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Evolutionary divergence of the smooth snake (Serpentes, Colubridae): The role of the Balkans and Anatolia

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Abstract

The smooth snake *Coronella austriaca* (Laurenti, 1768) is distributed across the western Palearctic throughout north-central, southern Europe and western Asia. So far, only few phylogenetic studies, based on mitochondrial DNA, have been carried out on this species focusing mainly on the Iberian Peninsula and northern Europe, leaving unstudied some of important areas of its distribution. This study aims to cover this gap and to explore the phylogenetic relationships between the populations of in the Eastern Mediterranean and to detect possible historical refugia. With this objective, total genomic DNA was extracted from samples originated from the Balkans, Anatolia and a large part of Europe. Two mitochondrial (cyt *b*, 16S) and six nuclear (BDNF, NKTR, RAG1, RAG2, MC1R and PRLR) markers were combined for phylogenetic and chronophylogenetic analyses. The different analyses confirmed previous phylogeographic hypothesis showing the presence of several well-supported clades distributed in Anatolia, Central Europe, Eastern Europe, Western Europe, the Balkans and the Iberian Peninsula and cryptic diversity within Anatolia and the Balkans. Moreover, dating analysis

confirmed the diversification of the species during the Pleistocene, as well as the role of the Southern Balkans and Anatolia region as refugia during this period.

KEYWORDS

Black Sea region, Colubridae, *Coronella*, Mediterranean, nuclear markers, refugia

1 | INTRODUCTION

The Mediterranean region is undoubtedly considered as a natural setting for evolutionary and ecological studies and has been included in the 25 most important biodiversity hot spots in the world (Myers et al., 2000). In the Eastern Mediterranean basin and the Black Sea region, three areas had a key role in the evolutionary history of reptiles: the Balkan Peninsula, Anatolia and the Caucasus region. Throughout the last several million years, all regions were exposed to complex geomorphological and topographical changes, which resulted in the formation of huge mountainous ranges a well-known barrier for small vertebrates (Hewitt, 2011; Tarkhnishvili, 2014). Such barriers, together with habitat changes, were probably important for the Miocene/Pliocene speciation events and for forming intraspecific genetic variability during the Pleistocene glacial cycles. Both of these factors were essential for the currently observed species and the genetic diversity of the Eastern Mediterranean biota (Poulakakis et al., 2015).

The European smooth snake, *Coronella austriaca* Laurenti 1768 (Colubridae), is a small, non-venomous snake with a large geographic range (Speybroeck et al., 2016; Strijbosch, 1997). At the west-east axis, it's distribution extends from the Iberian Peninsula to the Ural Mountains and northwestern Kazakhstan (Arnold et al., 1978; Sindaco et al., 2013) and reaches the eastern parts of Western Palearctic, such as Anatolia and Iranian Mountain ranges (Jablonski et al., 2019). At the south-north axis, it extends from Mediterranean peninsulas to Åland island, between Sweden and Finland (60°N, 20°E), where the northernmost population of the smooth snake is found (Galarza et al., 2015; Leigh & Bryant, 2015). The only big Mediterranean island the species is found is in Sicily (Engelmann, 1993; Sillero et al., 2014; Strijbosch, 1997). Despite its extended distribution throughout the Western Palearctic, only two subspecies are currently recognized; *Coronella austriaca austriaca* from most of the range (type locality Vienna, Austria) and *Coronella austriaca acutirostris*, Malkmus, 1995 in the northwestern part of the Iberian Peninsula (type locality: Serra da Estrela, northern Portugal). A third morphological subspecies, *C. a. fitzingeri* (Bonaparte, 1840), was recognized from southern Italy and Sicily due to its smaller size and less dorsal patterns (Tortonese & Lanza, 1968), but today, it is usually synonymized with *C. a. austriaca* (Razzetti et al., 2006; Speybroeck et al., 2016).

Currently, only a few molecular studies involving the geographically restricted interest have been carried out for *C. austriaca* (e.g. United Kingdom (Bond et al., 2005; Pernetta et al., 2011), Finland, the northern end of its distribution (Galarza et al., 2015), Iberian Peninsula (Llorente et al., 2012; Santos et al., 2008) and Poland (Sztencel-Jabłonka et al., 2015)), showing the existence of three main colonization routes across Europe arising from the south with an increased diversity in the Iberian Peninsula with three mitochondrial DNA lineages that may have originated during the Messinian Salinity Crisis (MSC; ~5.59–5.33 Mya) (Krijgsman et al., 1999).

A single study (Jablonski et al., 2019) has attempted to describe the phylogenetic relationships and phylogeography differences among the populations of the smooth snake across the whole region of its distribution. The authors focused on the southern and eastern parts of the species distribution based on a single mitochondrial gene (cytochrome *b*; cyt *b*) and revealed the existence of 14 distinct mitochondrial clades with unclear mutual relationships, characterized by complex genetic structure and relatively deep divergences. Most of these clades are distributed in the southern (Anatolia, Balkans, Iberian Peninsula and Sicily) and eastern parts (Caucasus and Iran) of the species habital range. Similar phylogeographic patterns are also observed in molecular studies performed using several European snake species with a wide range as *Natrix* spp. (Kindler et al., 2013, 2017; Kindler, de Pous, et al., 2018; Kindler, Graciá, et al., 2018) *Zamenis* spp. (Salvi et al., 2018) and *Hierophis* spp. (Mezzasalma et al., 2018; Rato et al., 2009).

To date, the investigation of the phylogeny and phylogeography of the *C. austriaca* rely solely on mitochondrial DNA (mtDNA) has identified different lineages and possible refugial areas across the distribution of *C. austriaca*, and suggesting that the Eastern Mediterranean region has served as a shelter for many reptile species during the Pliocene and Pleistocene periods. Here, we further investigate the phylogeographic history of *C. austriaca* based on multiple gene analysis approach. Our study combines

novel information, with published mitochondrial sequences from multiple regions of the Western Palearctic. Using both mtDNA markers and nuclear DNA (nDNA), we aimed to resolve the phylogenetic relationships among the different populations of *C. austriaca* mostly from the Balkans and Anatolia and provide an updated phylogeographic scenario to explain its current distribution and intraspecific diversity.

2 | MATERIAL & METHODS

2.1 | Sampling, DNA extraction

Total genomic DNA was isolated from 166 samples of *C. austriaca*, emphasizing on the unstudied areas of the Eastern Mediterranean region (Balkans, Anatolia) combined with specimens from Caucasus, Italy and central Europe (Table S1). Blood, buccal swabs and muscle were used as a DNA source preserved in absolute ethanol. For DNA extraction, we used the ammonium acetate protocol (Bruford et al., 1998). In addition, six specimens were extracted and sequenced from four related snake species from the family of Colubridae (*Coronella girondica* (Daudin, 1803), *Hierophis gemonensis* (Laurenti, 1768), *Elaphe quatuorlineata* (Lacapede, 1789) and *Zamenis situla* (Linnaeus, 1758)) and were used as outgroups.

To cover as much as possible of the distribution area of the species, we also used 115 available mitochondrial sequences from GenBank. Details for each specimen (geographic origin, voucher number, accession numbers) are given in Tables S1 and S2.

Double-stranded PCR was performed to amplify partial sequences of eight gene fragments; two mitochondrial [the cytochrome *b* (cyt *b*) and the large subunit of ribosomal RNA (16S rRNA)] and six nuclear (prolactin receptor [PRLR], natural killer tumour recognition [NKTR], brain-derived neurotrophic factor [BDNF], recombination-activating gene 1 [RAG1], recombination-activating gene 2 [RAG2] and melanocortin receptor 1 [MC1R]). The primers used and the PCR conditions are shown in Tables S3 and S4. Double-stranded DNA sequencing was conducted in automated sequencer ABI3730XL in CeMIA Company using the Big-Dye Terminator v.3.1 Cycle Sequencing kit®, following the manufacturer's protocol and using the same primers as in the PCRs. For the mtDNA genes, all specimens were targeted. For the nuclear gene fragments, at least four representatives from each major clade revealed in mtDNA phylogenetic tree (see below) were chosen. In the cases where less specimens formed a major clade in mtDNA tree, we analysed all of the samples.

2.2 | Alignment, genetic distances, substitution model

Sequences were edited in Codon Code Aligner (v. 3.7.1; Codon Code Corporation®), and the homology of the targeted loci was verified with BLAST algorithm. Alignment for each gene fragment was performed using the ClustalX v. 2.0 program (Larkin et al., 2007) in MEGA v. 6 (Tamura et al., 2013) and corrected by eye. Sequences of code genes were translated into proteins prior to further analysis to ensure the absence of stop codons. PHASE v. 2.1.1 (Stephens et al., 2001) was used for nuclear gene fragments as it is implemented in DnaSP v. 5.10.01 (Librado and Rozas, 2009) prior to alignment, to statistically infer the allelic sequences. Uncorrected pairwise genetic distances (*p*-distance) among specimens and the major phylogenetic clades were estimated for each gene separately using MEGA.

The selection of the best partition scheme and the most appropriate nucleotide substitution model for each scheme were carried out indepently for each type of genome (mitochondrial and nuclear loci) and for each phylogenetic analysis (Maximum likelihood [ML], Bayesian Inference [BI] in MrBayes, and BI BEAST) using PartitionFinder2 v. 2.1 (Lanfear et al., 2017). The mtDNA alignment was subdivided into four pre-defined blocks; three of them corresponding to each codon position for cyt *b* and the fourth to 16S rRNA, as it is a non-coding genetic locus. The nuclear loci were subdivided in 18 pre-defined blocks corresponding to the 1st, 2nd and 3rd codon positions for the genes PRLR, NKTR, BDNF, RAG1, RAG2 and MC1R. Data blocks had linked branch lengths and the model selection was based on the Bayesian Information Criterion (BIC) (Sullivan & Joyce, 2005), ignoring the evolutionary models that contain both gamma distribution and invariable sites (Yang, 2006). The optimal combination of block sequences was performed with the 'greedy' algorithm.

2.3 | Phylogenetic analyses & data sets

To investigate the phylogenetic relationships between the different populations of *C. austriaca*, four data sets were created as follows: the first (cyt *b* data set) includes the cyt *b* sequences from our study plus the available sequences from Genbank (Figure 1). The second (mtDNA data set) includes our mitochondrial sequences (16S rRNA and cyt *b* fragments) plus the available sequences from Genbank for both genes (Figure S6). The third data set (nDNA data set) includes the sequences of nuclear genes and the fourth (concatenated DNA data set) includes the concatenated sequences from the two

FIGURE 1 The map shows the correspondence of the clades (colours) of the cyt *b* data set with the geographical location of the samples of which they consist
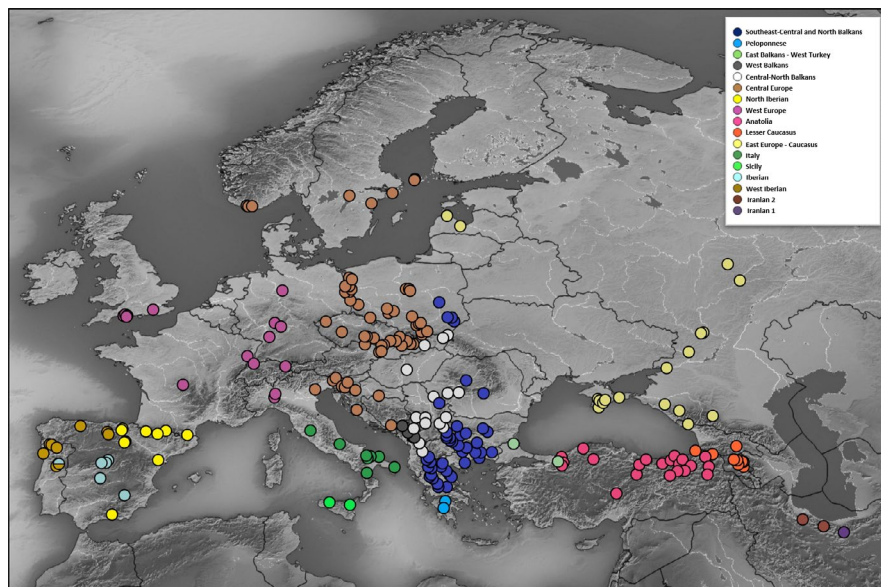
mitochondrial and the six nuclear gene fragments. Both ML (Felsenstein, 1981) and BI (Rannala & Yang, 1996) were applied for each data set.

ML analysis was performed with RAxML GUI v. 1.5 (Silvestro & Michalak, 2011; Stamatakis, 2006). Thorough bootstrap selection with 50 runs and 1000 replications was used for the statistical support of the tree. BI analysis was performed with MrBayes v. 3.2.7 (Huelsenbeck & Rannala, 2004) with four runs and five independent chains (MCMC chains) for each run. The number of generations was set at 10^7 in each chain with one tree stored every 100 generations, producing a total of 10^5 trees, whereas 25% of these were rejected (burnin' phase), as this is the period until the probability value (−lnL) is stabilized. The majority rule consensus tree was derived from the ex-post distribution of the optimal trees produced, while the posterior probabilities were calculated as the percentage of samples (trees) for statistical topology support that led to a specific topology. Values above 95% indicate statistical significance. The convergence of different runs and the Stability of Likelihood (−lnL) tests were performed on the basis of all diagnostic tests suggested by the MrBayes program manual. The average standard deviation of the frequency of each branch (ASDF) and the average value of the PSRF (Potential Scale Reduction Factor) of the different parameters were first examined. In addition, using the Tracer software: MCMC Trace Analysis Tool Version v 1.7.1 (Rambaut et al., 2014), we examined the graphs of probability values (ordinal: −lnL) of the number of generations (aborted) and active runs, sample size (ESS) for each parameter and execution. In cases where no convergence was observed between the different runs and/or probability non-stabilization, more generations were added.

