	20.9	17.3	17.2	18.1	19.3	19.8	16.6	20.8	*n.a./n.a.*

Table 3
Genetic distances of the mtDNA (cyt *b*/16S rRNA; below diagonal–left) and the nDNA genes (Pod55/Pod15b/MC1R; above diagonal–right) among the major subclades of *Podarcis tauricus ionicus*. Values in diagonal (italics) are within subclade sequence divergence for the five loci (cyt *b*/16S rRNA/Pod55/Pod15b/MC1R).

Podarcis tauricus ionicus subclade	(a)	(b)	(c)	(d)	(e)
(a) Zakynthos and Kefallonia Islands	*0.6/0.5/0.0/0.2/0.0*	0.1/0.3/0.3	0.2/0.5/0.6	0.0/0.2/0.3	0.2/0.3/0.3
(b) Western central Greece (Sterea Ellada)	5.3/3.2	*0.7/0.2/0.1/0.0/0.1*	0.2/0.5/0.4	0.0/0.2/0.2	0.1/0.1/0.1
(c) Northeastern Peloponnisos	8.7/2.6	9.2/3.6	*0.1/0.0/0.2/0.8/0.0*	0.2/0.2/0.4	0.2/0.4/0.5
(d) Central & Southeastern Peloponnisos	7.4/2.5	7.6/3.1	7.7/1.2	*0.2/0.0/0.0/0.0/0.1*	0.1/0.1/0.2
(e) Northern & Southwestern Peloponnisos, Northwestern Greece, Kerkyra Island, South-southwestern Albania	7.7/2.5	6.9/2.7	7.4/1.5	6.2/1.6	*0.5/0.2/0.2/0.1/0.0*

All mtDNA phylogenetic analyses (NJ, MP, ML, and BI) produced trees with very similar topologies (Suppl. Figs. S2 and S3). Unweighted parsimony analysis produced more than 10,000 equally parsimonious trees with a length of 1370 steps (consistency index CI = 0.366, retention index RI = 0.934). The large number of equally parsimonious solutions was largely due to terminal branch swapping. Maximum likelihood analysis resulted in a topology with $\ln L = -7367.62$. Bayesian inference resulted in a topology with mean $\ln L = -8348.59$. Identical topologies were recovered for each of the four runs with the full dataset, and the 50% majority-rule consensus tree of the 75×10^3 trees remaining after burn-in is presented in Supplementary Fig. S2 (full topology), as well as, in Supplementary Fig. S3 (collapsed to main clades topology). According to the resulted tree, the specimens of the genus *Podarcis* used in the present study are clustered into several main clades, with the majority of the morphologically recognized species being monophyletic. The phylogenetic relationships among them are mostly unresolved with the exception of four groupings; the *Podarcis tauricus* species subgroup, the *P. erhardii* species subgroup, the *P. siculus* – *P. waglerianus* pair, and the *P. pityusensis* – *P. lilfordi* pair. In the focal *P. tauricus* species subgroup, which appears monophyletic with moderate to high statistical support, five well-supported clades with unresolved phylogenetic relationships among them are recognized corresponding to the morphologically recognized taxa *P. melisellensis*, *P. milensis*, *P. gaigeae*, *P. t. ionicus* and *P. t. tauricus* - *P. t. thasopulae*. According to the mtDNA topology *P. tauricus* is polyphyletic, although with low statistical support, due to the more closely relationship of *P. t. ionicus* to *P. gaigeae*. On the other hand, *P. t. ionicus* is a monophyletic taxon, with five well-supported subclades (*a* – *e*; see Suppl. Fig. S3 for their corresponding distribution). However, the among-subclades relationships are partly resolved (only *a* and *b* have sister group relationship with high statistical support, whereas the relationship between *c* and *d* is supported by moderate to high statistical value. The other two subspecies of *P. tauricus* (*P. t. tauricus* and *P. t. thasopulae*) are grouped together in the same clade, containing specimens from a wide range of *P. tauricus* distribution (Fig. 1A–C) with low genetic diversity (Suppl. Fig. S2). The clade of *P. gaigeae* includes specimens from the island of Skyros and the islets Sarakino, Plateia, Koulouri, Exo Diavatis, Lakonisi, Skyropoula, Valaxa, Palamari, Rineia, and Piperi, with the subspecies inhabiting the latter (*P. g. weigandi*) not being phylogenetically distinct from *P. g. gaigeae* (Suppl. Fig. S2). The clade of *P. melisellensis* is divided into three, geographically distinct, subclades, with unresolved phylogenetic relationships among them (Suppl. Figs. S2 and S3). Two of them are originating from Dalmatian islands and islets (first subclade from Glavat and Lastovo, and the second from Jabuka, Brusnik, Biševo, and Vis), whereas the third one contains specimens from Montenegro, Bosnia & Herzegovina, Croatian coastal area (Koromačno-Istria, Cavtat-Dalmatia) and some Dalmatian islands (Korčula Island, Purara islet, and Hvar Island). Finally, the clade of *P. milensis* consists of specimens from the island group of Milos (Milos Island, Kimolos Island, and Antimilos and Agios Efstathios islets).

