The wall lizards of the Balkan peninsula: tackling questions at the interphase of phylogenomics and population genomics

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Abstract

Wall lizards of the genus *Podarcis* (Sauria, Lacertidae) are the predominant reptile group in southern Europe, including 24 recognized species. Mitochondrial DNA data have shown that, with the exception of *P. muralis*, the *Podarcis* species distributed in the Balkan peninsula form a species group that is further sub-divided into two subgroups: the one of "*P. tauricus"* consisting of *P. tauricus*, *P. milensis*, *P. gaigeae*, and *P. melisellensis*, and the other of "*P. erhardii"* comprising *P. erhardii*, *P. levendis*, *P. cretensis*, and *P. peloponnesiacus*. In an attempt to explore the Balkan *Podarcis* phylogenomic relationships, assess the levels of genetic structure and to re-evaluate the number of extant species, we employed phylogenomic and admixture approaches on ddRADseq (double digested Restriction site Associated DNA sequencing) genomic data. With this efficient Next Generation Sequencing approach, we were able to obtain a large number of genomic loci randomly distributed throughout the genome and use them to resolve the previously obscure phylogenetic relationships among the different *Podarcis* species distributed in the Balkans. The obtained phylogenomic relationships support the monophyly of both aforementioned subgroups and revealed several divergent lineages within each subgroup, stressing the need for taxonomic re-evaluation of *Podarcis*' species in Balkans. The phylogenomic trees and the species delimitation analyses confirmed all recently recognized species (*P. levendis*, *P. cretensis*, and *P. ionicus*) and showed the presence of at least two more species, one in *P. erhardii* and the other in *P. peloponnesiacus*.

Key words: Admixture, Biodiversity, Cryptic species, ddRADseq, Genome-wide SNPs, *Podarcis*, Species Delimitation, Taxonomy

1. Introduction

The wall lizards of the genus *Podarcis* Wagler, 1830 (Lacertidae) are currently represented by 24 species (Senczuk et al., 2019; Uetz et al., 2019), forming the most taxonomically diversified reptile group in southern Europe. Their distribution ranges from northwestern Africa through the Iberian and the Italian peninsulas to the Balkans, the northwestern Asia Minor and the Crimean Peninsula (Arnold, 1973). The taxonomy of *Podarcis* is continuously being subject of revision, especially at the species level, due to the extensive intraspecific variability (Arnold et al., 1978). The first taxonomic studies based on DNA sequence data (Harris and Arnold, 1999; Oliverio et al., 2000) divided the genus into several species groups with the relationships among them being considered mainly unresolved.

The present study is focusing on the "Balkan" species group. Despite being restricted to the Balkan peninsula and therefore being, geographically speaking, a well-defined group, its monophyly is not unambiguously supported when all the *Podarcis* species groups are included into a phylogeny (Psonis et al., 2017). This "Balkan" species group is phylogenetically subdivided into two distinct species subgroups (Poulakakis et al., 2005a; Poulakakis et al., 2005b; Psonis et al., 2017): (a) the "*P. erhardii*" subgroup that includes *P. cretensis* , *P. erhardii*, *P. levendis*, and *P. peloponnesiacus*; and (b) the "*P. tauricus*" species subgroup, consisting of *P. gaigeae*, *P. melisellensis*, *P. milensis*, and *P. tauricus*. Recently, *P. tauricus* was subdivided into two geographically distinct species *P. tauricus* and *P. ionicus*, but without the necessary update in the description of the new species *P. ionicus* (Psonis et al., 2017). The distributions of the two species subgroups are overlapping in the continental area of the southern Balkans, mostly in the Peloponnese and in the eastern part of continental Greece where the species subgroups are found

in sympatry. In contrast, the insular species on the Aegean, Ionian and Adriatic islands are allopatrically distributed both at the subgroup, as well as at the species level.

Most of the previous attempts to investigate the interspecific relationships of the Balkan wall lizards were based on mitochondrial DNA (mtDNA) of all species of this group (Poulakakis et al., 2003; Poulakakis et al., 2005b) or on a combination of mtDNA and nuclear DNA (nDNA, i.e., nuclear gene fragments and microsatellites), in a subset of this group (Psonis et al., 2017; Spilani et al., 2019). The aforementioned studies revealed several cases of hidden diversity (e.g. *P. cretensis, P. ionicus*). However, the phylogenetic relationships among the majority of the currently recognized species remained mostly unresolved, leaving uncertainties regarding their evolutionary history. Moreover, the exact number of species inhabiting the Balkans is not known due to cryptic diversity (e.g. within *P. peloponnesiacus*, *P. erhardii*, *P. cretensis* and *P. ionicus*).

The modern high-throughput sequencing technologies provide the potential to address previously intractable questions in evolution and ecology even in non-model organisms, and to study complex biological patterns in phylogenetics (e.g. DaCosta and Sorenson, 2016; Leaché et al., 2015; Nieto-Montes de Oca et al., 2017). Double-digest Restriction site associated DNA sequencing (ddRADseq) has become one of the most useful reduced-representation genome sequencing methods for phylogenetic studies (Peterson et al., 2012). This method is useful because it produces abundant, anonymous data from throughout the genome that can be used for phylogenetic, population genetic and phylogeographic inferences, and thus resolve difficult phylogenetic relationships that arise in groups where closely related species have diversified rapidly (e.g.; Psonis et al., 2018; Santonastaso et al., 2017; Senczuk et al., 2019; Uetz et al., 2019). The Balkan wall lizards constitute an ideal case for the application of ddRADseq due to the existence of polytomies attributed to rapid diversification (Psonis et al., 2017), a common

phenomenon observed in Lacertidae (Pavlicev and Mayer, 2009) and especially in *Podarcis* (Oliverio et al., 2000).

This approach (ddRADseq) was successfully applied to the *P. tauricus* species subgroup, (Psonis et al., 2018), that enabled the resolution of their phylogenetic relationships, with *P. ionicus* being the sister taxon to *P. tauricus*, *P. gaigeae* being the most closely related species to both of them, followed by *P. milensis* and finally by the phylogenetically most distant *P. melisellensis.* A few representatives of the other subgroup (*P. erhardii*) were used as outgroup. Here, we expanded the ddRADseq dataset from 46 of Psonis et al. (2018) to 116 specimens, including representatives from all the major phylogenetic clades of the "Balkan" species group of *Podarcis*, aiming to i) resolve the phylogenomic relationships within the *P. erhardii* subgroup, ii) infer the phylogenomic relationships of all *Podarcis* representatives in the Balkans, iii) assess the levels of genetic structure and admixture within each subgroup, and iv) propose an updated view on the current taxonomy of *Podarcis* in this region.

2. Materials and methods

2.1. Taxon sampling and data collection

We collected data of 116 specimens representing all species of the genus *Podarcis* in the Balkans, including almost all major clades or subclades within species revealed in previous phylogenetic studies (Podnar et al., 2004; Poulakakis et al., 2005a; Poulakakis et al., 2005b; Psonis et al., 2018; Psonis et al., 2017; Spilani et al., 2019). More precisely, we used 22 *P. cretensis*, 36 *P. erhardii*, five *P. gaigeae*, 11 *P. ionicus*, five *P. levendis*, two *P. melisellensis*, six *P. milensis*, seven *P. muralis*, 10 *P. peloponnesiacus*, and 12 *P. tauricus.* The raw data for the *P. tauricus* species subgroup samples were retrieved from Psonis et al. (2018) (**Table S1**). Albeit

not a member of the Balkan species group, *Podarcis muralis* was also included in the dataset given that it is a widespread European species, present in the Balkan peninsula, and sometimes in sympatry with *P. erhardii*. Furthermore, specimens of *Lacerta trilineata* and *Hellenolacerta graeca* were used as outgroups. Detailed description of the dataset and the sampling localities are given in **Table S1** and **Figure 1**.