2.4 | Species tree and divergence time estimation

For the species tree analysis and estimation of the divergence times, we used the * BEAST package of BEAST2 v. 2.4.7 (Bouckaert et al., 2014), which is based on coalescent theory (Rannala & Yang, 2003) as a prior knowledge of how different gene trees converged in the past, taking into account both the active population size (present and ancestral) and the differentiation times of branches (Heled & Drummond, 2010). The required import file (xml) was created using the BEAUti program (v. 2.4.7). Nucleotide substitution models were given a priori based on PF analysis. The Relaxed Clock Log Normal was used to estimate the molecular clock, the Multi Species Coalescent model was defined as the Birth-Death Process and the Piecewise Linear and constant root as the population size model. The analysis was performed three times for 10^8 generations, sampling every 5000 generations. All *BEAST runs were performed on CIPRES Science Gateway v. 3.3 (available at: http://www.phylo.org/). The results from the different runs were combined with the LogCombiner v 2.4.7, which is included in the BEAST2, eliminating 10% of the tree files as burnin. Convergence was evaluated with Tracer. Species trees with the splitting times and 95% confidence level was estimated in TreeAnnotator v. 2.4.7, which is included in the BEAST2. The tree was visualized with FigTree v. 1.3.1 (Rambaut, 2006–2009).

The analysis was performed in concatenated DNA data set, in which the sequences were assigned into potentially different evolutionary entities based on the distinct major phylogenetic groups that emerged from the concatenated gene trees of ML and BI analyses. For the estimation of divergence times, three sets of

external calibration age constraints were used based on Burbrink and Lawson (2007) and Salvi et al. (2018). The first set of calibration points (C.p.1) were the mean time of differentiation between *Z. situla* and *H. gemonensis* (39.36 Mya), the differentiation between *C. austriaca* and *Z. situla* species (27.38 Mya) and the split between *Z. situla* and *E. quatuorlineata* at 28.00 Mya (Burbrink & Lawson, 2007). The second and the third sets of calibration points (C.p. 2 and C.p. 3) were based on Salvi et al. (2018), who used two different mutation rates for the calibration of their trees. Based on this, the C.p. 2 and C.p. 3 were the differentiation of *Coronella* species at 5.66 and 10.11 mya, respectively, the splitting of *Zamenis*, *Coronella* and *Hierophis* at 17.28 and 30.61 Mya, respectively, and the splitting between *Coronella* and *Zamenis* at 9.04 and 18.20 Mya, respectively. In all cases, σ (Sigma - standard deviation) was set to 1.

2.5 | Reconstruction of the ancestral geographical distribution of potential phylogenetic groups of *C. austriaca*

The ancestral range was reconstructed using Lagrange (Dispersal-Extinction-Cladogenesis, DEC model) implemented in the program RASP 3.2 (Yu et al., 2014). The concatenated gene tree of all genes which derived from BI analysis was used as the input tree for DEC analysis. The distribution of the different phylogenetic groups as they emerged from the BI analysis, divided into 10 broad areas, based on geographic and phylogenetic criteria. These areas were the Southern Balkans (Area A) with Greece, Bulgaria, North Macedonia and Albania, Central-North Balkans (Area B) with the rest Balkan regions, Central Europe (Area C) with Hungary, Czech, Slovenia and Slovakia, North Europe (Area D) with Sweden, Anatolia (Area E) with Turkey, Lesser Caucasus (Area F) with Armenia, East Europe-Caucasus (Area G) with Caucasus and Crimea, Italian peninsula (Area H) with Italy and Sicily and the Iberian Peninsula with Spain (Area I) and Portugal (Area J). Furthermore, concerning the large distance between the areas, different dispersal constraints were set between them. Regarding the divergence time estimations, three time slices were set corresponding to the pre- and post-last glacial period (Dahl-Jensen et al., 2013; Rasmussen et al., 2006). The three time slices were defined as: 0–0.012 Mya, 0.012–0.115 Mya and 0.115–1.13 Mya.

2.6 | Haplotype network reconstruction

Haplotype networks are important tools to visualize intraspecific evolution and make inferences about

biogeography and history of populations (Leigh & Bryant, 2015). Here, we used Median Joining Network (MJN) method (Bandelt et al., 1999) for each gene fragment. MJN method is best suited for closely related sequences and mitochondrial sequences (Huson & Scornavacca, 2011). For nuclear gene fragments, we also applied MJN method because of the low-divergence data sets. Different traits were used in each data set according to the available sequences for each gene fragment. The traits were determined based on the phylogenetic clades that emerged from the different phylogenetic trees, where each trait represents one or more clusters of sequences which are related phylogenetically. The different traits are symbolized with different colours in the output (Figure 6, Figures S4 and S5).

3 | RESULTS

Regarding the cyt *b* data set, a total of 1086 base pairs (bp) were aligned, of which 301 were variable (~28%) and 220 parsimony informative (~20%) (336 and 256, respectively, when the outgroups included). For the mtDNA data set, a total of 780 bp were aligned (407 bp for 16S rRNA and 373 bp for cyt *b*), 163 (~21%) and 120 of which were variable and parsimony informative, respectively (228 and 182 when the outgroups included). For the nuDNA data set, a total of 3302 bp were aligned (624 bp for BDNF, 550 bp for PRLR, 336 bp for Rag2, 587 bp for MC1R, 743 bp for Rag1 and 462 bp for NKTR), 133 sites (~4%) and 31 (<1%) of which were variable and parsimony informative, respectively (182 and 39 when the outgroups included).

The mean genetic distances (*p*-distances) among the major phylogenetic groups ranged from 0.8% to 3.3% for the 16S rRNA gene and from 1.8% to 9.5% for the cyt *b* (Table 1). Concerning nuDNA loci, the mean genetic distances for the ingroup sequences were below 1% (see Table 2).

The best-fit partitioning scheme for each downstream analysis and the selected nucleotide substitution models are given in Table S6.

## 3.1 | Mitochondrial and concatenated trees

The phylogenetic analyses (ML and BI) of the cyt *b* and mtDNA data set produced trees with similar topologies. ML and Bayesian inference analyses resulted in topologies with lnL = −4072 and −4222, respectively. For the data set of nuclear gene fragments (nuDNA data set), the analyses revealed topologies with lnL = −5842 and −5871,

TABLE 1 The cyt *b* genetic *p*-distances % between and within (inside the brackets) the main clades of *Coronella austriaca*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
SE-Cental & North Balkans	(0.3)														
C-N Balkans	2.1	(0.4)													
West Balkans	2.2	2.3	(0.4)												
E. Europe	4.5	4.6	4.2	(0.9)											
Peloponesse	2.2	2.3	1.8	4.3	(0.4)										
E. Balkan-W. Turkey	4.4	4.0	3.5	5.1	3.5	(0.0)									
C. Europe	5.4	6.2	5.9	5.8	6.2	7.8	(0.7)								
Anatolia	4.0	3.7	4.1	5.2	3.6	4.8	7.0	(2.0)							
Italy	6.4	6.1	5.5	7.1	6.0	6.7	9.1	6.6	(0.7)						
Lesser Caucasus	4.9	4.7	4.8	3.9	4.4	5.7	6.3	5.2	7.1	(2.4)					
Sicily	5.2	4.6	4.6	6.2	5.1	6.4	8.7	6.4	6.9	6.1	(0.6)				
West Iberian	4.3	4.2	5.0	5.7	4.8	6.1	8.2	5.7	6.7	5.7	4.4	(0.5)			
Iberian	6.9	6.3	6.1	7.6	6.4	7.1	9.5	6.7	7.5	7.5	6.7	6.5	(1.9)		
N. Iberian	5.6	5.1	5.8	6.9	5.8	7.1	9.3	6.2	7.3	6.7	5.1	5.1	7.4	(1.0)	
W. Europe	5.0	4.4	4.5	6.0	5.1	6.5	7.8	5.6	5.2	6.6	5.3	5.5	7.3	6.8	(0.1)
Coronella girondica	11.4	11.8	12.0	12.7	12.7	14.0	13.2	12.2	14.1	12.1	12.1	10.8	13.1	12.2	11.5

TABLE 2 The concatenated nuclear DNA fragments genetic *p*-distances % between and within (inside the brackets) the main clades of *Coronella austriaca*

SE-Central & North Balkans	(0.3)										
Centra-North Balkans	0.2	(0.1)									
West balkans	0.2	0.1	(0.0)								
East Europe-Caucasus	0.4	0.3	0.2	(0.7)							
Peloponnese	0.2	0.1	0.1	0.5	(0.1)						
Central Europe	0.2	0.2	0.1	0.3	0.1	(0.1)					
Anatolia	0.2	0.1	0.1	0.3	0.1	0.2	(0.1)				
Italy	0.2	0.1	0.1	0.4	0.2	0.1	0.1	(0.2)			
Lesser Caucasus	0.2	0.1	0.1	0.4	0.2	0.2	0.1	0.2	(0.1)		
Sicily-N. Iberian	0.3	0.2	0.2	0.6	0.3	0.2	0.2	0.2	0.3	(0.3)	
West Iberian	0.3	0.2	0.1	0.2	0.2	0.3	0.1	0.0	0.2	0.2	(0.1)
Coronella girondica	0.3	0.3	0.3	0.6	0.3	0.3	0.3	0.4	0.3	0.5	0.3
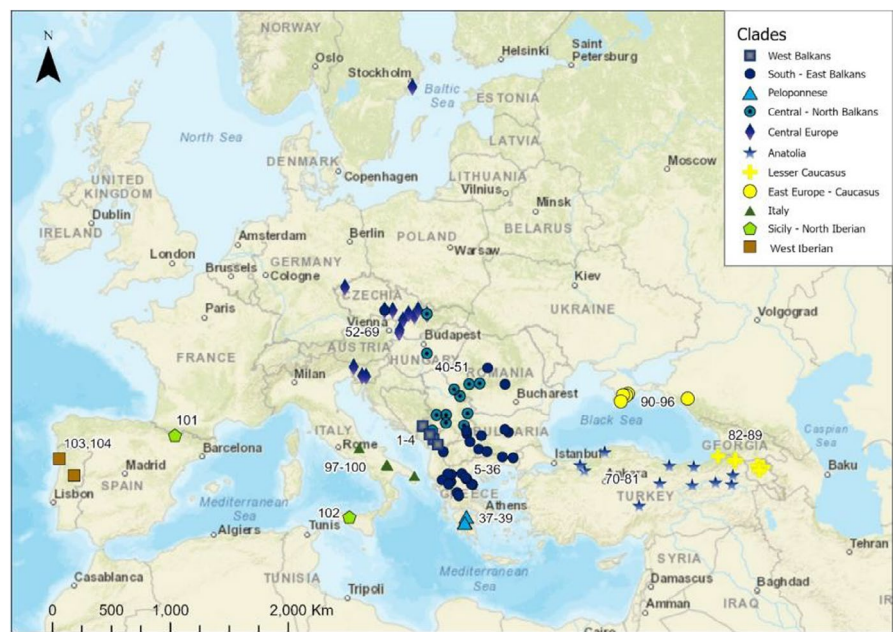
FIGURE 2 The map shows the correspondence of the clades (symbols) and the phylogenetic groups (colours) of concatenated DNA data set with the geographical location of the samples of which they consist. The numbers correspond the clustering based on the MapID of the specimens (Table S8)

respectively. For the concatenated data set, both analyses produced also similar topologies with lnL = −10307 and −9554, respectively.

For BI analysis, all MCMC diagnostic metrics indicated that the iterations of BI analysis reached convergence and stationarity. The average standard deviation of split frequencies (when this value approaches zero, the tree samples are more similar) was 0.006 for the mtDNA data set, 0.002 for the data set of nuclear genes, and 0.01 for the concatenated data set (mitochondrial and nuclear genes), the plot of generation versus log-likelihood of the data had characteristic 'white-noise' morphology after burn-in. In addition, for all parameters, the Potential Scale Reduction Factor (PSRF) values were 1.000 in both data sets and the

minimum Estimated Sample Sizes (ESS) values were well over 200 for all parameters in both data sets.