All phylogenetic (NJ, MP, ML, and BI) analyses of the nuDNA dataset produced phylogenies (Suppl. Fig. 4) that are in agreement with the mitochondrial ones (Suppl. Figs. S2 and S3) with $\ln L = -3943.03$ for ML and $\ln L = -4084.80$ for BI, but with incongruences in statistical support.

Respectively, all phylogenetic (NJ, MP, ML, and BI) analyses of the concatenated dataset (mtDNA and nuDNA) produced a more resolved phylogenetic tree (Fig. 2; $\ln L = -10{,}299.76$ for ML and $\ln L = -10{,}192.86$ for BI). Based on this tree and the results obtained from the mtDNA data (a) there is high to moderate statistical support that *P. melisellensis* is the root taxon of the *P. tauricus* species subgroup, (b) in the *P. erhardii* species subgroup, *P. erhardii* is the root taxon and *P. peloponnesiacus*, *P. levendis*, and *P. cretensis* are close related with the latter two being sister taxa, (c) *P. tiliguerta* is more closely related to the *P. lilifordi* – *P. pityusensis* pair and (d) *P. muralis* and *P. bocagei* have sister-taxon relationship.

3.2. Species tree

In the multilocus coalescent species tree analysis the ESS values were good (>224) with $\ln L = -9788.00$. The species tree (Figs. 3 and 4) is partly in agreement with the concatenated tree (Fig. 2) supporting (a) the monophyly of *P. tauricus* species subgroup, (b) the monophyly of *P. t. ionicus*, (c) *P. erhardii* being the root taxon of the homonym species subgroup, (d) *P. tiliguerta* being closely related to the sister taxa *P. lilfordi* and *P. pityusensis*, (e), and (f) the sister taxon relationship between *P. muralis* and *P. bocagei*. On the other hand, the majority of the nodes that are close the root have low statistical support. The same applies for the phylogenetic relationships among the major clades of the *P. tauricus* species subgroup, as well as among the *P. t. ionicus* subclades. Finally, the sister taxon relationship between *P. cretensis* and *P. levendis* is not supported.

3.3. Species delimitation

According to the estimated phylogenies, nine major clades and subclades are present within the *P. tauricus* species subgroup. In the BP&P analyses all nine of them were estimated as distinct species with the posterior probabilities ranging from 0.91 to 1.00, accounting all three prior combination schemes that supported the above solution of nine species with moderate, absolute, and good statistical support, respectively (Table 5). The rest of the solutions of either six, seven or eight species were estimated to have low statistical support. The analysis through STACEY produced high ESSs values (>290) with $\ln L = -5832.81$, resulted also in nine different species with almost absolute statistical support (p.p. = ~1.00). It is worth noticing that these nine clades/subclades were also estimated as different species within the *P. tauricus* species subgroup based on the single locus (here mtDNA) species delimitation analysis of PTP. In addition, PTP proposed to consider as different species the three *P. melisellensis* subclades, as well as the lineage from Kalamata within the subclade *e* of *P. t. ionicus*. In sum-

Table 4
Genetic distances of the Pod55 and Pod15b (within brackets) genes (below diagonal–left) and the MC1R gene (above diagonal–right) among the major clades. Values in diagonal (italics) are within lineages sequence divergence for the three loci (Pod55/Pod15b/MC1R), whereas dashes indicate the absence of the taxon from the gene sampling, and n.a. indicate inability to compute inter–lineage divergence due to unitary representative of the clade.