Total genomic DNA (gDNA) of 70 specimens (**Table S1**) was extracted either from muscle tissue or blood using the DNeasy Blood & Tissue Extraction kit (Qiagen®, Hilden, Germany). The quality and quantity of the extracted DNA was evaluated using both agarose gel electrophoresis (TAE 1.5%) and the Qubit® 2.0 Fluorometer (Invitrogen®, Carlsbad, California, USA).

The ddRADseq library was prepared based on the protocol described by Peterson et al. (2012) and the sequencing was performed on an Illumina HiSeq 2000 lane (Illumina Inc., San Diego, California, USA) (100-bp, single end reads). Raw Illumina reads were processed using pyRAD v. 3.0 (Eaton, 2014) pipeline, applying three different clustering threshold values (Wclust equal to 0.85, 0.90, and 0.95) as this parameter has been shown to affect phylogenetic relationships (Leaché et al., 2015). For more details, see the supplementary information (**Supplementary Notes S1 and S2**).

2.2. Additional data filtering

Acknowledging missing data as one of the main caveats of ddRADseq, we attempted to assess their impact and take it under consideration when we interpret our results. Aiming at increasing the potential to gather more phylogenetic information between more divergent taxa (in terms of quartet informativeness) about splits deeper in a tree (e.g. seeEaton et al., 2017), instead

of discarding all loci with missing data above a particular threshold (e.g. as implemented in pyRAD) we retained loci that are phylogenetically informative for parts of the tree in an attempt to keep the information contained in the data without however jeopardizing the integrity of our results. To accomplish that, two additional filters were applied to the outputs generated by pyRAD (as in Psonis et al., 2018) in order to i) assess the impact of missing data on all the analyses that followed and ii) extract the prominent signal of our data regarding the phylogenomic relationships and the status of the studied taxa.

In a more detail, with the first filter, identical sequences were grouped together in each locus avoiding in that way incorrect inference, since the resulting topology of identical sequences is completely random. In the next step, loci that contained less than four unique sequences (i.e., not counting identical sequences) were removed, since no topological relationships can be inferred with three or less unique sequences. Furthermore, in order to assess the impact of missing data on phylogenetic inference, as well as to determine the minimum amount of data that carry sufficient phylogenetic signal for resolving the topology, we constructed a set of supermatrices by selecting subsets of loci according to the minimum number of unique sequences per locus (termed as “min_taxa”). An important note here is that the min_taxa filter that was additionally performed by us, is completely different and must not be confused with the MinCov filter of pyRAD; although intuitively they appear to have similar behavior in practice, they provide a quite different result. MinCov=N retains the loci that are present for more than N samples, whereas min_taxa=N is more phylogenetic informative-oriented as it retains the loci that have at least N *unique* sequences. The sets were selected such as to most closely contain a percentage of loci in respect to the min_taxa=4 dataset i.e., 100% (min_taxa=4), 50% (min_taxa=9), 25% (min_taxa=14), and 12.5% (min_taxa=18). Overall, 12 datasets were

assembled (three clustering thresholds × four min_taxa filters), each of which hereinafter is mentioned using the clustering threshold and min_taxa filter parameters e.g. 0.85_4, 0.85_9 etc.

A second filtering step was applied to all 12 datasets for each locus as to contain at least the minimum number of variable (min_var) and parsimony informative (min_parinfo) sites that can resolve the topology of the given number of taxa (n_tax) in each locus. This resulted in the removal of loci with less than the minimum number of variable and parsimony informative sites and therefore the retention of only phylogenetically informative loci. These filters took the value of log2(n_tax) with n_tax being the number of taxa for each locus and represents the minimum number of informative/variable sites that can resolve the topology.

2.3. Phylogenomic analyses (gene trees and species tree)

The stability of the phylogenetic signal was evaluated by performing a Maximum Likelihood tree inference for each dataset, using ExaML (v.3.0.17; Kozlov et al., 2015). The resulting topologies were compared based on Robinson Foulds distances (RF distance; Robinson and Foulds, 1981). To elaborate on this issue, for each of the 12 datasets (clustering threshold × min_taxa filtering), 100 ExaML inferences were performed each using a random starting tree (random seed number) and a GTR+Γ model. The average pairwise RF distance among the 100 resulting trees was calculated using RAxML (v.8.2.9; Stamatakis, 2014) as a means to evaluate the topological congruence within each dataset. Then, using the best scoring trees of each setting average pairwise RF distances were calculated in order to assess the topological congruence between the 12 different datasets. Furthermore, using a custom R script that combined the R packages phangorn (Eaton et al., 2017) and rgl (http://rgl.neoscientists.org/about.shtml) a 3D visualization of the 70% consensus topology (i.e. among the 100 resulting ExaML trees) was

conducted. This was performed only for the best min_taxa filter solution in each clustering threshold ("selected datasets" hereinafter) as derived from the RF distances. The selected min_taxa values were 4, 9, and 9 for the 0.85, 0.90, and 0.95 clustering thresholds, respectively. Bootstrap values were calculated in RAxML with the bootstopping option of autoMRE (Pattengale et al., 2010) enabled. Subsequently, the bootstrap support was drawn onto the best-scoring tree of each one of the three ML topologies inferred using the three "selected datasets".

Bayesian Inference was conducted in ExaBayes (v.1.5; Aberer et al., 2014) for each of the "selected datasets". In all cases, the MCMC analysis ran for 500,000 generations using two independent runs with four chains each. The result was saved every 1,000 generations and the "burn in" period included the first 25% of samples. Convergence was evaluated using the standard deviation of split frequencies convergence option (sdsfConvergence; set at 5%) of ExaBayes, as well as by examining the effective sample sizes (ESSs) in Tracer (v.1.6; Rambaut et al., 2014).

Fully exploiting the power of the ddRADseq data (i.e., both sequences and genotypes), SNP based species trees were estimated using the multispecies coalescent method SVDQuartets (Chifman and Kubatko, 2014) as implemented in PAUP (v.4.0a152; Swofford, 2002) on the independent SNPs (i.e. one SNP per locus) of the three "selected datasets". Using a coalescent model, this method infers the topology among randomly sampled quartets of predefined species, and then a quartet method is used to assemble the sampled quartets into a species tree. The option of exhaustive search of quartet sampling was selected and the uncertainty in relationships was measured using non-parametric bootstrapping with 100 pseudo-replicates. It should be noted that for the species tree analyses, each distinct clade within species (i.e., *P. ionicus, P.*

peloponnesiacus and *P. erhardii*) was considered as putative species, whereas the non-*Podarcis* samples were excluded.

Coalescent-based SNAPP (SNP and AFLP Package for Phylogenetic Analysis; v.1.5; Bryant et al., 2012) analyses were used to test alternative species models for the two subgroups of *Podarcis* species in the Balkans i.e. the subgroup of *P. erhardii* and the subgroup of *P. tauricus*. Since SNAPP is computationally intensive, two specimens per phylogenetic subclade were chosen. To avoid model violations (SNAPP assumes no gene flow), we excluded admixed individuals; those with membership probability < 93% according to population structure analysis (see section 2.5), i.e. the two samples of *P. melisellensis*. Furthermore, and in order to analyze unlinked SNPs complying with SNAPP assumptions, the "selected datasets" were further filtered as to contain only one SNP per locus. Specimens were assigned to the following alternative species models; Five models for the subgroup of *P. erhardii*: i) 4_species (*P. erhardii, P. cretensis, P. levendis,* and *P. peloponnesiacus*) based on the current taxonomy, ii) 5_species (*P. erhardii, P. cretensis, P. levendis,* and two species in *P. peloponnesiacus*), iii) 6a_species (two species in *P. erhardii,* one in P. *cretensis,* two in *P. peloponnesiacus,* and one in *P. levendis*), iv) 6b_species (*P. erhardii,* two species in *P. cretensis,* two species in *P. peloponnesiacus,* and one in *P. levendis*), v) 7_species (two species in *P. erhardii,* two in *P. cretensis,* two in *P. peloponnesiacus,* and one in *P. levendis*), and two models for the subgroup of *P. tauricus*: i) 3_species (*P. tauricus, P. milensis,* and *P. gaigeae*), and ii) 4_species (*P. tauricus, P. ionicus, P. milensis,* and *P. gaigeae*).