For the cyt *b* and mtDNA data set, both the ML and BI analysis showed almost the same topology and revealed the existence of six major phylogenetic groups (Figures 2 and 3). The most geographically extended is the 'Balkan, Central Europe and Anatolia' group from the Balkans, Turkey and localities in proximity to the Balkans, which subdivided into seven major clades (Central Europe, Central-North Balkans, Southeast-Central & North Balkans, Peloponnese [South Balkans], East Balkans, East Balkan-West Turkey and Anatolia [Anatolia 1 & 2 in mtDNA phylogenetic tree]). Five of them are from the Balkans, one is from Central Europe and the last (two

FIGURE 3 The BI tree of cytochrome *b*. Posterior probabilities (pp) and bootstraps support values (bp) are shown by the numbers left to the nodes as pp/bsv. The symbol '–' indicates that there is no significant support for this node (<0.95 posterior probability, <60 bootstraps). In parethenthesis the name of the clades in the corresponding tree of Jablonski et al., (2019). The clades "Iberian" "East Balkan-West Turkey" are not existing in the Bayesian Inference phylogenetic tree of concatenated DNA data set
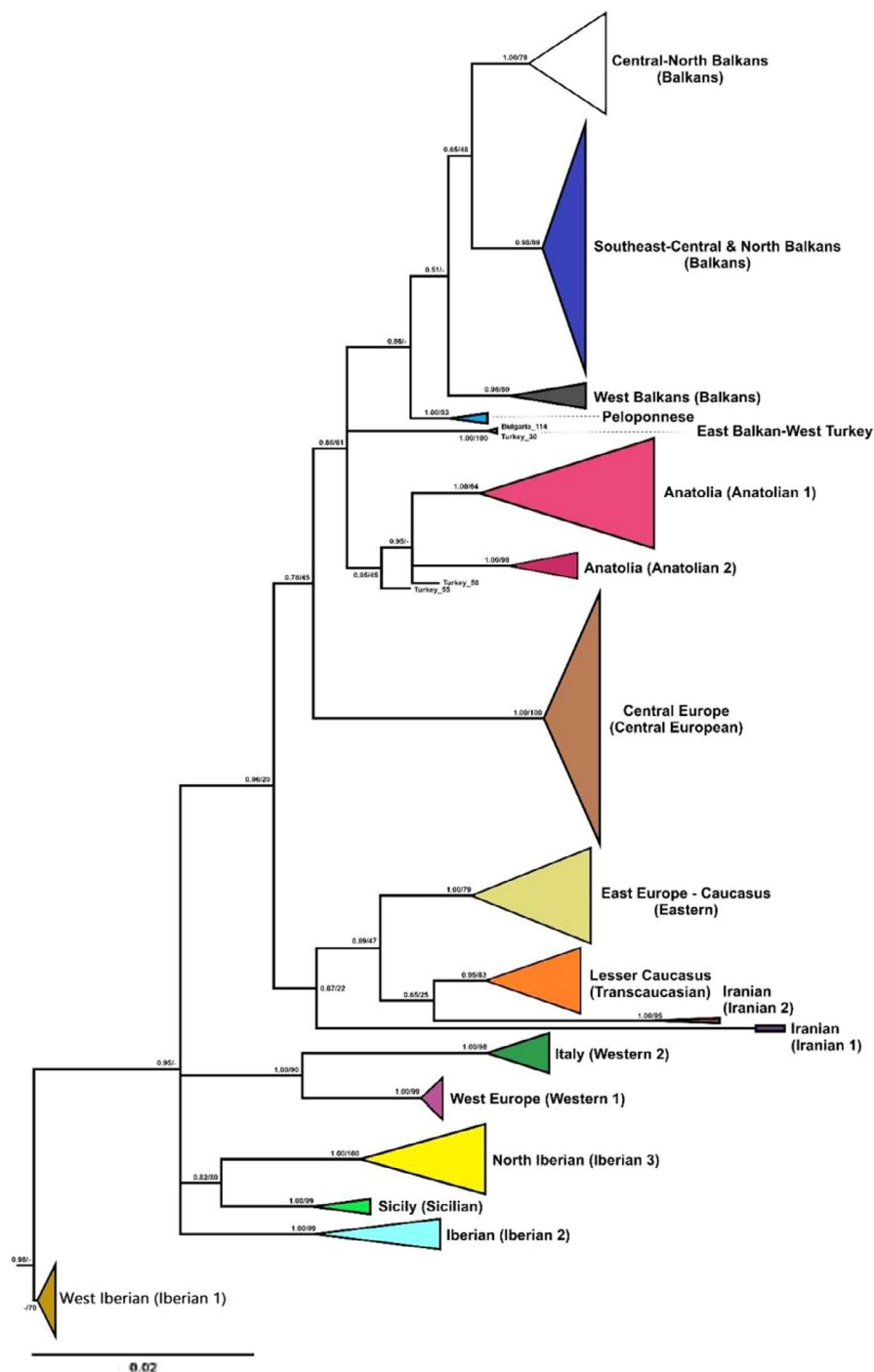
in mtDNA phylogenetic tree) from Turkey. The second phylogenetic group 'Caucasus group' consists of specimens from Armenia, eastern Anatolia, Russia, Georgia, Crimea and Iran (sequences from Iran are included in cyt *b* data set only) and is divided into five main clades, geographically located around the Lesser Caucasus region (Armenia, eastern Anatolia), north of Caucasus (Crimea, Georgia, Russia) and Iran. The third phylogenetic group ('Italy') consists of two clades, one with the specimens from Italy (excluding Sicily) and the other with the specimens from Western Europe (France, Austria, UK). The

last three phylogenetic groups are the group of 'West Iberian' with specimens mainly from Portugal that belong to *C. a. acutirostris*, which coincides with the Clade I of Santos et al. (2008), the 'Iberian' group with specimens from Spain which coincides with the Clade II of Santos et al. (2008), and the 'North Iberian-Sicily' phylogenetic group, which further subdivided into two main clades, one from north Iberia and the other from Sicily. Both cyt *b* and mtDNA phylogenetic trees reveal that the specimens from Sicily are more related to those from North Iberia, but only in the mtDNA tree this relation is statistically

significant. Regarding the specimens from West Iberian, these are separated from the other phylogenetic groups, but the phylogenetic relationships among them remain unclear. In terms of the statistical support in both analyses, the clades within the different phylogenetic groups are well supported, in contrast with the statistical support for the corresponding relationship between the different phylogenetic groups which is not significant.

For the concatenated DNA data set, ML and BI analyses produced trees with similar topology, which is similar to the topology of mitochondrial gene trees (Figure 4). The main difference among the trees of mtDNA-cyt *b* and the trees produced by concatenated data set is the sister group relationship of the two separate clades from Anatolia in the concatenated tree. As in the case of mtDNA tree, the phylogenetic relationships between the major groups and

within the group from Balkans, Turkey and localities in proximity to Balkans remained unsolved. Almost all specimens from Turkey, except for those located near the Turkey–Greece and Turkey–Georgia–Armenia borders, form a clade that consists of two subclades and at least two different lineages that extend along the Anatolia in an East-West direction, recovering a clear population structure. In contrast, the clade formed by the specimens from Central Europe has no structure. Also, in the concatenated data set, it has to be noticed that the 'East Balkans-West Turkey' and 'Iberian' clades are absent due to the lack of nuclear data for these specimens.

Concerning the nuDNA data set, *C. austriaca* and *C. girondica* form a bush-like monophyletic group in both type of analyses, in which *C. girondica* seems to be also a member (Figure S2).
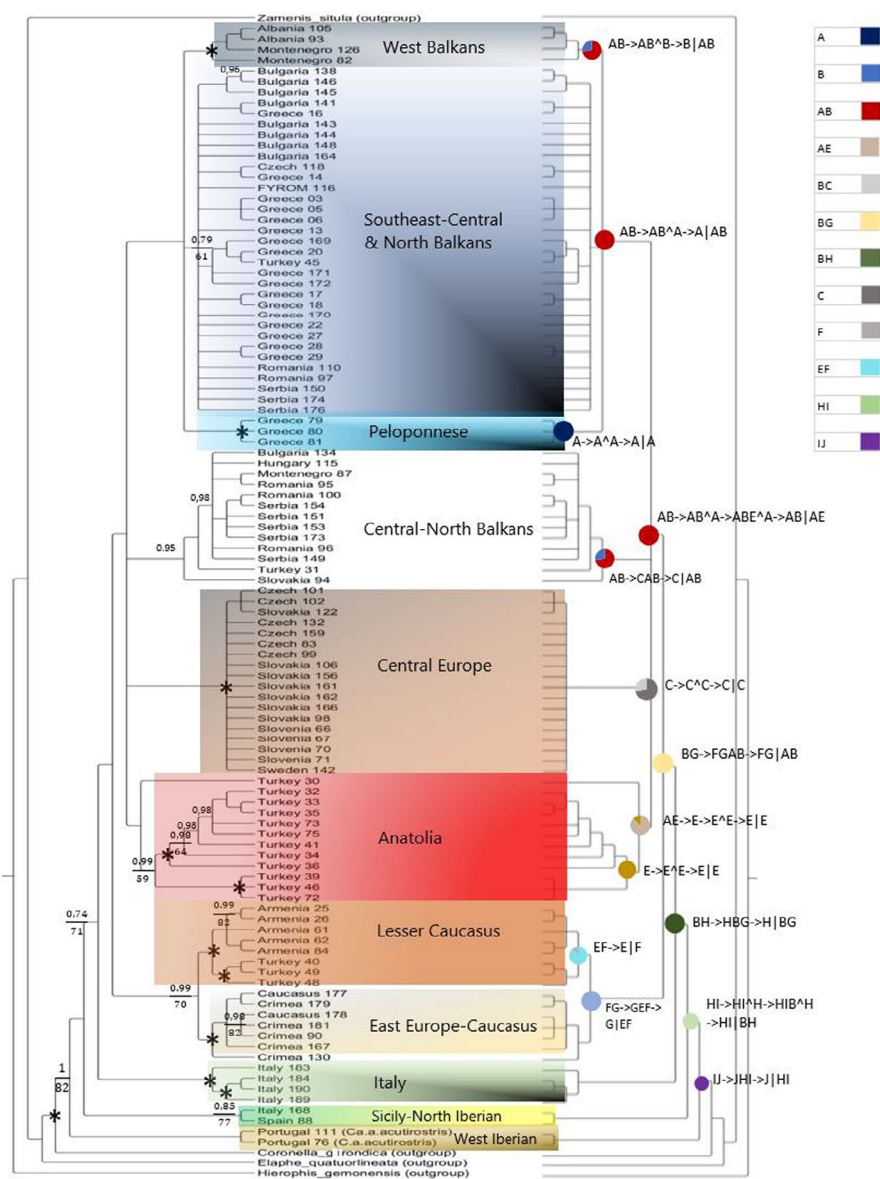
FIGURE 4 The Bayesian Inference phylogenetic tree of all genes (concatenated DNA data set). Posterior probabilities (pp) >0.7 and bootstraps support values (bsv) >55 are shown by the numbers above the branches as pp/bsv. The asterisk indicates significant statistical support (pp >0.95 and bsv >85). Pie diagrams show the probability of ancestral area combinations as they emerge from the LAGRANGE analysis and the most probable event route is given beside each one of these nodes. The symbol "^" indicates an event of migration while the vertical bar (|) indicates a vicariant event

3.2 | Species tree and divergence time estimation

The species trees analyses based on the three different sets of calibrations points revealed similar topologies (Figure 5), in which all the phylogenetic groups of concatenated gene tree were obtained. These phylogenetic groups are the 'Balkan, Central Europe and Anatolia', the 'Caucasus', the 'Italy', the 'N. Iberian-Sicily' and the 'West Iberian'. Although *C. austriaca* appeared as a monophyletic species (p.p. 1.00), the phylogenetic relationships of those phylogenetic groups remained unresolved as in the case of gene trees analyses. Considering the time of divergence of *C. austriaca*, its differentiation was estimated in Middle Pleistocene (1.13–0.95 Mya).

Based on the three sets of calibration points (C.p. 1, 2, and 3), the divergence time between *C. austriaca* and *C. girondica* is estimated at 3.99, 4.68 and 10.23 Mya, respectively. Within *C. austriaca*, the 'West Iberian' group (*C. a. acutirostris*) is branched off first at Early Pleistocene (1.13, 0.97 and 0.95 Mya, respectively). For the rest four groups, the phylogenetic relationships could be considered as unresolved (p.p. < 0.95) with the time of their differentiation to be placed in Middle Pleistocene (0.66, 0.61 and 0.45 Mya, respectively). Especialy for the different groups in Eastern Mediterranean and Anatolia region, the chronophylogenetic analysis showed that the separation between them took place during the Late Pleistocene. The statistical support for the exact relations between them is also not significant due to the short time of the disintegration of individual populations (Figure 5).

3.3 | Reconstruction of the ancestral geographical distribution of potential phylogenetic groups of *C. austriaca* and haplotype network reconstruction

According to the biogeographic reconstruction, the common ancestor of *C. austriaca* has been in Iberian Peninsula. The reconstruction revealed that *C. austriaca* reached Anatolia from the west through the Balkan Peninsula and from the east through the Caucasus region. Finally, Scandinavia has been reached through Central Europe colonization route. The results of the DEC analysis are presented in Figure 4.

Regarding the Median Joining Network analysis, the two mitochondrial markers had a clear structure in contrast with the nuclear genes' networks where the majority of the sequences created a cluster (Figures S4 and S5). The cyt *b* network is characterized with high numbers of nodes to represent inferred (unsampled) sequences, especially between the haplotypes of different phylogenetic

groups and high number of mutational steps between them (Figure 6).