Clade	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. *P. tauricus tauricus* & *Podarcis tauricus thasopulae*	*0.0/0.3/0.3*	0.3	0.5	0.3	0.5	0.9	0.8	0.8	0.2	0.8	0.5	0.8	0.8	0.7	0.8	0.7	0.9	2.0
2. *P. tauricus ionicus*	0.3 (0.6)	*0.1/0.2/0.3*	0.6	0.2	0.4	0.9	0.8	0.6	0.2	0.8	0.5	0.8	0.7	0.7	0.6	0.6	0.8	2.0
3. *P. gaigeae*	0.9 (0.9)	0.7 (0.9)	*0.0/0.0/0.0*	0.5	0.7	1.1	1.0	1.0	0.4	1.0	0.7	1.0	1.0	1.0	1.0	1.0	1.1	2.0
4. *P. milensis*	0.4 (0.7)	0.3 (0.7)	0.9 (1.0)	*0.0/0.0/0.1*	0.3	0.7	0.7	0.7	0.1	0.7	0.4	0.6	0.5	0.5	0.6	0.6	0.6	1.9
5. *P. melisellensis*	0.9 (1.3)	0.8 (1.1)	1.4 (1.1)	0.8 (1.4)	*0.2/1.1/0.2*	0.9	0.8	0.8	0.2	0.7	0.5	0.7	0.8	0.8	0.8	0.8	0.9	1.8
6. *P. cretensis*	1.0 (3.1)	1.3 (3.0)	1.9 (2.9)	1.2 (3.1)	1.1 (3.3)	*0.0/0.0/0.2*	0.4	1.0	0.7	1.3	0.9	1.0	1.3	1.3	1.3	1.2	1.4	2.6
7. *P. levendis*	1.1 (2.8)	1.4 (2.7)	2.0 (2.7)	1.3 (2.9)	1.2 (3.1)	0.1 (0.8)	*0.0/0.0/0.0*	0.9	0.6	1.2	0.7	1.0	1.2	1.2	1.2	1.2	1.1	2.4
8. *P. peloponnesiacus*	1.7 (3.1)	1.9 (2.8)	2.6 (3.0)	1.9 (3.1)	1.8 (3.2)	0.7 (1.0)	0.8 (0.9)	*0.0/0.4/0.0*	0.6	1.2	0.9	1.1	1.2	1.2	0.9	0.8	1.3	2.4
9. *P. erhardii*	1.2 (4.1)	1.5 (3.9)	2.0 (3.8)	1.3 (4.0)	1.3 (4.2)	0.2 (2.0)	0.4 (1.9)	0.9 (2.1)	*0.4/2.2/0.0*	0.6	0.3	0.6	0.6	0.6	0.6	0.5	0.6	1.8
10. *P. pityusensis*	1.7 (3.5)	1.8 (3.3)	2.5 (2.8)	1.8 (3.5)	1.7 (3.4)	0.7 (2.9)	0.8 (2.7)	1.4 (3.0)	0.9 (3.7)	*0.0/0.6/0.0*	0.3	0.5	0.9	1.2	0.9	0.9	1.0	2.2
11. *P. lilfordi*	1.6 (3.3)	1.8 (3.2)	2.5 (2.7)	1.8 (3.3)	1.7 (3.2)	0.6 (2.5)	0.7 (2.4)	1.3 (2.6)	0.8 (3.5)	0.0 (0.4)	*0.0/0.0/0.0*	0.2	0.6	0.9	0.6	0.5	0.6	2.0
12. *P. tiliguerta*	1.8 (3.8)	2.2 (3.7)	2.5 (3.2)	1.8 (3.5)	1.6 (3.7)	0.8 (2.9)	0.9 (2.6)	1.5 (2.8)	1.0 (3.9)	1.0 (1.4)	0.9 (1.0)	*0.7/1.1/0.3*	0.9	1.2	0.8	0.8	0.9	2.34
13. *P. siculus*	2.1 (3.3)	2.3 (2.7)	3.1 (3.0)	2.4 (3.2)	1.8 (2.8)	1.2 (2.7)	1.3 (2.5)	1.9 (2.6)	1.6 (3.5)	1.9 (2.4)	1.8 (2.2)	2.5 (2.4)	*n.a./n.a./n.a.*	0.4	0.8	0.9	0.5	2.
14. *P. waglerianus*	1.0 (3.1)	1.3 (2.5)	1.9 (2.9)	1.2 (3.1)	1.6 (2.6)	0.5 (2.3)	0.6 (1.9)	1.2 (2.2)	0.7 (3.3)	1.2 (2.0)	1.1 (1.8)	1.3 (2.2)	1.2 (0.4)	*n.a./n.a./n.a.*	1.0	0.9	0.7	2.4
15. *P. muralis*	2.0 (3.0)	1.8 (2.9)	2.5 (2.8)	1.8 (3.2)	2.1 (3.0)	1.5 (2.3)	1.7 (2.2)	2.3 (2.5)	1.9 (3.4)	2.2 (2.3)	2.2 (1.9)	2.7 (1.9)	2.2 (2.0)	1.6 (1.6)	*0.1/0.3/0.2*	0.5	0.7	2.2
16. *P. bocagei*	2.1 (3.4)	245 (3.1)	3.0 (2.9)	2.3 (3.4)	2.6 (3.0)	1.5 (2.7)	1.6 (2.1)	2.1 (2.5)	1.9 (3.7)	2.3 (2.2)	2.1 (1.8)	2.5 (2.0)	2.3 (2.0)	1.5 (2.0)	1.9 (1.1)	*0.0/0.4/0.0*	0.9	2.3
17. *P. filfolensis*	0.7 (2.8)	0.6 (2.7)	1.2 (2.6)	0.5 (2.7)	0.4 (3.0)	0.7 (2.0)	0.8 (1.6)	1.4 (1.8)	1.0 (3.1)	1.3 (2.1)	1.3 (1.7)	1.3 (2.2)	1.9 (2.1)	1.2 (1.7)	1.8 (1.6)	2.3 (1.8)	*0.0/0.0/0.6*	2.4
18. *Lacerta agilis*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*–/–/n.a.*