SNAPP uses a Yule prior with parameter lambda (λ) representing the speciation rate. For the λ prior we used a fixed values with the help of pyule script (https://github.com/joaks1/pyule height of the species tree at 0.1 expected substitutions per site based on available information of

maximum observed divergence between taxa (height = max divergence/2) and number of tips/species that varied from four to seven i.e. for seven species, λ = 16, for six species, λ = 14.5, for five species λ = 12.83, and for four species λ = 11. Mutation rates u and v were set to one and were not sampled, while intraspecific variance to 0.1 (10%, α = 1, β = 10, Rateprior = gamma) and coalescence rate was sampled with a starting value of 10. Path Sampling Analysis was run with chain length of 100,000, alpha = 0.3, 50% burn-in percentage and 24 steps. Following Leaché et al. (2014), Bayes factor delimitation (BFD*) was used as a model selection tool by subtracting the marginal likelihood estimate (MLE) values for pairs of models and then multiplying the difference by two [BF = 2 x (MLE_1-MLE_0)].

SNAPP analysis of the selected model was executed in BEAST (v. 2.6.2; Bouckaert et al., 2019) by performing two independent runs for each "selected dataset" with 90,000,000 MCMC length (sampling every 1,000). The obtained log files were analyzed with Tracer to verify that non-convergence was not the case and that satisfactory effective sample sizes had been obtained (ESS values > 200).

2.4. Divergence times estimation

A chronophylogenetic analysis was conducted using the dataset with the most congruent topology (i.e. Wclust=0.90 and min_taxa=9; see Results section and **Figure 2)** using two calibration points; the separation of Peloponnisos from the island of Crete at 5-5.5 Mya, which corresponds to the splitting of *P. cretensis* from *P. levendis* and *P. peloponnesiacus* (Poulakakis et al., 2005a) and the diversification of Lacertini at ~15 Mya (Mendes et al., 2016). The divergence times were estimated under a Bayesian framework using the MCMCTree program incorporated in PAML (v.4.9; Papoulia, 2017) using the relaxed molecular clock and the

approximate likelihood computation algorithm (Santonastaso et al., 2017). Priors for the rgene and sigma2 parameters were set as G(2, 20) and G(1, 10), respectively. Markov chains were sampled every 10th generation until 20,000 trees were collected, after a burn-in period of 20,000 generations. The analysis was performed two independent times to check for convergence. The obtained log files were analyzed 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 (ESS values > 200).

2.5. Population structure and admixture analyses

The underlying population structure was inferred by methods implemented in STRUCTURE (v.2.3.4, Pritchard et al., 2000). The correlated allele frequency with admixture model (F-model) was applied. Given the number of clusters *K,* this model pursues solutions that are, as far as possible, both in Hardy-Weinberg and Linkage equilibrium. K varied from one to ten while five replicate runs were performed for each *K*. Each run comprised 100,000 generations as burn-in period, followed by 500,000 MCMC iterations from which the data were collected. The inference of *K* was evaluated by the ΔK method (Evanno et al., 2005) using STRUCTURE HARVESTER (Earl and vonHoldt, 2012) and Structure_threader (Pina-Martins et al., 2017) on MSL_NHMC Cluster. In addition, CLUMPAK (Kopelman et al., 2015) was employed in order to conduct averaging, define whether there is one or multiple run modes that generate consensus solutions allowing for label switching and testing for convergence and finally to graphically compare the results of different *K* through DISTRUCT (Jakobsson and Rosenberg, 2007). The analysis was run in each “selected dataset” for all clustering thresholds i.e. 85, 90 and 95 after further filtering as to contain only one SNP per locus, complying with the model’s

assumptions of unlinked markers (i.e. same datasets as the ones used for species tree and species delimitation analyses of SVDQuartets and SNAPP, respectively).

To test for evidence of admixture among species or clades, we performed the four population test known as F4 statistics (Patterson et al., 2012; Reich et al., 2009) with fixed outgroup. F4 statistics are defined in terms of correlations of allele frequency differences involving four different populations and are defined as: $F4(A, B; C, D) = \langle (a - b)(c - d) \rangle$, where $\langle \cdot \rangle$ denotes the average over all genotyped sites, and a, b, c and d denote the allele frequency for a given site in the four populations A, B, C, and D. Without any admixture between C or D and A or B the statistic should be not statistically different from zero. Often, this statistic is used by putting a divergent outgroup as population A, for which we know for sure that there was no admixture into either C or D. With this setup, we can then test for evidence of gene flow between B and D (if the statistic is statistically positive, indicated by Z score > 3; $p < 0.0001$), or B and C (if it is statistically negative, indicated by Z score < -3; $p < 0.0001$). Since this analysis is best suited for testing different populations of the same species or closely related species, we performed all the different species combinations of three within either the *P. tauricus* species subgroup (vcf dataset 1) or the *P. erhardii* species subgroup (vcf dataset 2), using in both cases *P. muralis* as the fixed outgroup. Furthermore, based on the fact that *P. muralis* is frequently found syntopically with *P. erhardii* and that they also share both morphological and ecological similarities (Valakos et al., 2008), albeit their phylogenetic divergence, the above analyses were performed among *P. muralis* and the species of *P. erhardii* subgroup (vcf dataset 3) using *L. trilineata* as the fixed outgroup in F4 statistics. This allowed as to also evaluate the choice of *P. muralis* as suitable outgroup for the F4 statistics in the other two datasets. The tests were performed via the fourpop command of TreeMix (v. 1.13; Pickrell and Pritchard, 2012). The

input file for each test was generated as follows. First, using VCFtools (v. 0.1.16; Danecek et al., 2011) we filtered the pyRAD vcf formatted output in order to make the three aforementioned datasets, remove indels (--remove-indels) and keep only the bi-allelic SNPs (--min-alleles 2 --max-alleles 2). Then, the filtered vcf was converted to TreeMix input using Stacks 2 (v. 2.41; Rochette et al., 2019) and its subcommand *populations*, by providing a list of samples allocation to species or clades. In this step we also filtered the data by keeping only the first SNP per locus (--write-single-snp), we set the minimum percentage of individuals in a species/clade required to process a locus for that species/clade to 50% (-r 0.5), and finally, we set as minimum number of populations a locus must be present in to process a locus, the maximum number of species/clades of each dataset.