4 | DISCUSSION

To date, only very few studies have included sequences describing relationships among the smooth snake's populations of the Eastern Mediterranean in relation to the Central and Western European populations. These studies included one or two mitochondrial markers (Galarza et al., 2015; Jablonski et al., 2019; Sztencel-Jabłonka et al., 2015). One study (Jablonski et al., 2019) presented the whole-range phylogeographic hypothesis with 14 distict phylogenetic clades of *C. austriaca* based on cytochrome *b*. Our more comprehensive approach, in which both mitochondrial and nuclear DNA differentiation were combined under several chronological and phylogeographical analyses, revealed the Pleistocene divergence of *C. austriaca* and the presence of hidden intraspecific genetic diversity in the Balkans, Anatolia and Caucasus.

4.1 | Phylogenetic relationships of *Coronella austriaca*

In our research, the mitochondrial data revealed the existence of at least 14 major clades, which refer to five geographical areas and revealed hidden diversity in Anatolia, Balkans, Caucasus and Italy. This is in agreement with the recent study of Jablonski et al. (2019) that showed that there was a hidden diversity within *C. austriaca*, with the presence of 14 distinct clades in contrast to eight or 10 clades revealed in previous studies based on mitochondrial markers (Galarza et al., 2015; Santos et al., 2008; Sztencel-Jabłonka et al., 2015). The revealed phylogenetic relationships among the different clades of *C. austriaca* and their times of divergence provided strong evidence for the recent differentiation of populations of the species and the role of the Mediterranean peninsulas, Anatolia region and the Caucasus as refugia during the Pleistocene period. Our phylogenetic inference showed that the split between the main groups of the smooth snake (divergent Iranian population were not included; see Jablonski et al., 2019) occurred between Early Pleistocene (1.13–0.95 Mya depending the calibration points used) and the final of last glacial epoch (about 0.012 Mya). These results explain the low statistical support for the relevant phylogenetic positions of the different clades, as well as the absence of population structure inside the most recent clades. The recent history of *C. austriaca* is also supported by the fact that it is absent from islands, such as Sardinia, Corsica and
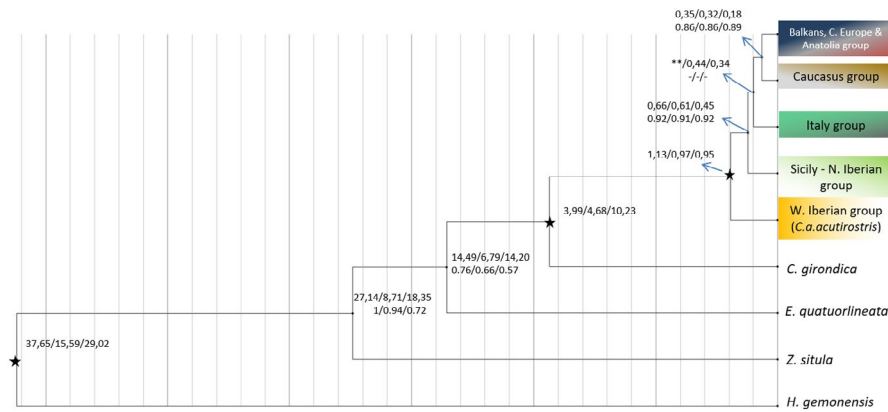
FIGURE 5 Species trees of *Coronella austriaca*. Above branches are the estimated times of divergence for each clade and below the branches the posterior probability for each one of the three different species tree analyses based on the three different sets of calibration points (C.p. l/C.p. 2/C.p.3). The star indicates that all the three analyses have posterior probability 1. Slash (-) at the posterior probability means that is below 0.5. **In the analysis based on the first set of calibration points (Cal 1), Italy group is most relative with the Sicily – N. Iberian group but the statistical support is not significant (0.42). More information provided in Figure S2
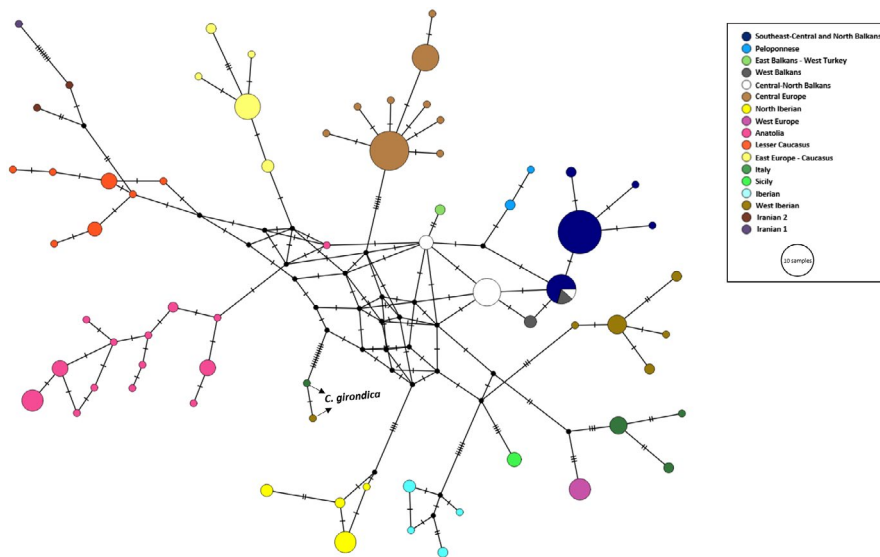
FIGURE 6 Median-joining network inferred from cyt *b* sequences of *Coronella austriaca*. Circle sizes are proportional to the number of sequences. Each colour represents a clade of cyt *b* data set. Black nodes represent inferred (unsampled) sequences by iteratively adding 'median' sequence vectors when these nodes shorten the total length of the network (Leigh & Bryant, 2015)

Crete, which they have not connected with continental areas since the end of Miocene (Advokaat et al., 2014; Dermitzakis, 1990). Similar distributional patterns, characterized by 'southern richness and northern purity' of genetic diversity and being formed during the Pleistocene, were also observed in other snake species. For example, the distribution ranges of *Hierophis* sp. (*H. gemonensis*, *Hierophis carbonarius* (Bonaparte, 1833) and *Hierophis viridiflavus* (Lacepede, 1789)) during the Pleistocene were fragmented into several, geographically restricted, suitable areas, which collectively represent distinct 'refugia within refugia' in both the Balkan and Italian peninsulas. These refugia might have played an important role during the Quaternary climatic oscillations in either inter- (especially between *H. carbonarius* and *H. viridiflavus*) and intraspecific differentiation

processes, shaping the current haplotype distribution of the European whipsnakes (Mezzasalma et al., 2018). In grass snakes (*Natrix natrix* (Linnaeus, 1758)), the highest mitochondrial diversity was found in the south of their range, where the putative glacial refugia were located (Kindler et al., 2013). In the last case, multiple microrefugia were located in Anatolia and the neighbouring Near and Middle East as well as in the Balkan peninsula (Kindler et al., 2013). The *cyt b* genetic distances between and within (0.28%–7.51% and 0%–0.62%, respectively) the main clades of *N. natrix* subspecies are similar with the observed distances in *C. austriaca*. Genetic diversity between *H. viridiflavus* and *H. gemonensis* was 2.4% (2%–3% between the main phylogenetic groups in *C. austriaca*) for *16S* and about 10% for *cyt b* (2%–7% between the main phylogenetic groups in *C. austriaca*).

4.2 | Phylogeographical pattern of *Coronella austriaca*

According to our chronophylogenetic analysis, the diversification of *C. austriaca* occurred in Pleistocene and afterwards, although the split between *C. austriaca* and *C. girondica* took place much earlier in Late Miocene and Early Pliocene. These are in agreement with previous published data (Santos et al. (2008), which suggested the presence of *C. austriaca* in the Iberian Peninsula since the Late Miocene. In addition, fossils from this area that belong to *Coronella* sp. dated back to Early Pliocene (Bailon, 1991), and phylogenetic evidence indicates diversification of the sister species *C. girondica* within the Iberian Peninsula during this period (Santos, Rato, Carranza, Carretero, de la Vega, et al., 2012; Santos, Rato, Carranza, Carretero, & Pleguezueloset al., 2012). Taking into consideration these results, it is possible that MSC in the western Mediterranean could be the main event that caused the separation between the two *Coronella* species. It is possible that *C. austriaca* expanded across the western Palearctic and reached the easternmost regions of its distribution during the Pliocene. Anyhow, records/remains of *Coronella* from the Pliocene and/or Early Pleistocene were indicated from northern Italy (*Coronella* sp.; Villa et al. (2018)), south-western Ukraine (*Coronella* aff. *austriaca*; Ratnikov (2009)), Hungary (*Coronella* cf. *austriaca*; Georgalis et al. (2019)), Slovakia, Poland, and Moldova (*C. austriaca*; (Holman, 1998; Ivanov, 2007; Ratnikov, 2002; Szyndlar, 1991)). However, its current distribution pattern is the result of the abrupt shifts in distribution ranges that were observed in Mediterranean peninsulas, Anatolia and Caucasus region during the Pleistocene, with multiple expansions and reductions.

4.3 | The Balkans

The Balkans have played a significant role as a biodiversity refugium during the Quaternary climatic fluctuations, rendering them as a significant center of post-glacial dispersal of species into central and northern Europe (Griffiths et al., 2004). In general, glacial refugia were highly important for the maintenance of biodiversity, especially for the species that shifted their ranges (Barbosa et al., 2017). The geographic, habitat and climatic heterogeneity within the glacial refugia have resulted in 'refugia within refugia' (Abellán and Svenning, 2014; Goméz and Lunt, 2007) that have often subdivided populations, leading to more complex biogeographical patterns. In the case of *C. austriaca*, there are four main clades according to the concatenated tree of all genes in the Balkans, the phylogenetic relationships of which are mainly unresolved. The genetic diversity

within and among these clades (4.8%–7.2% and 1%–2% *p*-distance between the clades for cyt *b* and 16S, respectively) and the time of their divergence indicate the very recent dispersal and colonization of those areas during the late Middle and the Upper Pleistocene from areas that probably played the role of local refugia. Although, the two out of the four Balkan clades have wide geographic distribution ('Southeast - Central & North Balkan' and 'Central - North Balkan' clades), the other two ('West Balkan' clade and 'Peloponnese' clade) are distributed in more restricted geographical regions, the West Balkans clade in Albania and Montenegro (with Hellenides and Dinarides as main mountain barriers) and the Peloponnese clade in southern Greece. During the Plio-Pleistocene period, the Peloponnese has been separated and re-connected with the mainland Greece, giving the space for the formation of local endemism and/or inner genetic diversity for a wide range of species, such as *Podarcis peloponnesiacus* (Bibron & Bory, 1833), *Anguis cephallonica* Werner, 1894, *Algyroides moreoticus* Bibron & Bory, 1833 and *Hellenolacerta graeca* (Bedriaga, 1886) which are distributed either only in Peloponnese or in Peloponnese and neighbouring Ionian Islands. Thus, the population from the peninsula suggests the existence of local genetic diversity within Greece and reveals the possible existence of an unknown Pleistocene refugia for *C. austriaca* in Peloponnese. The 'West Balkan' clade (Albania and Montenegro) is also phylogenetically distinct in all phylogenetic analyses, indicating the existence of another Balkan refugia, already mentioned by Jablonski et al. (2019). The south-western Balkans (Albania, Montenegro) probably also played important biogeographic role in the evolutionary history of other reptile species, such as *Anguis* (Jablonski et al., 2016), *Triturus* (Wielstra et al., 2013), *Vipera ursinii* (Bonaparte, 1835) (Mizsei et al., 2017; Zinenko et al., 2015), *Vipera ammodytes* (Linnaeus, 1758) (Ursenbacher et al., 2008), *Lacerta viridis* (Laurenti, 1768) (Marzahn et al., 2016), *Podarcis muralis* (Laurenti, 1768) (Salvi et al., 2018; Yang et al., 2022), *Dalmatolacerta oxycephala* (Schlegel, 1839), *Dinarolacerta mosorensis* (Kolombatović, 1886) (Podnar et al., 2014), *H. gemonensis* (Mezzasalma et al., 2015, 2018) and *Z. situla* (Linnaeus, 1758) (Salvi et al., 2018) during the Pliocene-Pleistocene ages.

Regarding the other two clades from Balkan Peninsula, the first ('Southeast - Central & North Balkans' clade) include all the specimens from mainland Greece and Bulgaria, the European part of Turkey and some specimens from Romania and Serbia, whereas the second ('Central - North Balkans' clade) consists of specimens from north-central and the east-central part of the Balkan peninsula (European Turkey, Romania, Serbia) and central Europe (Hungary, Slovakia) reflecting possible historical refugium in the Carpathian Basin.

Of particular note, cyt *b* gene tree revealed one more, small in size, clade with only two samples, one from western Turkey and the other from eastern Bulgaria. The monophyly of this clade provide insights into one more different lineage and a possible refuge in the easternmost point of the Balkans.