mary PTP estimated 12 distinct species within the *P. tauricus* species subgroup.

4. Discussion

This study provides a recapitulating phylogenetic reconstruction and assessment of the inter- and intra-specific relationships and diversity of the *P. tauricus* species subgroup. The data presented here feature complete taxon sampling, with representatives of all presently recognized species and most subspecies in the species subgroup. Specimens throughout the species' distribution ranges were incorporated and several phylogenetic and coalescent based methods were applied to generate gene trees, species trees and determine species delimitation. Among other things, our findings revealed several taxonomic discrepancies.

4.1. Phylogenetic relationships within the Balkan Wall lizards

Phylogenetic relationships between species groups in the genus remain largely unresolved. Failure to reconstruct them among the major clades of *Podarcis* could be due to rapid diversification early on in the evolutionary history of the genus, producing short but ancient branches with a low phylogenetic signal (Oliverio et al., 2000). This has already been cited as giving rise to the difficulty in resolving phylogenetic relationships in the Lacertidae family (Pavlicev and Mayer, 2009).

Two monophyletic species subgroups were revealed for the Balkan species group (*P. tauricus* and *P. erhardii*), in line with previous studies. However, although the *P. tauricus* subgroup appears to be more closely related to *P. erhardii*, this is not statistically supported by either a concatenated (Fig. 2) or a coales-