Moreover, in order to test if there is admixture between sympatric populations of different species, we used the three populations test, also known as F3 statistics (Patterson et al., 2012; Reich et al., 2009). More specifically, we tested if a population found in sympatry of two species is the product of admixture between the two species (using as sources other populations of each species). If the F3 statistic is significantly negative (indicated by Z score < -3; $p < 0.0001$) there is evidence that the test population is admixed between the two source populations. We tested three populations of our sampled locations where sympatric *Podarcis* are found. The first one was Feneos, Korinthos prefecture, Peloponnese including adjacent areas, such as Ancient Feneos and Doxas lake, where *P. peloponnesiacus* (East clade; sample 1003) and *P. ionicus* (subclade c; samples 716-718) co-exist. As source populations we used *P. ionicus* (subclade d) individuals from Lakonia and Arkadia prefecture (samples 886-887) and a *P. peloponnesiacus* individual from Argolida prefecture (East clade; sample 999). The second location was Prespes lakes (Megali Prespa lake), Florina prefecture, northwestern Greece, where

P. erhardii (Mainland clade; samples 982-983) and *P. tauricus* (samples 716-718) are found in sympatry. As source populations we used *P. erhardii* (Mainland clade) individuals from Larisa, Kozani, and Grevena prefectures (samples 143, 984, 989, 991, 993, 994) and *P. tauricus* individuals from Nestos prefecture (samples 864-865). Finally, the third location tested was the area around Karditsa, central continental Greece, including adjacent areas, such as Plastira lake, where *P. erhardii*, *P. tauricus*, and *P. muralis* were sampled (samples 017, 936, 990, 995, 998). Here we perform the F3 statistics for all three pairs of species, using as source populations *P. erhardii* (Mainland clade) individuals from Larisa, Kozani, and Grevena prefectures (samples 143, 984, 989, 991, 993, 994), *P. tauricus* individuals from Nestos prefecture (samples 864-865), and *P. muralis* individuals from Florina prefecture (samples 992, 996-997). The tests were performed via the threepop command of TreeMix, with the input file for each test generated as described above.

3. Results

3.1. ddRADseq data metrics

The Illumina sequencing of ddRADseq libraries resulted in an average of 1,117,943.311 quality reads per sample (after Phred quality filtering of 20) ranging from 51,794 to 7,776,532. The mean number of loci per sample for each dataset based on the three different Wclust values (equal to 0.85, 0.90, and 0.95) was 33,743 (range=11,857 – 129,961), 35,655 (range=12,062 – 84,484), and 53,182 (range=13,134 – 255,285) loci, respectively. The number of loci present in at least four samples (MinCov=4, paralogs removed) increased with higher clustering thresholds (40,255, 44,086, and 55,426, respectively for each dataset). However, as we needed to have at least four unique sequences per locus (min_taxa=4 after applying all the additional filters) for

phylogenetic purposes, loci numbers were reduced to 8,395, 7,850, and 5,425 respectively, resulting also in a slight increase of the percentage of missing data (80.75%, 81.62%, and 82.83%, respectively). By subsampling the datasets and retaining 50% (min_taxa=9), 25% (min_taxa=14), and 12.5% (min_taxa=18) of the initial amount of loci, the amount of gaps/undetermined characters was reduced as expected (71.56%, 72.06%, and 70.85%, respectively for min_taxa=9, to 63.65%, 63.52%, and 61.85%, respectively for min_taxa=14, and to 56.63%, 55.44%, and 52.62%, respectively for min_taxa=18). Summary statistics for all ddRADseq datasets are given in **Table 1**. Various parameters (i.e. sample representation, the percentage of samples per locus, gappyness, and percentage of variable sites per locus) for each Wclust-based dataset and its subsets assembled using the different selected min_taxa filter is plotted in **Figures S1** and **S2**.

The mean relative RF distances of each dataset that were used as a proxy of phylogenetic signal stability among the 100 ExaML are shown in **Table S2**, whereas the corresponding pairwise mean relative RF distances between the best scoring ExaML trees inferred from the 12 different datasets are given in **Table S3**. Based on the RF distances within each clustering threshold the best min_taxa filter was 4, 9, and 9 for the 0.85, 0.90, and 0.95 clustering thresholds, respectively. The three "selected datasets" for performing statistical phylogenomic analyses included 8,395 (Wclust=0.85, min_taxa=4), 4,061 (Wclust=0.90, min_taxa=9), and 2,174 (Wclust=0.95, min_taxa=9) ddRAD loci, bearing 80,75%, 72.06%, and 70.85% missing data, respectively. The discordance among the 100 ExaML trees for each of the three "selected datasets" is shown in **Figure S3** as a combination of a consensus phylogenetic tree and a network. The observed discrepancy within each "selected dataset" is concentrated in some of the

external splits of the tree that involve leaves belonging to *P. cretensis* from western Crete, as well as to *P. erhardii* from the Cyclades.

3.2. Phylogenomic relationships and molecular dating

The topology of the main clades and subclades of the Balkan *Podarcis* are displayed in **Figure 2** that summarizes the results of the ML and BI analyses and their statistical support for all three "selected datasets". For the BI analyses the number of required generations for converging according to the sdsfConvergence option were 1,000,000 for all three "selected datasets" and resulted in parameter estimations with high effective sample sizes (ESS>262, >1,223, and >480) and posterior probabilities (*ln*L=−3,749,828.69, −917,815.79, and −1,242,671.61). The common denominator in the resulting phylogenetic trees from all datasets (**Figure 2**), is the following: *P. erhardii* species subgroup was most closely related to *P. tauricus* species subgroup than to *P. muralis*. Within *P. tauricus* species subgroup five major clades were revealed that correspond to *P. gaigeae, P. ionicus, P. melisellensis, P. milensis*, and *P. tauricus* with their relationships being almost fully resolved. *Podarcis ionicus* also displayed high phylogenetic intraspecies differentiation with several distinct subclades. Within *P. erhardii* species subgroup the relationships among the morphologically recognized species were also fully resolved with *P. erhardii* being the first to branch off and with *P. peloponnesiacus* and *P. levendis* being sister taxa. Moreover, *P. peloponnesiacus, P. erhardii*, and *P. cretensis* are further differentiated into two highly (especially in the first two species) diverged subclades. A summary of the results of the ML and BI analyses and their statistical tests for all three "selected datasets" concerning the topology within *P. cretensis* and within *P. erhardii* are provided in **Figures S4** and **S5**, respectively.

The exhaustive quartet search of SVDQuartets method resulted in 3,856,755 quartets (common value in all "selected datasets"), which were used to infer the SNP based species trees. Concerning the major clades and subclades the resulting topologies for the 0.85_4 (best statistically supported tree compared to the rest) and 0.90_9 datasets partly match the most congruent one inferred from the concatenated datasets, with topological disagreements observed on the relationships among *P. peloponnesiacus* West – *P. peloponnesiacus* East – *P. levendis*, as well as among *P. gaigeae – P. tauricus – P. ionicus* (**Figure 3**). The SVDQuartets tree for 0.95_9 dataset resulted in a weakly supported topology with many incongruences compared to the rest, a result possibly induced by the low number of unlinked SNPs existing in this dataset.

The marginal likelihood estimates that were obtained for each model run in SNAPP analyses for the 0.90_9 dataset are reported in **Table S4**. Same results were obtained for the other two selected datasets (data not shown). The species delimitation model ranking in accordance to the BDF* methods is also displayed (**Table S4**). According to the results the species delimitation of choice is the one with seven species for the *P. erhardii* subgroup and with four species (*P. melisellensis* excluded, see M&M section) for the *P. tauricus* subgroup. In general, the species trees obtained with SNAPP were in accordance with the SVDQuartets results (**Figure 3)**. A worth noting difference was the sister group relationship of *P. peloponnesiacus* - East clade to *P. levendis* and not to *P. peloponnesiacus* - West clade (**Figure 3**). Furthermore, in agreement with the SVDQuartets analyses, but not with the gene tree analyses, in the SNAPP species tree of *P. tauricus* subgroup, *P. gaigeae* is a sister clade to *P. tauricus* and then to *P. milensis* and *P. ionicus* (**Figure 3**).