The current pattern of *C. austriaca* in the Balkans could be explained by the presence of several climatic refugia there during the last glacial periods and the complex geomorphology (biogeographic barriers) of the region, providing numerous environmental niches that preserved biodiversity during Pleistocene climate extremes (Griffiths et al., 2004). The mountain massifs, such as Hellenides in Albania, Kosovo, North Macedonia and Greece, Thrace-Macedonian Massife (Rhodopes) in Bulgaria and Greece, Dinarides in the western Balkans, were important barriers during the Pleistocene glacials, while the scattered valleys of the Balkans served as a shelter for many species (see eg. Jablonski et al. (2019)). The opposite process was taking place during the mid-glacial periods as *C. austriaca* tends to be a mountainous species in the south of its range (Arnold & Ovenden, 2002). The multiple events of range retractions and subsequent expansions have often caused long-term isolation, bottlenecks and extinction of marginal populations (Podnar et al., 2014). Based on the dispersal abilities of a species and its opportunities for post-glacial expansion to the north, a lineage could be characterized either as a 'post-glacial re-colonizer' (Hewitt, 1999, 2000), or as a 'refugial endemic' with limited expansion potential (Bilton et al., 1998; Kryštufek et al., 2007). In *C. austriaca*, both of these types are probably present in the Balkans, with 'Southeast - Central & North Balkans' and 'Central - North Balkans' clades being examples of a post-glacial colonizers and 'West Balkan' clade and 'Peloponnese' clade to be refugial endemic' with limited expansion potential. A similar pattern has been observed in other reptile taxa in the Balkans, such as *Podarcis tauricus* group (Psonis et al., 2018).

4.4 | Central and North Europe

The 'Central Europe clade' with populations from Austria, Croatia, Bosnia and Herzegovina, Czech Republic, Slovenia, Slovakia, Italy, Norway and Sweden (Figure 1) is sister clade to the Balkan clades. Previous research in *C. austriaca* based on mtDNA markers indicated that specimens from Poland grouped with specimens from Slovakia and Greece, supporting also the hypothesis that the colonization of Poland took place quite recently and rapidly (Sztencel-Jabłonka et al., 2015). In addition, Galarza et al. (2015) showed that the central lineage originating in the Balkans was the only lineage that reached Scandinavia. However, their sampling effort from the Balkans was low. Similar results have also arisen from Jablonski et al. (2019), where all the specimens from those countries as well as specimens from Croatia, Norway and Bosnia-Herzegovina formed a clade, which appeared as sister to the Balkan clades.

In our study, 'Central Europe' clade is characterized by shallow phylogenetic structure, confirming the previously published results (Jablonski et al., 2019). Based on those findings and the results of both chronological and biogeographic analyses, we can claim that central-east part of Central Europe and Scandinavia were colonized from the Balkan route after the last glacial period. The starting point of this lineage has to be a refuge place somewhere in the Carpathian Mountains, an arc extending from Central Europe to Eastern Europe, covering a distance of approximately 1500 km and ranking as the third longest mountain range in Europe. Its range extends through Romania, Ukraine, Poland, Hungary, Czech Republic, Slovakia and Serbia, the sequences of which are represented in the 'Central - North Balkans' and 'Central Europe' clades. An alternative starting point could be a refuge in Central Europe somewhere between the northern ice sheet and the Alpine glaciers of the last glaciation and most likely in a permanfrost region (Kindler, Graciá, et al., 2018). This pattern of colonization was also reported for other snakes, such as *Natrix* spp. (Kindler et al., 2013; Kindler, Graciá, et al., 2018) and *Zamenis longissimus* (Laurenti, 1768) (Musilová et al., 2010). The 'border line' between the Balkan and the western colonization routes of Europe seems to be a line which begins from Austrian Alps goes through Germany and reaches United Kingdom. The re-analysed cyt *b* data set, which includes also sequences from France, Austria, United Kingdom, Finland and Poland, revealed that the sequences from Austria are grouped with the sequences from United Kingdom and France, forming a sister clade to the 'Italy' clade, while the sequences from Poland, Czech, Slovakia and Finland clustered with the 'Central Europe' clade, which is phylogenetically closer to the Balkan clades. Regarding which clade of the Balkans gave birth to the 'Central Europe' clade, there is an uncertainty as none of the different analyses revealed a clear pattern. In addition, the statistical support in the concatenated trees is not significant, likely due to the short separation time.

4.5 | The Anatolian region

For many reptile species, Anatolia has repeatedly acted as either a barrier or a bridge between Asia and Europe (Arslan et al., 2018; Kornilios et al., 2011; Kurnaz, 2020; Sindaco et al., 2000). Owing to their complex orogeny,

Anatolian mountains have promoted a variety of climatic regions and vegetation types (Atalay, 2006; Kornilios et al., 2011), allowing both the long-term local survival of biota and repeated colonization from nearby areas (Ansell et al., 2011). During the Pleistocene, glacier development within Anatolia was limited to the higher mountain peaks, while the lowlands remained open, developing steppe communities (Ansell et al., 2011). For this reason, a 'refugia-within-refugia' pattern during the Pleistocene climatic oscillations has also been proposed for Anatolia, with several studies describing secondary contacts and hybridization events (Fritz et al., 2009; Guicking, Joger, & Wink, 2009; Kindler et al., 2013; Stöck et al., 2012; Vamberger et al., 2013). This high level of genetic diversity in Anatolia has also reported in several other organisms (Dubey et al., 2007; Korkmaz et al., 2014; Rokas et al., 2003) and reptile groups (Bellati et al., 2015; Kornilios et al., 2012, 2018, 2020; Rato et al., 2021; Sindaco et al., 2013; Tamar et al., 2016).

In the case of *C. austriaca*, the complex geology and geomorphology of Anatolia might have represented insurmountable barriers—especially in the Pontic region, shaping the genetic structure we observe today. Our phylogenetic analyses confirmed previous results (Jablonski et al., 2019) showing that Anatolia is characterized by significant diversity with at least two different clades. The first has a clear population structure in contrast to the populations of the Balkans and Central Europe. The lineages of the biggest clade that corresponds to this area ('Anatolia' clade) are extending along Turkey, creating a clear population structure from east to west. This clade shows up more related to the Balkan populations and form, all together, the Balkan phylogenetic group in both mitochondrial and concatenated trees, with the notice that the statistical support is significant only for the mt concatenated tree. The sequences from eastern Anatolia are related with Armenia sequences, formatting 'Lesser Caucasus' clade which is sister clade to 'East Europe-Caucasus' clade. These clades according to phylogenetic analyses and the two mitochondrial haplotype networks belong to a different phylogenetic group than 'Anatolia' clade. The time of differentiation of the populations of Anatolia is among the most recent among the different population groups. Nevertheless, the genetic structure is much more intense when compared to the Balkan clades, with many different lineages confirming the existence of 'refugia within refugia' pattern.

4.6 | The Caucasus region

Eastern Anatolia is the meeting point between the Balkan phylogenetic group (consisting of the Southeast-Central & North Balkans, Peloponnese, West Balkans, Central-North Balkans, East Balkans-West Turkey, Central Europe and Anatolia clades) and the 'Caucasus' phylogenetic group (Lesser Caucasus, East Europe-Caucasus clades) from the Caucasus region (Figure 2). According to BI and ML analysis for all data sets, two well-supported sister clades which are separated geographically from Caucasus were revealed. Specimens from eastern Turkey and Armenia form the first clade ('Lesser Caucasus' clade), while the other one is constituted with specimens from Crimea, Georgia, Russia and Latvia, covering a huge geographical region ('East Europe – Caucasus' clade). 'Lesser Caucasus' clade is one of the clades with the largest genetic diversity, formatting two well separated subclades with distinct geographic and genetic differentiation. The first subclade contains specimens from the eastern regions located in the highlands of Armenia and the other contains the specimens from Armenia. When Iranian sequences (Jablonski et al. (2019)) were included in the analysis (cyt *b* phylogenetic tree), the clade Iranian 2 (sensu Jablonski et al. (2019)), although with no statistical support, appeared as the most phylogenetically closer to 'Lesser Caucasus' clade. The remaining Iranian clade (1; sensu Jablonski et al. (2019)) have sister group relationship with whole East Europe & Caucasus group. This supporting the conclusion about the cryptic diversity even in the easternmost part of the distribution of the smooth snake, despite the fact that the statistical support for the relevant position of Iranian sequences is not significant. Considering the second sister clade, the unresolved relationships among the sequences from Latvia, Georgia, as well as the molecular dating results, which place the split of the 'Caucasus' group from the Balkan one at 18–35 Kya (Figure 5), suggest that the colonization of the eastern parts of *C. austriaca* distribution occurred very rapidly, starting from the Caucasus region. This is partially supported by the fact that fossil remains of *C. austriaca* dating of the Late Pleistocene age are relatively numerous in Crimea (Kovalchuk et al., 2020; Ratnikov, 2015; Vremir & Ridush, 2005) and also found in some sites of East European Plain (Ratnikov, 2009) and the northwestern Caucasus (Syromyatnikova et al., 2013, 2017). The geographic limits of this clade and the meeting point with the Balkan lineages at the north Europe remain unclear. More sampling effort is required, especially in the Eastern Europe and western Asia, for the clarification of the questions arose from the form of this clade. However, the complex genetic structure of the clades, which belong to the Eastern part of *C. austriaca* distribution, confirms the crucial role of Caucasus region for the recent expansion of the species (Tarkhnishvili, 2014).

4.7 | Western regions

For the clades from the western Europe ('Italy', 'West Europe', 'Iberian' [only in mtDNA trees], 'Sicily-North Iberian', 'West Iberian'), the phylogenetic status remains unchanged. Previously published data (Jablonski et al., 2019; Sztencel-Jabłonka et al., 2015) have shown that the specimens and populations from Western Europe form a group that includes all specimens of western origin, except the Iberian ones. This lineage (Western 1 clade in Jablonski et al. (2019)) probably originated from the Apennine and colonized a big part of Europe (Austria, France, Germany and United Kingdom). According to the topologies of both cyt *b* and the mt gene trees of the current study, Italian specimens (excluding Sicilian) form a separate clade which has sister group relationships to the 'West Europe' clade (Figure 2). The chronological analyses reveal that the separation of 'Italy group' occurred somewhere between 0.44 and 0.34 Mya, indicating that during the Pleistocene glacials, Apennines served as refugium for *C. austriaca*. Biogeographic analysis suggests an area between North Balkans and Italy (most probably Alps) as the birthplace of the Italian clade.

Of particular note, the 'Sicily' clade, which consists of specimens belonging to the morphological subspecies *Coronella austriaca fitzingeri* (that today is synonymized to *C. a. austriaca*), seems to be more closely related to North Iberian clade (Clade III in Santos et al. (2008)) (statistical support is significant only in ML trees) than to the Italian clade, a relationship that was also observed in a previous study (Santos et al. (2008). Interestingly, the chronophylogenetic analysis showed that the split occurred during the Pleistocene and not at the beginning of Pliocene as indicated in Santos et al. (2008). The question that arises is how *C. austriaca* colonized the island of Sicily, since there was no land connection between Iberian Peninsula and Sicily. A possible scenario could be that the relative population of Sicily emerged from a colonization event starting from Iberian Peninsula during the Pliocene and disappeared from continental Italy maybe due to competition or other ecological factors and failed to settle again the continental part after the formation of the Messina strait that occurred in Late Pleistocene (Di Stefano & Longhitano, 2009). Other studies have shown that other European reptiles inhabit only Sicily and the south of the continental part of Italian peninsula being excluded there as residual populations (*Zamenis lineatus* (Camerano, 1891) (Salvi et al., 2018), *Podarcis waglerianus* Gistel, 1868, (Salvi et al., 2021)), while the pattern of two separate colonization events of relative species, as well as the intraspecific variation during the Quaternary has also been observed for other reptiles (Poulakakis et al., 2005; Psonis et al., 2018). Our chronological analysis contradicts

this scenario due to the fact that the split between the 'Sicily-North Iberian' and Italian clade is much more recent (0.44–0.34 Mya). Alternatively, the most possible scenario according to our data is that this separate population maybe is a result of human-related transportation or a stepping stone event, but in both cases, more research is necessary. Nonetheless, the population of Sicily (named as *C. a. fitzingeri*) according to all studies on *C. austriaca* including ours, constitutes a distinct clade, associated more to the Iberian populations than the Italian ones, but more sampling effort from Italian peninsula and Sicily as well as ecological research is required for the resolving of the phylogeographic history of the Sicilian population of *C. austriaca*.

Regarding the 'West Europe' clade, according to the current and previous studies, it seems that there is a line starting from the Alpes, then passing through Austria and reaching Germany, which separates the populations of this clade from those of Central Europe and Balkan clades. The absence of any phylogenetic structure between specimens from Austria and United Kingdom supports the rapid colonization of Western Europe after the glacials of the Pleistocene, starting from the Alps, which also have been a barrier between the Apennine populations and the northern populations.

5 | CONCLUSION

In conclusion, the phylogenetic analyses included here revealed the existence of many phylogenetic clades and lineages throughout the distribution of *C. austriaca* and the important role of the region of Eastern Mediterranean as a refugium. The relatively low intraspecific diversity of *C. austriaca* and the recent evolutionary divergence among the populations indicate that this species has been significantly affected by the alternation of the Pleistocene events. The Quaternary Ice Age undoubtedly played an important role in Europe, primarily on the Balkans and the Caucasus, and secondarily on the Italian Peninsula. In addition, the 'refugia within refugia' pattern in Anatolia reveals new evidence of cryptic diversity within the species. However, more genetic/genomic data is needed to fully clarify the history of the smooth snake.