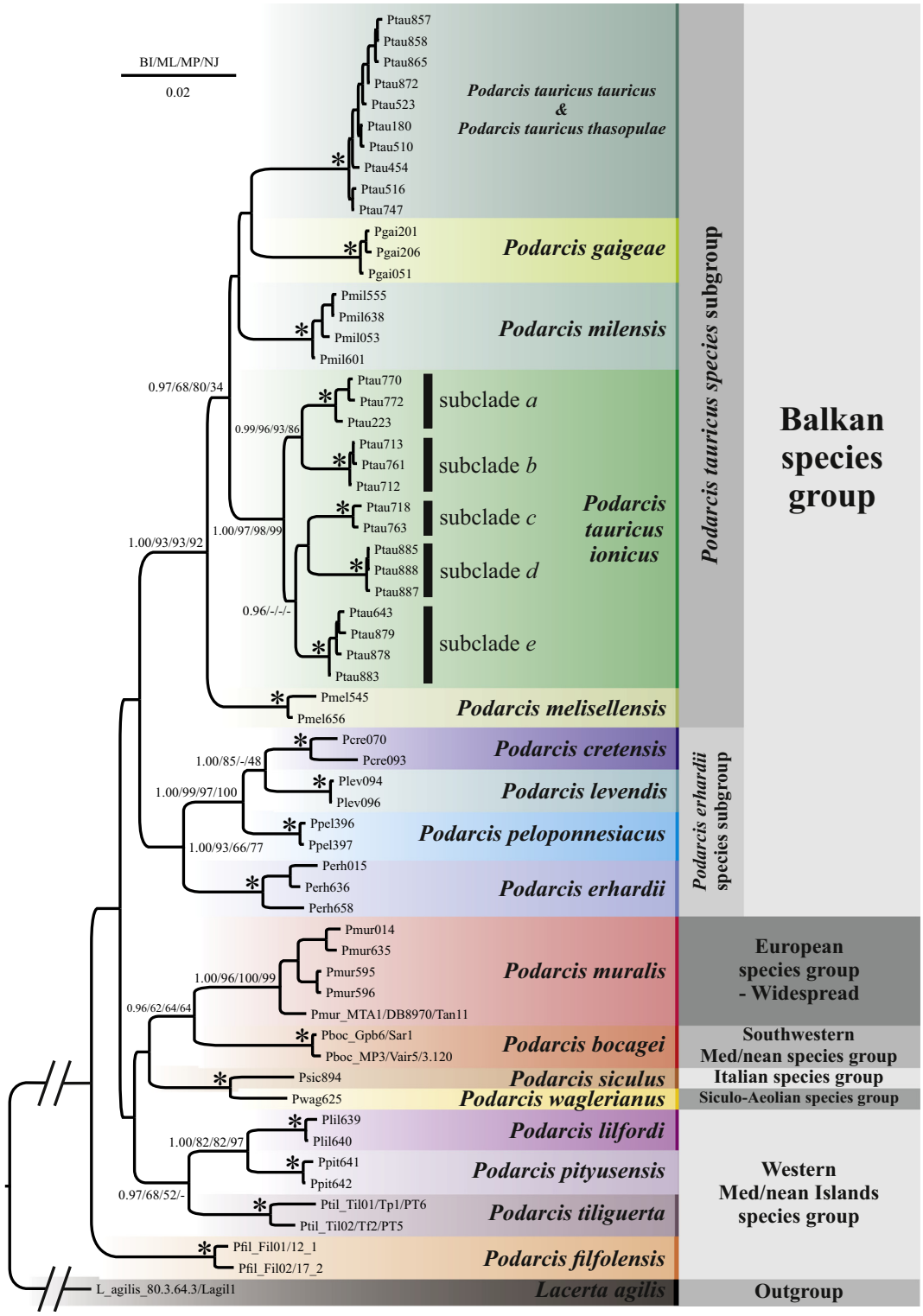

Fig. 2. Bayesian Inference tree based on the concatenated (mtDNA & nDNA) dataset focusing on the *P. tauricus* species subgroup. The posterior probabilities (>0.95) and bootstrap support (>50%) of all the phylogenetic methods used are given near the branches. No values, mean low statistical support and dashes mean different topology or polytomy. Asterisks indicate absolute support by all methods (BI/ML/MP/NJ).

cent approach (Fig. 3). This indicates that more markers should be implemented to evaluate their phylogenetic affinity. Within the *P. erhardii* species subgroup, phylogenetic relationships are fully resolved with high statistical support in the concatenated tree (Fig. 2), indicating *P. erhardii* as the root taxon of the species subgroup. Moreover, *P. cretensis* and *P. levendis* are sister taxa and both closely related to *P. peloponnesiacus*, resolving the phylogenetic relationships of these species, though the latter relationship is not well-supported in the species tree (Figs. 3 and 4).