The molecular dating analysis resulted in high posterior ESS values (> 225) for all parameters, and convergence was reached prior to 40,000 generations (lnL= -7.603×10^{32}).

According to the inferred dates (**Table 2**), the Balkan species subgroup of *Podarcis* started to diversify at the end of Serravallian Stage (Middle to Late Miocene) around 11.93 Mya with the split between the *P. erhardii* and *P. tauricus* species subgroups. The diversification/speciation within the species subgroups occurred from the Tortonian Stage (Late Miocene) to the Zanclean (Upper Pliocene).

3.4. Evidence of population structure and admixture

High levels of genetic structure were detected in all analyses of the different clustering threshold datasets with K values varying between 3 and 6 (**Figure S6**) with further sub-structuring in all cases. The best K of the clustering threshold 0.85_4 dataset equals to 6 with three equally likely modes that differ slightly in their clustering solutions, but eventually end up in the same groupings after subsequent analyses (e.g. in *P. tauricus* sub-grouping, *P. tauricus* is either allocated to a unique cluster or to a cluster with the remaining *P. tauricus* sub-group species, with subsequent analyses leading to its allocation to a separate cluster, **Figure S6A**). The best K of the 0.90_9 dataset is 4 with two equally likely modes (**Figure S6B**) while the best K of the 0.95_9 dataset clustering threshold is 3 with two equally likely modes (**Figure S6C**). As a consensus of all runs in all "selected datasets" and therefore similarity thresholds, it is evident that the clustering reflects not only the taxonomic status of the studied samples, but in some cases their geographic origin. In a more detail, the consensus clusters are: *P. cretensis, P. erhardii* populations of the Aegean islands (*P. erhardii* – Cyclades Islands), *P. erhardii* populations of the mainland Greece (*P. erhardii* Mainland), *P. ionicus, P. tauricus, P. muralis, P. gaigeae, P. milensis, P. peloponnesiacus* - East, and *P. peloponnesiacus* – West, where allocation is characterized by high q-values with only one sample of *P. erhardii* from mainland

Greece displaying admixed genealogy with the two *P. erhardii* clusters i.e. Mainland and Cyclades Islands in all datasets (**Figure S6**). In 0.95_9 dataset only, samples of *P. peloponnesiacus* East, appear admixed with members of the *P. erhardii* subgroup, fact that could be attributed to the lower number of independent SNPs in comparison to the other two datasets i.e. 0.85_4 and 0.90_9. Furthermore, the samples of *P. melisellensis* appear highly admixed in all analyses with their genome being equally allocated to two or three clusters after the analysis with CLUMPAK (**Figure S6**).

Finally, according to the F4 statistics there is no statistically significant evidence of gene flow (admixture) among any of the species trios tested, as suggested by the *Z* scores of the four population tests (**Table S5** to **S7**). Similarly, the F3 statistics tests found no evidence of admixture in target populations of sympatric species from the source populations used (**Table S8**).

4. Discussion

4.1. Impact of missing data and stability of phylogenetic signal

The effect of missing data on the inferred phylogenomic relationships was evaluated by using different values of the min_taxa filter that generated different sets of data, containing 100% (min_taxa=4), 50% (min_taxa=9), 25% (min_taxa=14), and 12.5% (min_taxa=18) of the recovered loci, after the application of all filters. According to the results, the different levels of missing data did not generate serious discordance regarding the retrieved phylogenomic relationships among the studied taxa (**Figure 2**) or the discovered population genomic structure (**Figure S6**). This is probably attributed to the fact that in our approach, the two additional filters that were applied to the datasets generated by pyRAD pipeline (i.e. the min_taxa, and the

min_parinfo - min_var filters), were oriented towards the retention of phylogenetic informativeness, allowing the preservation of the phylogenetic signal contained in the data. This is also the reason why it was possible to resolve deeper relationships i.e. relationships of taxa that diverged ~17mya (**Table 2**).

With the additional filters that were applied, instead of discarding all loci with missing data above a particular threshold, loci that are phylogenetically informative for parts of the tree were retained in an attempt to keep the information contained in the data without jeopardizing the integrity of the results, since including more missing data between more divergent taxa increases the potential to gather more phylogenetic information (in terms of quartet informativeness) about splits deeper in a tree (e.g. see Eaton et al., 2017). This is also supported by the findings of the present study where the "selected datasets" were the ones which either retained all loci (min_tax=4 in Wclust=0.85) or 50% of the loci (min_tax=9 in Wclust=0.90 and 0.95) and therefore the ones with the highest and second highest number of missing data, respectively (80.75%, 72.06%, and 70.85% missing data, respectively). At this point it is worth mentioning that in population genomic analyses conducted in datasets of all similarity thresholds without applying the two additional filters and only retaining data with less than 50% missing data (*vs* 71.2% missing data on average in our datasets), resulted in meaningless and biologically not acceptable clusters (e.g. clusters with 2-3 samples vs all the rest; results not shown).

In this line of thoughts, there is a number of studies that have demonstrated that phylogenetic analyses of the largest datasets with the highest amount of missing data provide similar topologies despite the presence of missing data. On the other hand matrices containing minimal missing data and relatively few SNPs produce topologies with extremely low bootstrap support with the opposite (i.e. more loci and more missing data) generally providing higher

bipartition supports both in empirical (e.g. Eaton and Ree, 2013; Emerson et al., 2010; Takahashi et al., 2014; Wagner et al., 2013; Wang et al., 2017) and simulated phylogenetic studies (Huang and Knowles, 2016; Leache et al., 2015; Rubin et al., 2012; Streicher et al., 2016; Wang et al., 2017). Furthermore, consistent results were produced when samples with very few loci were removed (i.e. one order of magnitude fewer) and all analyses were conducted with pyRAD Wclust=0.90 and the additional filters were applied as before (results not shown).

4.2. Insights into the "Balkan" *Podarcis* phylogeny

Despite years of study, DNA-based research, coupled with thorough character and taxonomic sampling taxa, is still yielding insights into the phylogenetic history of the wall-lizards in the Balkans. Using between few hundreds to few thousands base pair long DNA fragments and nearly complete or incomplete taxon sampling, several previous studies (Poulakakis et al., 2003; Poulakakis et al., 2005a; Poulakakis et al., 2005b; Psonis et al., 2018; Psonis et al., 2017; Spilani et al., 2019) provided a phylogeny of this group of lizards in the Balkans. However, uncertainties remained in the placement of several clades that were not strongly supported by all studies and all phylogenetic analyses. In one of the most recent studies (Psonis et al., 2018), a fully resolved tree for the *P. tauricus* subgroup was presented using the genome-wide ddRADseq SNPs data. In the present study, we have generated the most robust DNA phylogeny of wall-lizards in the Balkans by substantially increasing the amount of sequence data, enhancing taxon sampling, and applying comprehensive analytical methods to reconstruct and evaluate trees, removing the topological ambiguity in several key relationships within the entire Balkan *Podarcis* phylogeny.