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SUPPORTING INFORMATION

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

1 Supplementary Material

2 **Table S1** List of samples used in phylogenetic analyzes of the present study. The following are
3 given in order: the code of the sample at the Museum of Natural History of Crete (NHMC), the
4 country of origin of the sample, the participation of each sample (√) in the three datasets
5 (Dataset 1, Dataset 2 & 3), used for phylogenetic analyses reported. The name of each sample
6 on the trees is a combination of the country of origin and the last 2 digits of the NHMC.

	NHMC	Origin	Dataset 1		Dataset 2 & 3							
			Mt gene tree, networks		All genes (gene tree, Species Tree & nuclear gene networks)							
			16S	Cytb	16S	Cytb	BDNF	RAG2	RAG1	MC1R	NKTR	PRLR
C. austriaca	80.3.27.104	Albania	√	√	√	√	√					
C. austriaca	80.3.27.105	Albania	√	√	√	√	√	√		√	√	√
C. austriaca	80.3.27.93	Albania	√	√	√	√	√					
C. austriaca	80.3.27.24	Armenia	√	√	√	√	√					
C. austriaca	80.3.27.25	Armenia	√	√	√	√	√	√			√	√
C. austriaca	80.3.27.26	Armenia	√	√	√	√	√					
C. austriaca	80.3.27.61	Armenia	√	√	√	√	√	√	√			
C. austriaca	80.3.27.62	Armenia	√	√	√	√	√	√	√	√	√	√
C. austriaca	80.3.27.84	Armenia	√	√	√	√	√					
C. austriaca	80.3.27.113	Bulgaria	√		√		√					
C. austriaca	80.3.27.114	Bulgaria	√	√								
C. austriaca	80.3.27.134	Bulgaria	√	√	√	√	√	√		√	√	√
C. austriaca	80.3.27.135	Bulgaria	√	√	√	√	√					
C. austriaca	80.3.27.136	Bulgaria	√		√		√					
C. austriaca	80.3.27.137	Bulgaria	√	√	√	√	√					
C. austriaca	80.3.27.138	Bulgaria	√	√	√	√	√					
C. austriaca	80.3.27.139	Bulgaria	√		√		√					
C. austriaca	80.3.27.141	Bulgaria	√	√	√	√	√	√	√		√	√
C. austriaca	80.3.27.143	Bulgaria	√	√	√	√	√	√	√	√	√	√
C. austriaca	80.3.27.144	Bulgaria	√		√		√		√			
C. austriaca	80.3.27.145	Bulgaria	√	√	√	√	√	√	√	√	√	√
C. austriaca	80.3.27.146	Bulgaria	√	√	√	√	√	√	√	√	√	√
C. austriaca	80.3.27.147	Bulgaria	√		√		√					
C. austriaca	80.3.27.148	Bulgaria	√		√		√					
C. austriaca	80.3.27.163	Bulgaria	√	√	√	√	√					
C. austriaca	80.3.27.164	Bulgaria	√	√	√	√	√	√	√	√	√	√
C. austriaca	80.3.27.130	Crimea	√		√		√					
C. austriaca	80.3.27.167	Crimea	√	√	√	√	√	√	√	√	√	√
C. austriaca	80.3.27.90	Crimea	√	√	√	√	√	√	√	√	√	
C. austriaca	80.3.27.101	Czech	√	√	√	√	√	√	√	√	√	√
C. austriaca	80.3.27.102	Czech	√	√	√	√	√	√				
C. austriaca	80.3.27.103	Czech	√	√	√	√	√					
C. austriaca	80.3.27.112	Czech	√	√								
C. austriaca	80.3.27.117	Czech	√	√								
C. austriaca	80.3.27.118	Czech	√		√		√					

Species	Code	Country										
C. austriaca	80.3.27.132	Czech	√		√		√					
C. austriaca	80.3.27.159	Czech	√	√	√	√	√					
C. austriaca	80.3.27.160	Czech	√	√	√	√	√					
C. austriaca	80.3.27.83	Czech	√	√	√	√	√	√	√	√	√	√
C. austriaca	80.3.27.89	Czech	√	√								
C. austriaca	80.3.27.91	Czech	√	√	√	√	√					
C. austriaca	80.3.27.92	Czech	√	√	√	√	√					
C. austriaca	80.3.27.99	Czech	√	√	√	√	√	√	√	√		√
C. austriaca	80.3.27.116	FYROM	√		√		√	√				
C. austriaca	80.3.27.124	FYROM	√		√		√					
C. austriaca	80.3.27.10	Greece	√	√								
C. austriaca	80.3.27.11	Greece	√	√	√	√	√					
C. austriaca	80.3.27.13	Greece	√	√	√	√	√	√		√	√	
C. austriaca	80.3.27.14	Greece	√	√	√	√	√	√	√	√	√	√
C. austriaca	80.3.27.15	Greece	√	√	√	√	√					
C. austriaca	80.3.27.16	Greece	√	√	√	√	√					
C. austriaca	80.3.27.17	Greece	√		√		√	√	√	√	√	
C. austriaca	80.3.27.18	Greece	√	√	√	√	√	√	√	√		
C. austriaca	80.3.27.19	Greece	√	√	√	√	√					
C. austriaca	80.3.27.2	Greece	√	√								
C. austriaca	80.3.27.20	Greece	√	√	√	√	√					
C. austriaca	80.3.27.21	Greece	√	√								
C. austriaca	80.3.27.22	Greece	√	√	√	√	√					
C. austriaca	80.3.27.23	Greece	√	√	√	√	√					
C. austriaca	80.3.27.27	Greece	√	√	√	√	√					
C. austriaca	80.3.27.28	Greece	√	√	√	√	√					
C. austriaca	80.3.27.29	Greece	√	√	√	√	√	√	√	√	√	√
C. austriaca	80.3.27.3	Greece	√	√	√	√	√					
C. austriaca	80.3.27.4	Greece	√	√	√	√	√					
C. austriaca	80.3.27.5	Greece	√	√	√	√	√	√	√	√	√	
C. austriaca	80.3.27.6	Greece	√	√	√	√	√					
C. austriaca	80.3.27.79	Greece	√	√	√	√	√	√	√	√	√	√
C. austriaca	80.3.27.80	Greece	√	√	√	√	√	√	√	√	√	√
C. austriaca	80.3.27.81	Greece	√	√	√	√	√	√	√		√	√
C. austriaca	80.3.27.115	Hungary	√	√	√	√	√	√	√		√	√
C. austriaca	80.3.27.168	Italy	√	√	√	√	√			√		
C. austriaca	80.3.27.125	Montenegro	√		√		√					
C. austriaca	80.3.27.126	Montenegro	√		√		√					
C. austriaca	80.3.27.82	Montenegro	√	√	√	√	√	√		√	√	√
C. austriaca	80.3.27.87	Montenegro	√	√	√	√	√	√	√	√	√	√
C. austriaca	80.3.27.111	Portugal	√		√		√					
C. girondica	80.3.27.58	Outgroup	√	√								
C. austriaca	80.3.27.59	Portugal	√	√								
C. austriaca	80.3.27.60	Portugal	√	√								
C. austriaca	80.3.27.69	Portugal	√	√								
C. austriaca	80.3.27.76	Portugal	√	√	√	√	√					

			1	2	3	4	5	6	7	8	9	10
C. austriaca	80.3.27.77	Portugal	√									
C. austriaca	80.3.27.100	Romania	√	√	√	√	√					
C. austriaca	80.3.27.110	Romania	√	√	√	√	√	√	√	√	√	√
C. austriaca	80.3.27.133	Romania	√		√		√					
C. austriaca	80.3.27.95	Romania	√	√	√	√	√	√	√	√	√	√
C. austriaca	80.3.27.96	Romania	√	√	√	√	√					
C. austriaca	80.3.27.97	Romania	√	√	√	√	√					
C. austriaca	80.3.27.149	Serbia	√	√	√	√	√					
C. austriaca	80.3.27.150	Serbia	√	√	√	√	√					
C. austriaca	80.3.27.151	Serbia	√	√	√	√	√	√	√	√		√
C. austriaca	80.3.27.152	Serbia	√	√	√	√	√	√	√	√	√	√
C. austriaca	80.3.27.153	Serbia	√	√	√	√	√	√	√	√	√	√
C. austriaca	80.3.27.154	Serbia	√	√	√	√	√					
C. austriaca	80.3.27.155	Serbia	√	√	√	√	√					
C. austriaca	80.3.27.165	Serbia	√	√	√	√	√					
C. austriaca	80.3.27.63	Serbia	√	√	√	√	√	√				
C. austriaca	80.3.27.106	Slovakia	√	√	√	√	√					
C. austriaca	80.3.27.108	Slovakia	√	√								
C. austriaca	80.3.27.109	Slovakia	√	√								
C. austriaca	80.3.27.122	Slovakia	√	√	√	√	√					
C. austriaca	80.3.27.123	Slovakia	√		√		√					
C. austriaca	80.3.27.156	Slovakia	√	√	√	√	√					
C. austriaca	80.3.27.157	Slovakia	√	√	√	√	√					
C. austriaca	80.3.27.158	Slovakia	√	√	√	√	√					
C. austriaca	80.3.27.161	Slovakia	√	√	√	√	√	√	√	√	√	√
C. austriaca	80.3.27.162	Slovakia	√	√	√	√	√	√	√	√		√
C. austriaca	80.3.27.166	Slovakia	√	√	√	√	√	√	√	√	√	
C. austriaca	80.3.27.85	Slovakia	√	√	√	√	√					
C. austriaca	80.3.27.94	Slovakia	√	√	√	√	√					
C. austriaca	80.3.27.98	Slovakia	√		√		√					
C. austriaca	80.3.27.127	Slovenia	√		√		√					
C. austriaca	80.3.27.128	Slovenia	√		√		√					
C. austriaca	80.3.27.129	Slovenia	√		√		√					
C. austriaca	80.3.27.64	Slovenia	√	√								
C. austriaca	80.3.27.65	Slovenia	√									
C. austriaca	80.3.27.66	Slovenia	√	√	√	√	√	√		√		
C. austriaca	80.3.27.67	Slovenia	√	√	√	√	√					
C. austriaca	80.3.27.68	Slovenia	√	√								
C. austriaca	80.3.27.70	Slovenia	√	√	√	√	√					
C. austriaca	80.3.27.71	Slovenia	√	√	√	√	√					
C. austriaca	80.3.27.78	Slovenia	√									
C. austriaca	80.3.27.57	Spain	√	√								
C. austriaca	80.3.27.88	Spain	√	√	√	√	√	√	√			
C. austriaca	80.3.27.142	Sweden	√	√	√	√	√	√				
C. austriaca	80.3.27.30	Turkey	√	√	√	√	√					
C. austriaca	80.3.27.31	Turkey	√	√	√	√	√	√	√			√

Species	ID	Location										
C. austriaca	80.3.27.32	Turkey	√	√	√	√	√	√	√	√	√	√
C. austriaca	80.3.27.33	Turkey	√	√	√	√	√	√	√	√	√	√
C. austriaca	80.3.27.34	Turkey	√	√	√	√	√					
C. austriaca	80.3.27.35	Turkey	√	√	√	√	√	√		√	√	√
C. austriaca	80.3.27.36	Turkey	√	√	√	√	√	√	√	√	√	√
C. austriaca	80.3.27.39	Turkey	√	√	√	√	√					
C. austriaca	80.3.27.40	Turkey	√	√	√	√	√	√	√	√	√	√
C. austriaca	80.3.27.41	Turkey	√	√	√	√	√	√	√	√	√	√
C. austriaca	80.3.27.43	Turkey	√	√								
C. austriaca	80.3.27.45	Turkey	√									
C. austriaca	80.3.27.46	Turkey	√	√	√	√	√	√			√	
C. austriaca	80.3.27.47	Turkey	√	√								
C. austriaca	80.3.27.48	Turkey	√	√	√	√	√					
C. austriaca	80.3.27.49	Turkey	√	√	√	√	√	√	√		√	√
C. austriaca	80.3.27.51	Turkey	√	√	√	√		√				
C. austriaca	80.3.27.55	Turkey	√	√								
C. austriaca	80.3.27.72	Turkey	√	√	√	√	√					
C. austriaca	80.3.27.73	Turkey	√	√	√	√	√	√	√	√		
C. austriaca	80.3.27.74	Turkey	√	√								
C. austriaca	80.3.27.75	Turkey	√	√	√	√	√			√	√	
H. genomensis	80.3.25.101	Outgroup	√	√	√	√	√			√	√	
H. genomensis	80.3.25.18	Outgroup	√	√								
Z. situla	80.3.30.70	Outgroup	√	√	√	√	√			√		
E. quatuorlineata	80.3.31.15	Outgroup	√	√	√	√	√			√	√	
C. austriaca	80.3.27.169	Greece	√	√			√	√	√	√	√	√
C. austriaca	80.3.27.170	Greece	√	√				√				√
C. austriaca	80.3.27.171	Greece	√	√			√	√	√		√	
C. austriaca	80.3.27.172	Greece	√	√			√	√	√	√	√	√
C. austriaca	80.3.27.173	Serbia	√	√				√		√		√
C. austriaca	80.3.27.174	Serbia	√	√				√				√
C. austriaca	80.3.27.176	Serbia	√	√			√	√	√	√		√
C. austriaca	80.3.27.177	CAUCASUS NW	√	√				√		√	√	
C. austriaca	80.3.27.178	CAUCASUS NW	√	√				√	√	√	√	√
C. austriaca	80.3.27.179	Crimea	√	√				√	√	√	√	√
C. austriaca	80.3.27.180	Crimea	√	√				√	√	√		
C. austriaca	80.3.27.181	Crimea	√	√				√	√	√	√	
C. austriaca	80.3.27.182	Italy	√	√				√				
C. austriaca	80.3.27.183	Italy	√	√				√	√	√	√	
C. austriaca	80.3.27.184	Italy	√	√				√	√			
C. austriaca	80.3.27.185	Italy	√	√								
C. girondica	80.3.27.186	Outgroup	√	√				√	√	√	√	
C. austriaca	80.3.27.187	Italy	√	√								√
C. austriaca	80.3.27.188	Italy	√	√								
C. austriaca	80.3.27.189	Italy	√	√				√	√		√	√
C. austriaca	80.3.27.190	Italy	√	√				√	√	√	√	√

7   **Table S2** Sequences used in the study coming from the Genbank genetic library.