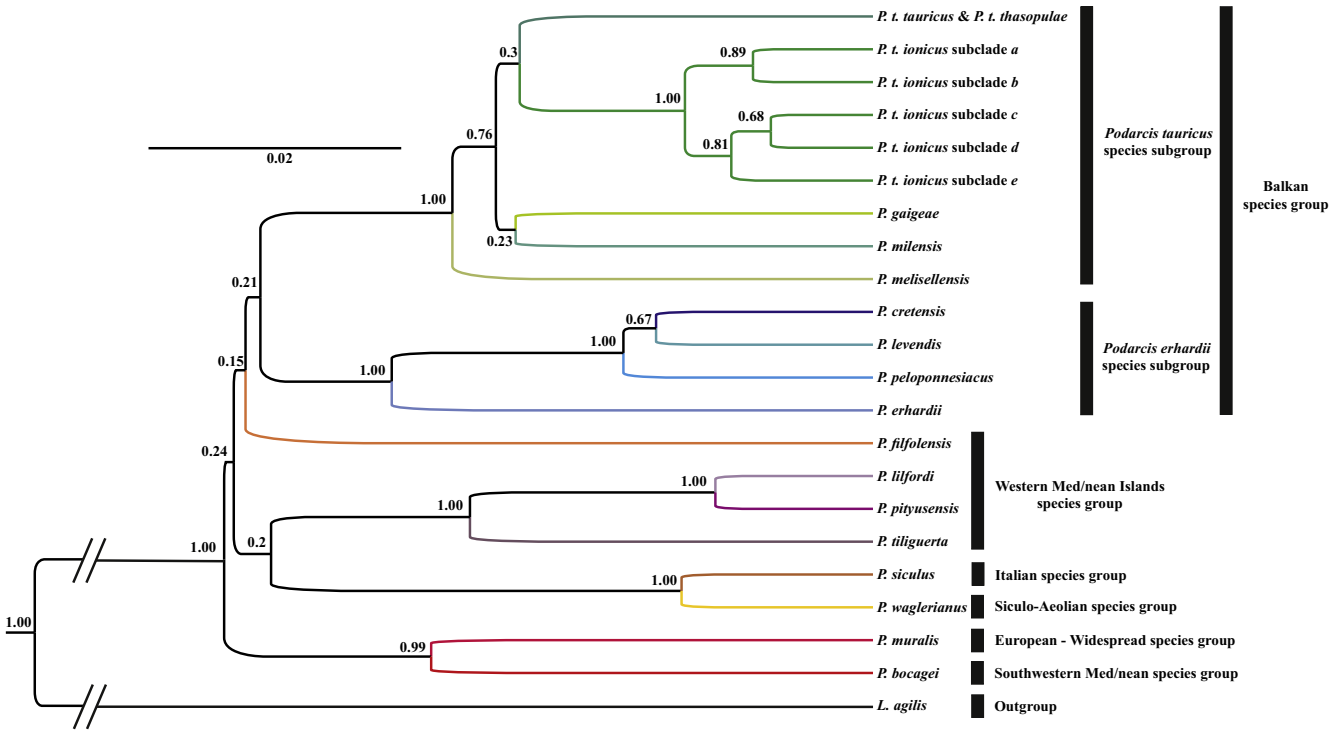

Fig. 3. The concensus multilocus coalescent species tree of the *P. tauricus* species subgroup and its conspecifics. The posterior probabilities are given near the branches.

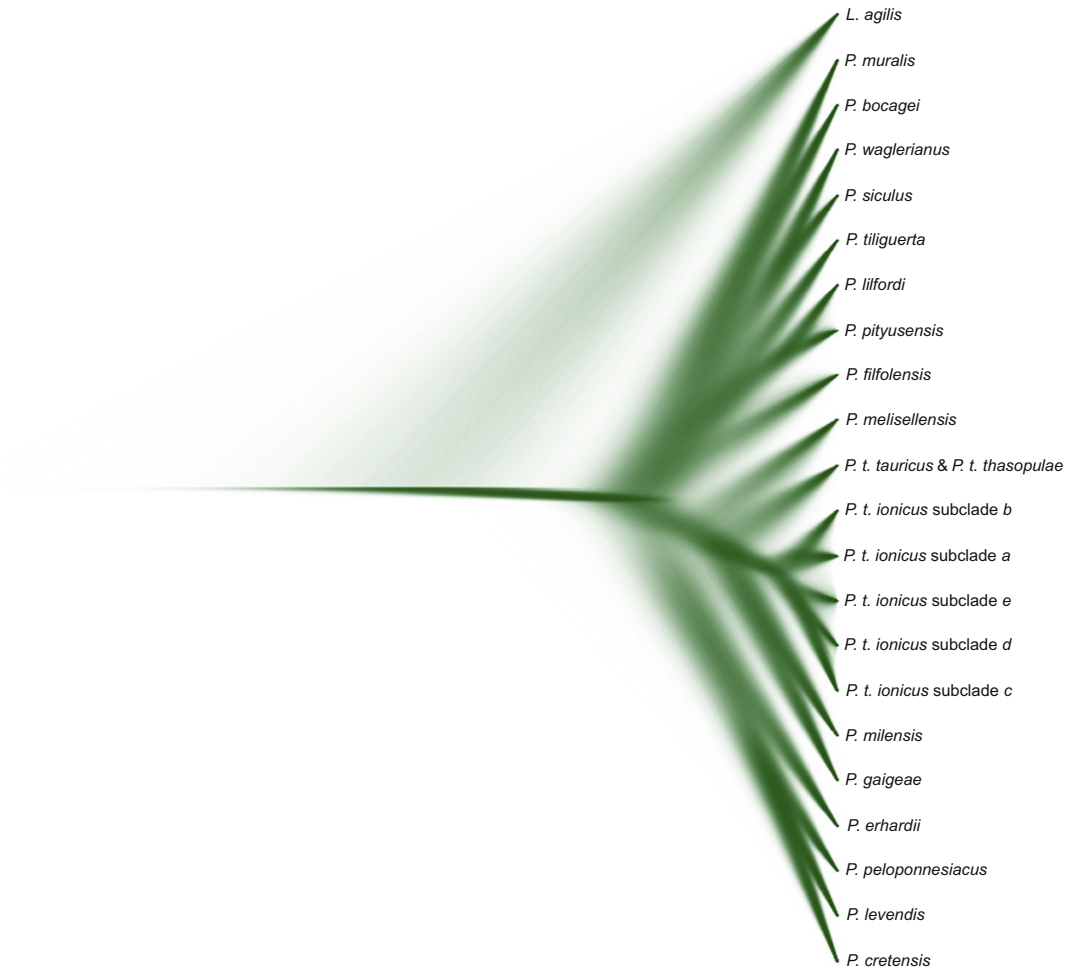

Fig. 4. Set of all trees (shown as a star tree) generated by the multilocus coalescent species tree analysis of the *P. tauricus* species subgroup and its conspecifics.