From a phylogenetic point of view, the two major subgroups occurring in the Balkans (those of *P. tauricus* and *P. erhardii*) appeared to be more closely related to each other than to *P. muralis* that belongs to a separate group (Arnold et al., 2007; Buades et al., 2013; Carranza et al., 2004; Harris et al., 2005; Harris and Arnold, 1999; Vasconcelos et al., 2006). Starting from the *P. erhardii* species subgroup, of which the phylogenomic relationships have been fully resolved, *P. peloponnesiacus* and *P. levendis* are sister species, with *P. cretensis* being the most closely related to them, followed by *P. erhardii*. The relationships among the first three species has been a difficult problem to resolve using mtDNA or a few nDNA markers alone, with previous studies reporting conflicting topologies, with low to moderate statistical support (Poulakakis et al., 2003; Poulakakis et al., 2005b; Psonis et al., 2017; Spilani et al., 2019). Such polytomies could be attributed to the rapid diversification of several *Podarcis* species that produced low phylogenetic signal (Psonis et al., 2017) and/or to non-contemporary (given the observed allopatric distribution) admixture (interspecies gene flow) among the three species. The latter (admixture) is not supported by our population genomics analyses that is congruent with the results of a previous study (Spilani et al., 2019), in which no evidence of admixture was observed based on microsatellite data. To be noted, though, in the species tree analysis *P. levendis* is sister taxon either to the eastern (SNAPP analyses of 0.85_4 and 0.90_9; see **Figure 3** legend) or to the western (SVDQuartets analysis although not well supported; **Figure 3**) clade of *P. peloponnesiacus*. This incongruence between the concatenated genes tree and the species tree methods could be attributed to the different type of data and models used, as well as assumptions made by the two different families of tree reconstruction methods. However, the most stable topology overall, is the one showing that *P. peloponnesiacus* is monophyletic (**Figure 2**).

Phylogenomic analyses revealed significant intraspecies differentiation within all species of the *P. erhardii* subgroup, but not in *P. levendis*. Each species comprises two very divergent clades, following a west/east axis of diversification. However, the relationships within each clade and subclade could not be unambiguously reconstructed as only some of them were well supported. This could be attributed to either ongoing gene flow (interbreeding) among some of the populations, or to a very recent divergence and incomplete lineage sorting, leading to low phylogenetic resolution. The only clade that showed a biogeographically interpretable pattern is that of *P. erhardii* in the Cyclades (**Fig. S5**), with three groups of lineages that, partly, follow a geographical pattern of islands in close proximity sharing a common geological history (Dermitzakis, 1990; Lambeck, 1996; Meulenkamp, 1985; Perissoratis and Conispoliatis, 2003) and thus levels of isolation (see also Hurston et al., 2009; Poulakakis et al., 2005b; Santonastaso et al., 2017). Those are a) the western, b) the northwestern, and c) the southeastern Cyclades. This grouping of island populations is only partially congruent with previously published mtDNA-based findings (Poulakakis et al., 2005b), in which island groupings also exhibited low statistical support. Interestingly, specimens sampled from Santorini Isl. and Nea Kameni islet show high affinity with lizards from both southeastern and northwestern Cycladic groups, which is consistent with the possibility of rare, anthropogenic long-distance dispersal events.

Overall, the concatenated genes tree based phylogenomic relationships within the *P. tauricus* species subgroup replicate the results of Psonis et al. (2018) where in a fully resolved species tree, *P. ionicus* is the sister taxon to *P. tauricus*, and *P. gaigeae* being the most closely related species to both of them followed by *P. milensis*. The most phylogenetically distant is the species of *P. melisellensis.* In general, all datasets analyzed in the present study resulted in similar phylogenomic trees with those in Psonis et al. 2018 with slight differences mainly under

the 95% similarity (Wclust) threshold (vs 85% and 90%) that resulted in a significantly lower number of loci. This is also evident in the incongruences observed between the concatenated genes trees and the species trees produced by both methods employed in this study with the different datasets. No sign of admixture among any of the species was detected by our population genomics analyses. According to the phylogeographic history of the subgroup (Psonis et al., 2018) the divergence of these species (Late Miocene-Early Pliocene) was followed by consecutive retractions and expansions of their distributions during the glacial and interglacial Pleistocene periods. Currently, the species are not overlapping geographically, with a potential exception of a very small area (that needs to be verified with *in situ* observations) in central Albania (north of Tirana), where *P. ionicus* and *P. melisellensis* (subclade/subspecies *fiumana*) could share or compete for the same habitat.

Population genomic analyses revealed high levels of population structure in all datasets, where clustering reflected the taxonomic status of the studied samples and in some cases the geographic origin of samples (i.e. *P. erhardii* populations of the Aegean islands and the mainland, *P. peloponnesiacus* East and West). However, having highly divergent taxa in the dataset, in combination to the low number of analyzed samples from those groupings, resulted in some not well resolved cases, as the case of *P. melisellensis*. Overall, population genomic analyses are in agreement with the phylogenomic analyses, except in the cases of *P. cretensis* and *P. ionicus* where no further substructuring was observed, thus not reaching at the same resolution level.

Furthermore, the population genomic results were in agreement with those of previous studies (i.e. Psonis et al., 2018; Spilani et al., 2019), indicative of the robustness of the results of the present study and of the informativeness of the data at hand. However, it was not possible to

reach the same level of resolution in the case of *P. cretensis* or of *P. tauricus,* fact that could be attributed to the significantly lower number of samples used in the ddRAD *versus* the microsatellite datasets.

4.3. Taxonomic evaluation and reconsiderations

In the present study using ddRADseq data, we recorded several divergent clades within some of the recognized *Podarcis* species distributed in the Balkans. Species delimitation suggests the number of delimited species to be varying from 10 to 11, excluding *P. muralis* and *P. melisellensis*, with 7 species delimited within *P. erhardii* subgroup (two species in *P. erhardii*, one or two in *P. cretensis* [very close BF (ln) value], two in *P. peloponnesiacus*, and one in *P. levendis*) and four species within *P. tauricus* subgroup (*P. tauricus*, *P. ionicus*, *P. milensis*, and *P. gaigeae* (**Table S4**). Our findings support the first observations of high levels of substructure made by previous studies (Poulakakis et al., 2003; Poulakakis et al., 2005a; Poulakakis et al., 2005b; Psonis et al., 2018; Psonis et al., 2017; Spilani et al., 2019). All the accumulated genetic evidence suggests that the taxonomy of the *Podarcis* taxa of the Balkan peninsula needs several taxonomic changes and a reconsideration of the number of species that are present.

For *P. tauricus* subgroup, our results confirm the previously published ones (Psonis et al., 2018; Psonis et al., 2017) with the presence of five very well supported species that diverged at the Late Miocene and Early Pliocene (8.24-4.91 Mya): i) *P. tauricus*, ii*) P. gaigeae*, iii) *P. milensis*, iv) *P. melisellensis*, and v) *P. ionicus.*

In the *P. erhardii* subgroup, its current taxonomy of four recognized species that diverged also at the Late Miocene and Early Pliocene (9.41-4.89 Mya) is not adequate given the observed molecular phylogeny and differentiation on genomic data. Our data supported the differentiation

of *P. cretensis* and *P. levendis* at the species level, both diverged at ~5 Mya. Both species are quite well studied using multilevel data [morphological data (Lymberakis et al., 2008); genetic data (mtDNA, nuDNA, microsatellites) (Lymberakis et al., 2008; Poulakakis et al., 2003; Poulakakis et al., 2005b; Spilani et al., 2019) and representative specimens from their whole distribution area. All findings clearly indicate that *P. levendis* is indeed a distinct species, but with limited genetic variability and substructure. This is a crucial piece of information that should be taken into account in future conservation plans and evaluations. On the other hand, total evidence also suggests the distinction of *P. cretensis* at the species level with high intraspecific structure, in contrast to *P. levendis*, with the existence of two major groups of allopatric lineages that were significantly divergent, without reaching an unambiguous conclusion on whether it reaches that of the species level (present study and Spilani et al., 2019). The species delimitation results of the present study only slightly support [based on the BF (ln) values] the splitting of *P. cretensis* into two species, hence there is lack of confidence to make a final decision. Future morphological comparison between the two clades may provide additional evidence. We agree, though, with the conclusion of Spilani et al. (2019) that these two lineages should be considered as separate conservation units.