Genbank sequences				
Accession numbers		Name in the study	Region	Author
16S	Cytb			
KM435290.1	EU022645	E17116_2_Russia	Vysha, Republic of Mordovia Russia	Santos et al., 2008
KM435291.1	KM435297	ABKAZIA050	Ajaria	Galarza et al., 2015
KM435292.1	KM435298	ALFINSTR01	Åland, Finström, Finland	Galarza et al., 2015
KM435293.1	KM435302.1	Georgia_Aj	Georgia: Ajaria	Galarza et al., 2015
KM435295.1	KM435303	Latvia	Latvia	Galarza et al., 2015
EU022626	EU022651	E27104_0_Austria	Vorarlberg Austria	Santos et al., 2008
EU022629	EU022652	E27104.4_Spain	Sierra Nevada, Granada	Santos et al., 2008
EU022630	EU022654	E3010.4_France	Canigou, France	Santos et al., 2008
EU022632	EU022658	E3010_5_Spain	Puertos de Beceite, Castellón Spain	Santos et al., 2008
EU022633	EU022668	E2026.8_Portugal	Northern Portugal	Santos et al., 2008
EU022637	EU022670	E3026_16_Etna_Caf	Etna, Adrano, Sicily	Santos et al., 2008
EU022639	EU022675	Spain_Albac	Riopar, Albacete	Santos et al., 2008
EU022639	EU022675	E2405.2_Spain	Riopar, Albacete	Santos et al., 2008
EU022640	MH382907	1515_Slovakia	Slovakia: Borský Mikuláš	Jablonski et al., 2019
	MH382908	1605_Slovakia	Slovakia: Jasenie	Jablonski et al., 2019
	MH382909	1713_Slovakia	Divina Slovakia	Jablonski et al., 2019
	MH382910	2393_Slovakia	Beňuš-Gašparovo Slovakia	Jablonski et al., 2019
	MH382911	2475_Portugal	Portela do Homen Portugal	Jablonski et al., 2019
	MH382912	2479_Bosnia_Herzegovina	Bosnia and Herzegovina	Jablonski et al., 2019
	MH382913	2487_Czech	Řadovy Czech Republic	Jablonski et al., 2019
	MH382914	497JAB_Romania	Ciuguzel - Cicard Romania	Jablonski et al., 2019
	MH382915	649JAB_Portugal	Senhora da Boa Estrela Portugal	Jablonski et al., 2019
	MH382916	741JAB_Bulgaria	Asenovgrad Bulgaria	Jablonski et al., 2019
	MH382917	1014JAB_North_Macedonia	Korab Mts. N. Macedonia	Jablonski et al., 2019
	MH382918	1056JAB_Czech	Studenec Czech Republic	Jablonski et al., 2019
	MH382919	1067JAB_Italy	Monte Rufeno Italy	Jablonski et al., 2019
	MH382920	1182JAB_Crimea	Bataľnoe Crimea	Jablonski et al., 2019
	MH382921	1450JAB_Slovakia	NPR Šúr Slovakia	Jablonski et al., 2019
	MH382922	1647JAB_North_Macedonia	Galichica Mts. Macedonia	Jablonski et al., 2019
	MH382923	2037JAB_Montenegro	Durmitor Mts. Montenegro	Jablonski et al., 2019
	MH382924	2038JAB_Montenegro	Durmitor Mts. Montenegro	Jablonski et al., 2019
	MH382925	2073JAB_Slovenia	NP Triglav Slovenia	Jablonski et al., 2019
	MH382926	2075JAB_Slovenia	Trenta Slovenia	Jablonski et al., 2019
	MH382927	2076JAB_Slovenia	Trenta Slovenia	Jablonski et al., 2019
	MH382928	2379JAB_Crimea	Pchelinoe Crimea	Jablonski et al., 2019
	MH382929	2380JAB_Crimea	Simferopol Crimea	Jablonski et al., 2019
	MH382930	2404JAB_Czech	Hoštětín Czech Republic	Jablonski et al., 2019
	MH382931	2443JAB_Romania	Gernik Romania	Jablonski et al., 2019
	MH382932	2459JAB_Turkey	Ortaçımağıl Turkey	Jablonski et al., 2019
	MH382933	2460JAB_Turkey	Küçükotlukbeli Turkey	Jablonski et al., 2019
	MH382934	2464JAB_Turkey	Between Niksar and Başçiftlik Turkey	Jablonski et al., 2019

MH382935	2467JAB_Turkey	Güneyce Turkey	Jablonski et al., 2019
MH382936	2468JAB_Turkey	Söğüteli Turkey	Jablonski et al., 2019
MH382937	2469JAB_Turkey	Between Mecidiye and Yarbaşı Turkey	Jablonski et al., 2019
MH382938	2470JAB_Turkey	Between Mecidiye and Yarbaşı Turkey	Jablonski et al., 2019
MH382939	2471JAB_Turkey	Çilhoroz Turkey	Jablonski et al., 2019
MH382940	2472JAB_Turkey	Çilhoroz Turkey	Jablonski et al., 2019
MH382941	2473JAB_Turkey	Çilhoroz Turkey	Jablonski et al., 2019
MH382942	2481JAB_Bulgaria	Zhilentsi Bulgaria	Jablonski et al., 2019
MH382944	2485JAB_Bulgaria	Petrohan Bulgaria	Jablonski et al., 2019
MH382943	2484JAB_Bulgaria	Maglenishki rid Bulgaria	Jablonski et al., 2019
MH382945	2489JAB_Bulgaria	Senokos Bulgaria	Jablonski et al., 2019
MH382946	2492JAB_Bulgaria	Zdravets Bulgaria	Jablonski et al., 2019
MH382947	2493JAB_Bulgaria	Vitata Bulgaria	Jablonski et al., 2019
MH382948	Ca1Iran	Varewasht Mts. Iran	Jablonski et al., 2019
MH382949	Ca2Iran	Damash Iran	Jablonski et al., 2019
MH382950	Ca3Iran	Veresk Iran	Jablonski et al., 2019
MH557091	1709_Slovakia	Svetlice Slovakia	Jablonski et al., 2019
KX694868	ROM_26536_Georgia	Tbilisi Georgia	Alencar et al. 2016
KP756615.1	KP756615_1_Poland	Poland	Sztencel-Jabłonka etal., 2015
KP756616.1	KP756616_1_Poland	Poland	Sztencel-Jabłonka etal., 2015
KP756617.1	KP756617_1_Poland	Poland	Sztencel-Jabłonka etal., 2015
KP756618.1	KP756618_1_Poland	Poland	Sztencel-Jabłonka etal., 2015
KP756619.1	KP756619_1_Poland	Poland	Sztencel-Jabłonka etal., 2015
KP756620.1	KP756620_1_Poland	Poland	Sztencel-Jabłonka etal., 2015
KP756621.1	KP756621_1_Poland	Poland	Sztencel-Jabłonka etal., 2015
KP756622.1	KP756622_1_Poland	Poland	Sztencel-Jabłonka etal., 2015
KP756623.1	KP756623_1_Armenia	Armenia	Sztencel-Jabłonka etal., 2015
KP756624.1	KP756624_1_Armenia	Armenia	Sztencel-Jabłonka etal., 2015
KM435299	ALJOMALA56	Åland, Jomala Finland	Galarza et al., 2015
KM435300	FRROUFFA53_France	Bollenberg France	Galarza et al., 2015
KM435301	FRROUFFA55_France	Bollenberg France	Galarza et al., 2015
KM435303	LATJELGA58	Latvia	Galarza et al., 2015
KM435304	MACEDONI42	Resen North Macedonia	Galarza et al., 2015
KM435305	RUSTAVR51	Russia: Stavropol	Galarza et al., 2015
JQ904296	DB2710_Portugal	Northern Portugal Portugal	Santos et al., 2008
JQ904297	DB2682_Spain	Miraflores de la Sierra, Madrid Spain	Santos et al., 2008
EU022642	E17116_3_Russia	Kalininskiy, Rostov Russia	Santos et al., 2008
EU022643	E17116_5_Russia	Volkonschino, Penza Russia	Santos et al., 2008
EU022644	E17116_6_Russia	Matveyevskiy, Rostov Russia	Santos et al., 2008
EU022646	E3010_1_Italy	Treviso Italy	Santos et al., 2008

EU022647	E27104_3_Greece	Kazarma, Kardhista Greece	Santos et al., 2008
EU022648	E27104_2_UK	Hampshire United Kingdom	Santos et al., 2008
EU022649	E1511_9_France	Tigouleix, Creuse France	Santos et al., 2008
EU022650	E1110_10_France	Merindal, Creuse France	Santos et al., 2008
EU022653	E2007_1_Spain	Sierra Nevada, Granada Spain	Santos et al., 2008
EU022655	E18124_1_Spain	Canejan, Lleida Spain	Santos et al., 2008
EU022656	E2606_1_Spain	Arguijo, Soria Spain	Santos et al., 2008
EU022657	E2606_3_Spain	Puerto Piqueras, Soria Spain	Santos et al., 2008
EU022659	E27014_5_Spain	Valle de Anson, Navarra Spain	Santos et al., 2008
EU022660	E2505_2_Spain	Lasarte, Alava Spain	Santos et al., 2008
EU022661	E2026_4_Spain	Escoriaza, Alava Spain	Santos et al., 2008
EU022662	E2026_1_Portugal	Serra da Estrela Portugal	Santos et al., 2008
EU022663	E2026_7_Portugal	Northern Portugal Portugal	Santos et al., 2008
EU022664	E2026_2_Portugal	Serra da Estrela Portugal	Santos et al., 2008
EU022666	E2026_3_Spain	Covanera, Burgos Spain	Santos et al., 2008
EU022667	E2026_11_Spain	Sedano, Burgos Spain	Santos et al., 2008
EU022669	E27104_1_Portugal	Pitoes Portugal	Santos et al., 2008
EU022671	E3026_17_Etna_Caf	Etna, Saifio, Sicily Italy	Santos et al., 2008
EU022672	E1406_30_Spain	El Espinero, Segovia Spain	Santos et al., 2008
EU022673	E2026_5_Spain	Sierra de Guadarrama, Madrid Spain	Santos et al., 2008
EU022674	E2026_6_Spain	Montes de Toledo, Toledo Spain	Santos et al., 2008
AY486930	AY486930_1_Turkey	Ilgaz Turkey	Nagy et al., 2004

8

9   **Table S3.** The primers used to amplify the genes fragments along with information about their

10   sequences, the size of their products and their source of origin.