Table 5
Species delimitation results for the *P. tauricus* species subgroup based on BP&P software assuming nine species and using different prior schemes. The posterior probabilities are average values of two independent mcmc runs using different seed numbers.

Prior scheme	$\theta \sim G(1, 10) - \tau^0 \sim G(1, 10)$		$\theta \sim G(2, 2000) - \tau^0 \sim G(2, 2000)$		$\theta \sim G(1, 10) - \tau^0 \sim G(2, 2000)$	
Number of possible species	Posterior probability	Prior probability	Posterior probability	Prior probability	Posterior probability	Prior probability
6	–	–	–	–	<0.01	0.18
7	0.01	0.18	–	–	<0.01	0.18
8	0.11	0.13	<0.01	0.13	~0.10	0.13
9	0.88	0.06	~1.00	0.06	~0.90	0.06
Candidate species	Posterior probability					
P. milensis	1.00		1.00		1.00	
P. t. tauricus	1.00		1.00		1.00	
P. gaigeae	1.00		1.00		1.00	
P. melisellensis	1.00		1.00		1.00	
P. t. ionicus Subclade a	0.99		1.00		0.99	
P. t. ionicus Subclade b	0.93		1.00		0.96	
P. t. ionicus Subclade c	0.97		1.00		0.98	
P. t. ionicus Subclade d	0.95		1.00		0.94	
P. t. ionicus Subclade e	0.91		1.00		0.91	
P. t. ionicus Subclades b & e	0.05		–		0.05	
P. t. ionicus Subclades d & e	0.03		–		0.02	
P. t. ionicus Subclades a & d	–		–		0.01	

4.2. Phylogenetics relationships within the Podarcis tauricus species subgroup

Though largely unresolved, phylogenetic relationships among the current morphological taxa (Figs. 2 and 3) within the *P. tauricus* species subgroup were unexpected. No support was found for the previously published topologyb (Poulakakis et al., 2005a, b), which indicated a sister-taxon relationship between *P. milensis* and *P. gaigeae*, with *P. tauricus* as the root taxon of the species subgroup. Instead, the *P. tauricus* species subgroup is subdivided into five major clades, with *P. melisellensis* as the root taxon of the subgroup. Three of the clades correspond to three of the four morphological species in the species subgroup (*P. melisellensis*, *P. milensis*, and *P. gaigeae*). The other two clades include specimens of *P. tauricus*, without evidence that they are clustered together in a monophyletic group. The first of those clades corresponds to the subspecies *P. t. ionicus*, whereas the second consists of the other two subspecies (*P. t. tauricus* and *P. t. thasopulae*), which appear to be phylogenetically indistinguishable (Suppl. Fig. 2). Therefore, the taxonomy of *P. tauricus* at the species and subspecies level is not valid (see below). Genetic distance between the above two clades is comparable to the species level distances in *Podarcis* (Tables 2 and 4), as well as in other Lacertidae [e.g. *Lacerta* (Sagonas et al., 2014) and *Phoenicolacerta* (Tamar et al., 2015)].

It is worth noting here that *P. tauricus* has been considered a species complex with high genetic diversification (Poulakakis et al., 2005a), especially due to the high genetic divergence found within *P. t. ionicus* (Figs. 2–4, Tables 3 and 5). Five geographically distinct subclades were recovered within *P. t. ionicus*. Based on the mtDNA tree, their phylogenetic relationships were partly resolved. The subclades from the southern Ionian Islands (subclade *a*) and western central Greece (subclade *b*) have a sister-subclade relationship, whereas the other three subclades are clustered together, with the subclade from Albania, northwestern Greece and the western Peloponnisos (subclade *e*) being the most basal, and the subclades from the northeastern (subclade *c*) and south-central Peloponnisos (subclade *d*) forming a sister group. The genetic distances between them (Table 3) are relatively high (e.g. for the cyt *b* fragment they range from 5.4% to 9.2%), reaching and in some cases exceeding those among the morphologically recognized species of *P. levendis*, *P. cretensis*, and *P. peloponnesiacus*.