The Peloponnesian wall-lizard, *P. peloponnesiacus* (diverged at ~5 Mya) has also been quite well studied in the same way as *P. cretensis* and *P. levendis*, but with smaller sampling coverage in respect to its distribution. The current phylogenomic data supported the presence of two very divergent groups of lineages, diverged at ~4.4 Mya, congruent to the results of Spilani et al. (2019). Putting all the findings together, i.e. the high levels of differentiation along with the lack of any evidence of admixture and their geographic distinctiveness, renders their recognition as two separate species highly probable. However, we are reluctant to describe a new species

because a) due to sampling gaps, it is not clear which is the actual distribution of each of the *P. peloponnesiacus* lineages, and b) currently, there is lack of evidence, whether these two clades conform to the taxonomy at the subspecies level that includes *P. p. peloponnesiacus*, *P. p. lais*, and *P. p. thais*. A future study under an integrated taxonomic approach that will fill the sampling gaps and evaluate the taxonomy at the subspecies level (genetics, morphology and distribution) is suggested to be conducted before a final decision (elevation to species level with possible synonimization or description of a new species) regarding the formal taxonomic changes is taken.

Finally, *P. erhardii* (diverged at ~9.4 Mya) is also subdivided into two clearly divergent groups of lineages (one distributed in the Cyclades Islands and the other in the continental Greece) that could be considered as distinct, allopatric species, with their time of divergence estimated at ~6.8 Mya. However, *P. erhardii* has not be equally well studied in the past, neither on the amount and diversity of markers used, nor on its distribution sampling coverage. Particularly, there is a very imbalanced sampling coverage (that follows the populations' density of the species) between the continental and the insular populations of the species with the first being under-sampled and with the majority of samples collected within the Greek territory. Furthermore, only mtDNA markers have been implemented for phylogenetic reconstruction with emphasis on the insular populations (Hurston et al., 2009; Poulakakis et al., 2003; Poulakakis et al., 2005b), whereas microsatellites have been used to study the population structure of selected insular populations (Hurston et al., 2009). Although our genomic data suggested the distinctiveness of these two deeply divergent clades at the species level, the absence of evidence from other sources of data i.e. genetic markers (multigene markers and microsatellites), morphology, ecological distribution, do not permit us to proceed with any taxonomic

modification in *P. erhardii*, but only to postulate the presence of two species within it. A representative sampling of the continental populations, alongside with a thorough evaluation of the taxonomy at the subspecies level (several subspecies are expected to be invalid) will allow making decisions whether a new species is going to be described.

In total, our data in conjunction to the previously published ones have challenged the current taxonomy of *Podarcis* in the Balkans, increasing the real number of species from seven in 2005 (Poulakakis et al., 2005a) to nine in 2008 (Lymberakis et al., 2008), 10 in 2018 (Psonis et al., 2018; Psonis et al., 2017) and to 11 or 12 (depending on the status of *P. cretensis*) in the current study. This makes the genus *Podarcis* the most speciose vertebrate genus in the Balkans, in which all species were phylogenetically distinct from one another and, as such, should be treated as different conservation units. This could be of high priority if we take into account the fact that two exotic species of *Podarcis* (*P. vaucheri*, and *P. siculus*) have invaded the southern Balkans (Adamopoulou, 2015; Spilani et al., 2018). The proven negative consequences that some of them (e.g. *P. siculus*) may induce to native species (Downes and Bauwens, 2002) underscore the need for high alert.

With 86 species, Greece hosts one of the richest herpetofaunas in Europe. Thirteen of the species are endemic, whereas for 13 more, Greece hosts the only European populations (Pafilis, 2010). These numbers will be increased in 89 and 16, respectively with the recognition of *P. ionicus*, and the new species in *P. peloponnesiacus*, and in *P. erhardii*, confirming the importance of the southern Balkans as an area with high species and genetic diversity with many cases of cryptic species, when compared to higher latitude areas of the European continent (Hewitt, 2011), and which as part of the Mediterranean basin is one of the world's top biodiversity hotspots (Myers et al., 2000).

Overall, it becomes obvious that current taxonomy does not properly reflect the phylogenomic relationships and the population genomics of the wall-lizard's species in the Balkans. According to our findings and in order to reflect their evolutionary relationships, the taxonomy of several *Podarcis* species in the Balkans should be changed (*P. peloponnesiacus, P. erhardii,* and *P. ionicus*). Among them, only the case of *P. ionicus* is quite simple with the elevation of one of the subspecies of *P. tauricus* (*P. t. ionicus*) to the species level. For the other two, the description of the new species demands, among others, a morphological description that needs a more thorough systematic work with a plethora of populations and specimens per population which is not the goal of the current work. For these reasons, the updated description for *P. ionicus* is given in **Supplementary Note S3.**

Future directions

Finally, future multi-gene, morphological and ecological (distribution models) studies are needed for the evaluation of the taxonomy in species and subspecies of *P. erhardii* and *P. peloponnesiacus* in the southern Balkans. Moreover, although distinct species, *P. milensis* needs more thorough intraspecific analyses since genetic/genomic data are totally lacking for two of the three described subspecies. The same is true for *P. melisellensis* in Dalmatian coasts, in which only two of the three subspecies have been studied using mtDNA. Finally, *P. muralis* demands extremely more efforts due to its widespread distribution ranging from northern Spain, much of the mainland Europe to the Balkans (excluding most of the Aegean islands) and northwestern Anatolia, Turkey (Böhme et al., 2009).

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Data accessibility

The ddRADseq data are available on the NCBI SRA in demultiplexed form. All new data generated for this study, together with the ddRADseq data of Psonis et al. (2018), and the sequence alignments of Psonis et al. (2017) are listed under project number (*To Be Provided*). The post pyRAD filtering scripts used by the authors can be accessed in github (https://github.com/ddarriba/ddrad-seq).

Author Contributions

NP (Psonis) collected samples, performed laboratory work, analyzed data and wrote the manuscript. AA collected samples, performed laboratory work, analyzed the data, wrote parts of and commented on the manuscript. EK collected samples, performed laboratory work, and commented on the manuscript. AS and DD advised on phylogenetic analyses on ddRADseq data and commented on the manuscript. PL collected or provided samples, wrote a part and commented on the manuscript. NP (Poulakakis) designed and supervised the research, collected samples, analyzed data and refined the manuscript. All authors read and improved the final manuscript.

Conflict of Interest

The authors have no conflict of interest to declare.

Supporting Information

Figure S1. Taxa (individuals/samples) representation of the three (Wclust) clustering threshold datasets (A. 0.85, B. 0.90, and C. 0.95) of the ddRADseq data, before applying the

min_taxa, min_var, and min_parinfo filters. It informs about the percentage of loci (y axis) that are present in a specific percentage of samples (x axis).

Figure S2. Percentage of taxa (individuals/samples) per locus, gappyness and percentage of variable sites regarding the ddRADseq data before and after applying the following values of Wclust clustering threshold and min_var, min_parinfo, and min_taxa=4 filter, respectively: A. 0.85, B. 0.90, and C 0.95.

Figure S3. 3D visualization of the topological congruence among the 100 resulted ExaML trees for the "selected datasets" assembled using A. Wclust=0.85 and min_taxa=4, B. Wclust=0.90 and min_taxa=9, C. Wclust=0.95 and min_taxa=9. The 70% consensus topology is drawn as a tree and the incongruence (<70%) as a network amongst branches. The values on the branches declare the number of the different topologies supporting the consensus one. Branches without values correspond to 100% support.

Figure S4. Summary of the results produced by the ML and BI analyses for all assembled datasets concerning the topology within *Podarcis cretensis*. Bootstrap test and the BI analyses were conducted only using the three "selected datasets" (indicated with purple text color). The depicted topology corresponds to the most congruent phylogeny amongst the different datasets.

Figure S5. Summary of the results produced by the ML and BI analyses for all assembled datasets concerning the topology within *Podarcis erhardii*. Bootstrap test and the BI analyses were conducted only using the three "selected datasets" (indicated with purple text color). The depicted topology corresponds to the most congruent phylogeny amongst the different datasets.

Figure S6. Bar plots of STRUCTURE population genomic analyses that depict the q-values of the different Ks and modes in each K detected after CLUMPAK analysis. **A.** for the 0.85_4 dataset, **B.** for the 0.90_9 dataset, **C.** for the 0.95_9 dataset.

Table S1. List of specimens (in taxon name alphabetical order) examined in the present study with their corresponding sample codes, taxon names, representing clade/subclade, voucher numbers, country/region/locality names, and reference of the study in which they were used.

Table S2. Mean relative RF distances within the four different min_taxa filters for the three clustering threshold datasets.

Table S3. Pairwise mean relative RF-distances between the best scoring ExaML trees inferred from the 12 different datasets as defined by the three clustering thresholds and the four min_taxa filters.

Table S4: Marginal Likelihood Estimates (MLEs) of the alternative species delimitation models. Ranking: ranked by their MLE, BF (ln) for each pair of species delimitation models comparison.

Table S5. F4 statistics calculations testing for admixture among the species of the *Podarcis tauricus* species subgroup using *P. muralis* as fixed outgroup.

Table S6. F4 statistics calculations testing for admixture among the species of the *Podarcis erhardii* species subgroup using *P. muralis* as fixed outgroup.

Table S7. F4 statistics calculations testing for admixture among *Podarcis muralis* and the species of the *P. tauricus* species subgroup using *Lacerta trilineata* as fixed outgroup.

Table S8. F3 statistics calculations testing for admixture in populations of sympatric *Podarcis* species.

Note S1. Details on ddRADseq protocol and Illumina sequencing.

Note S2. Details on the use of pyRAD.

Note S3. The updated description of *Podarcis ionicus*.

Tables

Table 1. Summary statistics for the ddRADseq datasets of the Balkan species group used. Loci and SNPs statistics are in italics and bold, respectively.

Statistic	Bioinformatics Pipeline step	Wclust = 0.85	Wclust = 0.90	Wclust = 0.95
Retained reads that passed quality filtering - NQual (avg ± sd)[a]	pyRAD step 2 (filtering)	1,000,965 ± 846,524	964,067 ± 818,807	920,197 ± 779,586
Mean depth of clusters with depth greater than NQual (avg ± sd)	pyRAD step 3 (within-sample clustering)	58.5 ± 26.5	59.1 ± 26.7	63.53 ± 28.9
Number of loci per sample (avg ± sd)	pyRAD step 5 (consensus sequences)	*33,743 ± 16,414*	*35,655 ± 14,597*	*53,182 ± 28,509*
Number of loci per sample with depth greater than NQual (avg ± sd)	pyRAD step 5 (consensus sequences)	*10,179 ± 4,023*	*10,433 ± 3,447*	*11,189 ± 4,326*
Number of loci per sample with depth greater than NQual and paralogs removed (avg ± sd)	pyRAD step 5 (consensus sequences)	*9,071 ± 2,539*	*9,418 ± 3,127*	*10,558 ± 4,067*
Number of sites across loci per sample with depth greater than NQual and paralogs removed (avg ± sd)	pyRAD step 5 (consensus sequences)	805,468 ± 314,658	836,468 ± 277,842	938,168 ± 361,651
Number of polymorphic sites across loci per sample with depth greater than NQual and paralogs removed (avg ± sd)	pyRAD step 5 (consensus sequences)	2,220 ±1,222	2,358 ± 1,138	2,655 ± 1304
Number of loci with at least MinCov samples containing data[b]	pyRAD step 6 (across-sample clustering)	*40,255*	*44,086*	*55,426*
Number of loci with at least MinCov samples containing data and paralogs removed	pyRAD step 7 (alignment and paralog filtering)	*35,098*	*38,948*	*50,737*
Total variable sites	pyRAD step 7 (alignment and paralog filtering)	233,719	216,956	183,184
Sampled unlinked SNPs	pyRAD step 7 (alignment and paralog filtering)	**30,064**	**32,845**	**41,352**
Sampled unlinked bi-allelic SNPs	pyRAD step 7 (alignment and paralog filtering)	**19,052**	**20,117**	**20,413**
Number of loci after Min_taxa=4 filtering[c]	Further filtering by authors	*8,395*	*7,850*	*5,425*
Number of loci after Min_taxa=9 filtering[c]	Further filtering by authors	*4,639*	*4,061*	*2,174*
Number of loci after Min_taxa=14 filtering[c]	Further filtering by authors	*2,650*	*2,155*	*979*
Number of loci after Min_taxa=18 filtering[c]	Further filtering by authors	*1,447*	*1,063*	*330*

[a] NQual equals to 14, 9, and 5 for the 0.85, 0.90, and 0.95 Wclust filtered datasets, respectively

[b] MinCov equals to 4 for all three Wclust filtered datasets

[c] This step also includes the filtering for the number of variable and parsimony informative sites, respectively (see text in Materials and Methods section for further information).

Table 2. Results of the molecular dating analysis of the Balkan Podarcis using the dataset constructed with Wclust threshold of 0.90 and min_taxa filter of 9. The split numbering corresponds to the one depicted in Figure 2. Values within square brackets indicate the 95% Highest Posterior Density (HPD). Split D was used as a calibration point (see text).

Split	Inferred Date (in Mya)
A	13.75 [15.70 – 9.57]
B	11.93 [15.12 – 7.11]
C	9.41 [14.07 – 5.46]
D	5.25 [5.5 – 5.00]
E	5.01 [5.42 – 4.36]
F	4.89 [5.41 – 4.00]
G	4.40 [5.22 – 3.10]
H	6.83 [12.60 – 4.74]
I	8.24 [14.00 – 4.80]
J	6.41 [12.46 – 4.54]
K	5.44 [10.48 – 4.26]
L	4.91 [7.99 – 3.93]
M	4.35 [5.25 – 3.13]
N	3.85 [4.94 – 2.45]
O	3.20 [4.56 – 1.61]
P	2.13 [3.9 – 0.45]

Figure captions

Figure 1. Map showing the sampling localities of the present study for each *Podarcis* species.

Figure 2. Summary of the results produced by the ML and BI analyses for all assembled datasets concerning the topology of the main clades and subclades of the Balkan *Podarcis*. Bootstrap test and the BI analyses were conducted only using the three "selected datasets" (indicated with purple text color). The depicted topology corresponds to the most consensus phylogeny amongst the different datasets.

Figure 3. Balkan *Podarcis* species trees inferred for all three selected datasets (A: 85_4, B: 90_9, C: 95_9). The values on the branches correspond to statistical support (SVDQuartets bootstrap / SNAPP posterior probabilities). Note that SNAPP analyses were performed separately for the *P. erhardii* and *P. tauricus* species subgroups excluding *P. muralis* and *P. melisellensis* (see Materials and Methods section). n.s.: non-supported topology by SNAPP; n.s.[1]: SNAPP show absolute support for *P. milensis* being the sister group of *P. gaigeae* / *P. tauricus*; n.s.[2]: SNAPP show absolute support for *P. peloponnesiacus* - East being the sister group of *P. levendis*; n.a.: non-accounted due to taxon exclusion from SNAPP analysis.