4.3. How many species? A proposed taxonomy of the Podarcis tauricus species subgroup in the light of multilocus phylogeny and species delimitation

In concordance with gene tree estimations, Bayesian species delimitation approaches clearly support a scheme of nine species within the *P. tauricus* species subgroup. PTP analysis estimated 12 distinct species within the *P. tauricus* species subgroup. Nevertheless, these results should be treated with caution, as PTP may overestimate the number of species in cases of uneven sampling between putative species (Zhang et al., 2013), as e.g. with *P. melisellensis*.

Based on the aforementioned results and the fact that the two clades of *P. tauricus* are morphologically, geographically, and phylogenetically distinct, the taxonomic status of *P. tauricus* should be revised by splitting the species into two separate taxa. The first taxon, *Podarcis tauricus* (Pallas, 1814) with type locality in northern Crimea, includes the populations currently assigned to the subspecies *P. t. tauricus* and *P. t. thasopulae*, together being synonymized under monotypic *Podarcis tauricus* without further subspecific division. The second taxon corresponds either to a distinct evolutionary species (de Queiroz, 2005), *Podarcis ionicus* (Lehrs, 1902), with type locality in the island of Kerkyra (Corfu Isl.), displaying high levels of intraspecific genetic diversity (five Deep Conspecific Lineages; DCL, Table 3), or to a species complex comprising of up to five Unconfirmed Candidate Species (UCS) (Padial et al., 2010). For the time being and until extensive genetic, morphological and ecophysiological examination is carried out, we propose referring to this second taxon as the *P. ionicus* species complex. According to Padial et al. (2010), the provisional names for *P. ionicus* phylogenetic subclades *a* to *e* should be: (a) *Podarcis ionicus* [Ca1 Poulakakis et al., 2005a], (b) *Podarcis ionicus* [Ca2 Psonis et al., 2016], (c) *Podarcis ionicus* [Ca3 Psonis et al., 2016], (d) *Podarcis ionicus* [Ca4 Poulakakis et al., 2005a], and (e) *Podarcis ionicus* [Ca5 Lehrs, 1902]. The Greek island endemics *P. milensis* and *P. gaigeae* did not display any phylogenetic structure and geographic pattern. Intra-specific phylogeny of the specimens inhabiting different islands and islets was in both cases bush-like, indicating either a recent colonization/fragmentation history or high gene flow among them. Given that *P. g. weigandi* and *P. g. gaigeae* could not be distinguished from each other, the subspecific taxonomy of *P. gaigeae* should be revised by synonymizing the current subspecies under monotypic *P. gaigeae* with no further subspecific

division, a proposal that has already been highlighted by Poulakakis et al. (2005b). For *P. milensis*, more samples from the rest of its subspecies (*P. m. adolfjordansi* and *P. m. gerakuniae*) should be included in future analyses if safe conclusions are to be reached. Finally, *P. melisellensis* is represented by three lineages that have already been recognized at the subspecies level: *P. m. melisellensis*, *P. m. fiumana*, and a third undescribed lineage corresponding to the *Lastovo* subclade.

5. Conclusions

According to the present study, the *P. tauricus* species subgroup is a monophyletic unit that includes five major clades, with unresolved intra-clades phylogenetic relationships and *P. melisellensis* as the root taxon. Monophyly of *P. tauricus* sensu stricto is not supported, with *P. t. ionicus* displaying high levels of hidden genetic diversity, comprising five subclades with partially resolved phylogenetic relationships. Genetic diversification within *P. t. tauricus*, *P. gaigeae*, and *P. milensis* was found to be low, whereas the taxonomic validity of the subspecies *P. t. tauricus* - *P. t. thasopulae* and *P. g. gaigeae* – *P. g. weigandi* is unfounded; they should be regarded as synonyms. Finally, species delimitation approaches clearly support the existence of nine distinct species within the *P. tauricus* species subgroup. The main focus of further research should be on the *P. ionicus* species complex as defined above, comprising five Unconfirmed Candidate Species. This will necessitate detailed population-genetic analysis of nuclear markers in contact zones, as well as of the morphology and ecology typifying these UCS. Finally, though not the focus of the present study, it is clear that despite having used three nuDNA and two mtDNA loci, phylogenetic relationships among the species of the entire genus remain largely unresolved. To gain a fuller understanding, future investigations should consider selecting numerous and more appropriate molecular markers.

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Appendix A. Supplementary material

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