Gene	Primer name	Type	Product size	Sequence (5' → 3')	Reference
16S	coron67F coron462R	forward reverse	~400 bp	TATAGACCTGTATGA CAGATC ACG TAG GAC	Present work Present work
cytb	L14724NAT H15307_mod	forward reverse	~420 bp	GACCTGCGGTCCGAAAAACCA GCTCAAAAKGATATTTGTCCTCA	Guicking *et al,*. 2006 Unpublished
BDNF	BDNF_F BDNF_R1	forward reverse	~713 bp	ACCATCCTTTTCCTKACTATGG CTATCTTCCCCTTTTAATGGTC	Vieites *et al*., 2007 Vieites *et al*., 2007
Rag 1	Rag1_14F Rag1_856R	forward reverse	~810 bp	GGGAGATATTGACACCATCC CTAGATCTCAAAGCCAGCAG	Present work Present work
Rag 2	PY1_f PY1_r	forward reverse	~430 bp	CCCTGAGTTTGGATGCTGTACTT AACTGCCTRTTGTCCCCTGGTAT	Gamble *et al*., 2008 Gamble *et al*., 2008
MC1R	MC1R_F MC1R_r	forward reverse	~700 bp	GGCNGCCATYGTCAAGAACCGGAACC CTCCGRAAGGCRTAAATGATGGGGTCCAC	Pinho *et al*., 2010 Pinho *et al*., 2010
NKTR	NKTR f19 NKTR r18	forward reverse	~630 bp	GATGACATGGAGATYTGYACTCC CTYCTDGAYCGACTTCTTGAGTGACT	Townsend *et al*., 2011 Townsend *et al*., 2011
PRLR	PRLR f1 PRLR r3	forward reverse	~600 bp	GACARYGARGACCAGCAACTRATGCC GACYTTGTGRACTTCYACRTAATCCAT	Townsend *et al*., 2008 Townsend *et al*., 2008

11

12 **Table S4** PCR conditions per primer

Gene	16S	Cytb	BDNF	Rag 1	Rag2	MC1R	NKTR	PRLR
Primers	coron67F	L14724NAT	BDNF_F1	Rag1_14F	PY1_f	MC1R_F	NKTR f19	PRLR f1
	coron462R	H15307_mod	BDNF_R1	Rag1_856R	PY1_r	MC1R_r	NKTR r18	PRLR r3
MgCl2	1.5 mM	1.5 mM	1.5 mM	1.5 mM	3 mM	3 mM	1.5 mM	3 mM
Number of Cycles	35	35	35	35	35	35	35	35
Denaturion	94 ºC/1'	94 ºC/1'	94 ºC/1'	94 ºC/1'	94 ºC/1'	94 ºC/1'	94 ºC/1'	94 ºC/1'
Annealing	52 ºC/1'	52 ºC/1'	55,6 ºC/1'	63 ºC/1'	56 ºC/1'	62 ºC/1'	50 ºC/1'	51,7 ºC/1'
Extension	72 ºC/1	72 ºC/1'	72 ºC/1'	72 ºC/1'	72 ºC/1'	72 ºC/1'	72 ºC/1'	72 ºC/1'

13

14 **Table S5** The mt (cyt *b*, 16S) genetic p-distances % between and within (inside the brackets)
15 the main phylogenetic groups of *Coronella austriaca*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
SE-Central & North Balkans	(0.3)															
Central-North Balkans	1.5	(0.5)														
West Balkans	1.8	1.7	(0.1)													
Eastern Europe	2.9	3.0	3.4	(0.6)												
Peloponnese	1.5	1.7	1.3	3.0	(0.2)											
E.Balkans-W.Turkey	3.1	3.1	2.8	3.4	2.7	(0.0)										
Central Europe	3.2	3.3	2.8	3.4	3.5	4.1	(0.3)									
Anatolia 1	2.6	2.6	2.9	3.3	2.5	3.1	3.8	(0.9)								
Anatolia 2	2.7	2.7	2.5	3.1	2.5	2.8	2.9	2.2	(0.1)							
Italy	3.8	4.0	3.5	3.6	4.0	4.3	4.6	4.2	4.1	(0.5)						
Lesser Caucasus	3.0	3.2	3.3	2.5	2.9	3.9	3.7	3.4	2.9	4.3	(1.0)					
Sicily	3.0	2.9	3.2	3.0	3.3	4.0	4.3	3.8	3.9	3.9	3.8	(0.3)				
West Iberian	2.7	2.8	3.5	2.7	3.1	3.5	3.6	3.3	3.2	3.3	3.2	2.4	(0.2)			
Iberian	3.7	3.8	3.8	3.4	4.0	4.4	4.4	4.0	4.0	4.0	4.5	3.6	3.0	(2.1)		
North Iberian	3.7	3.7	4.2	3.6	4.0	4.7	5.1	4.4	4.3	4.6	4.5	3.1	3.0	4.4	(1.0)	
West Europe	3.2	3.1	3.1	3.0	3.6	4.0	4.1	3.5	3.5	2.7	4.1	2.9	2.6	3.6	4.1	-
Coronella girondica	6.8	7.7	7.2	7.3	8.4	8.9	8.1	8.3	7.8	8.1	8.2	7.6	6.5	8.2	8.0	7.0

16

17 **Table S6** Partition subsets and nucleotide substitution model selected through PartitionFinder 2.
18 a) For Bayesian inference analyses of dataset 1, b) For Bayesian Inference analysis of dataset 2
19 c) For BEAST analysis, d) For RaxML analysis of dataset 2 e) For RaxML analysis of dataset 1.

a)	Subset	Best Model	# sites	Partition names
	1	HKY+I	407	16S
	2	K80+G	249	cytb_pos3, cytb_pos1
	3	GTR+G	124	cytb_pos2

b)	Subset	Best Model	# sites	Partition names
	1	GTR+G	463	16S, cytb_pos1
	2	HKY	124	cytb_pos2
	3	GTR+G	124	cytb_pos3
	4	K80+I	1750	nktr_pos3, rag2_pos3, prlr_pos2, bdnf_pos3, prlr_pos3, rag1_pos3, mc1r_pos2, prlr_pos1, nktr_pos2, rag1_pos1
	5	JC	416	bdnf_pos1, bdnf_pos2
	6	F81	626	rag2_pos2, rag1_pos2, nktr_pos1, rag2_pos1
	7	F81	196	mc1r_pos3
	8	HKY+G	196	mc1r_pos1

c)	Subset	Best Model	# sites	Partition names
	1	GTR+I	338	16S
	2	HKY+G	373	cytb
	3	JC+I	624	BDNF
	4	HKY+I	1019	MC1R, PRLR
	5	HKY+I	1541	Rag2, Rag1, NKTR

d)	Subset	Best model	#sites	Partition names
	1	GTR+I	865	16S, rag1_pos3, nktr_pos3, cytb_pos1
	2	GTR+I	752	mc1r_pos3, cytb_pos2, prlr_pos2, prlr_pos1, prlr_pos3
	3	GTR+G	124	cytb_pos3

	Subset	Best model	#sites	Partition names
	4	GTR+I	1958	mc1r_pos2, rag1_pos2, bdnf_pos3, rag2_pos3, rag2_pos2, bdnf_pos2, rag2_pos1, bdnf_pos1, nktr_pos1, nktr_pos2, rag1_pos1
	5	GTR_G	196	mc1r_pos1

	Subset	Best model	#sites	Partition names
e)	1	GTR+G	656	cytb_pos1, cytb_pos3, 16S
	2	GTR+G	124	cytb_pos2

20

21 **Table S7** Divergence time estimates in million years based on rates from calibration I (Cal I;
22 ~1.3 % substitutions per million years, s/my), calibration II (Cal II; ~0.8 % s/my) from Salvi et
23 al., (2018) and on calibration points from Burbring and Lawson (2007) with the *Coronella*
24 taxon-set; 95% HPD intervals are provided in brackets. *Zamenis: Zamenis situla, Elaphe:*
25 *Elaphe quatuorlineata.*

Node	Cal1 (Salvi et al. 2018)	Cal2 (Salvi et al. 2018)	Cal3 (Burbring & Lawson)
	Age and 95% HPD (Ma)		
Coronella spp.	4.65 (2.80-6.49)	10.22 (8.16-12.15)	
Coronella austriaca	0.96 (0.41-1.60)	0.96 (0.15-2.16)	1.13 (0.41-1.99)
(*Zamenis,* (*Elaphe, Coronella* spp.))	8.37 (6.46-10.25)	17.89 (15.94-19.89)	27.14 (24.13-30.12)

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Table S8 Matching between MapID, NHMC Code, Clade and Phylogenetic group where belong every specimen of dataset 2 based on the concatenated gene trees.

NHMC Code	Clade	Phylogenetic group	MapID	Cluster Map
NHMC80.3.27.105	West Balkans	Balkan	1	1-4
NHMC80.3.27.82	West Balkans	Balkan	2	1-4
NHMC80.3.27.126	West Balkans	Balkan	3	1-4
NHMC80.3.27.93	West Balkans	Balkan	4	1-4
NHMC80.3.27.169	Southeast Balkans-Central & North Balkans	Balkan	5	5-36
NHMC80.3.27.144	Southeast Balkans-Central & North Balkans	Balkan	6	5-36
NHMC80.3.27.20	Southeast Balkans-Central & North Balkans	Balkan	7	5-36
NHMC80.3.27.16	Southeast Balkans-Central & North Balkans	Balkan	8	5-36
NHMC80.3.27.164	Southeast Balkans-Central & North Balkans	Balkan	9	5-36
NHMC80.3.27.141	Southeast Balkans-Central & North Balkans	Balkan	10	5-36
NHMC80.3.27.14	Southeast Balkans-Central & North Balkans	Balkan	11	5-36
NHMC80.3.27.22	Southeast Balkans-Central & North Balkans	Balkan	12	5-36
NHMC80.3.27.118	Southeast Balkans-Central & North Balkans	Balkan	13	5-36
NHMC80.3.27.150	Southeast Balkans-Central & North Balkans	Balkan	14	5-36
NHMC80.3.27.172	Southeast Balkans-Central & North Balkans	Balkan	15	5-36
NHMC80.3.27.145	Southeast Balkans-Central & North Balkans	Balkan	16	5-36
NHMC80.3.27.116	Southeast Balkans-Central & North Balkans	Balkan	17	5-36
NHMC80.3.27.28	Southeast Balkans-Central & North Balkans	Balkan	18	5-36
NHMC80.3.27.138	Southeast Balkans-Central & North Balkans	Balkan	19	5-36
NHMC80.3.27.5	Southeast Balkans-Central & North Balkans	Balkan	20	5-36
NHMC80.3.27.6	Southeast Balkans-Central & North Balkans	Balkan	22	5-36
NHMC80.3.27.3	Southeast Balkans-Central & North Balkans	Balkan	23	5-36
NHMC80.3.27.148	Southeast Balkans-Central & North Balkans	Balkan	24	5-36
NHMC80.3.27.18	Southeast Balkans-Central & North Balkans	Balkan	25	5-36
NHMC80.3.27.110	Southeast Balkans-Central & North Balkans	Balkan	26	5-36

| NHMC80.3.27.170	Southeast Balkans-Central & North Balkans	Balkan	27	5-36
NHMC80.3.27.176	Southeast Balkans-Central & North Balkans	Balkan	28	5-36
NHMC80.3.27.13	Southeast Balkans-Central & North Balkans	Balkan	29	5-36
NHMC80.3.27.143	Southeast Balkans-Central & North Balkans	Balkan	30	5-36
NHMC80.3.27.171	Southeast Balkans-Central & North Balkans	Balkan	31	5-36
NHMC80.3.27.27	Southeast Balkans-Central & North Balkans	Balkan	32	5-36
NHMC80.3.27.174	Southeast Balkans-Central & North Balkans	Balkan	33	5-36
NHMC80.3.27.146	Southeast Balkans-Central & North Balkans	Balkan	34	5-36
NHMC80.3.27.97	Southeast Balkans-Central & North Balkans	Balkan	35	5-36
NHMC80.3.27.17	Southeast Balkans-Central & North Balkans	Balkan	36	5-36
NHMC80.3.27.29	Southeast Balkans-Central & North Balkans	Balkan	37	5-36
NHMC80.3.27.80	Peloponnese	Balkan	38	37-39
NHMC80.3.27.79	Peloponnese	Balkan	39	37-39
NHMC80.3.27.81	Peloponnese	Balkan	40	37-39
NHMC80.3.27.115	Central-North Balkans	Balkan	21	40-51
NHMC80.3.27.149	Central-North Balkans	Balkan	41	40-51
NHMC80.3.27.96	Central-North Balkans	Balkan	42	40-51
NHMC80.3.27.134	Central-North Balkans	Balkan	43	40-51
NHMC80.3.27.151	Central-North Balkans	Balkan	44	40-51
NHMC80.3.27.154	Central-North Balkans	Balkan	45	40-51
NHMC80.3.27.100	Central-North Balkans	Balkan	46	40-51
NHMC80.3.27.95	Central-North Balkans	Balkan	47	40-51
NHMC80.3.27.173	Central-North Balkans	Balkan	48	40-51
NHMC80.3.27.87	Central-North Balkans	Balkan	49	40-51
NHMC80.3.27.153	Central-North Balkans	Balkan	50	40-51
NHMC80.3.27.94	Central-North Balkans	Balkan	51	40-51
NHMC80.3.27.142	Central Europe	Balkan	52	52-56
NHMC80.3.27.71	Central Europe	Balkan	53	52-56

NHMC80.3.27.67	Central Europe	Balkan	54	52-56
NHMC80.3.27.66	Central Europe	Balkan	55	52-56
NHMC80.3.27.70	Central Europe	Balkan	56	52-56
NHMC80.3.27.106	Central Europe	Balkan	57	57-69
NHMC80.3.27.83	Central Europe	Balkan	58	57-69
NHMC80.3.27.159	Central Europe	Balkan	59	57-69
NHMC80.3.27.156	Central Europe	Balkan	60	57-69
NHMC80.3.27.132	Central Europe	Balkan	61	57-69
NHMC80.3.27.122	Central Europe	Balkan	62	57-69
NHMC80.3.27.102	Central Europe	Balkan	63	57-69
NHMC80.3.27.166	Central Europe	Balkan	64	57-69
NHMC80.3.27.101	Central Europe	Balkan	65	57-69
NHMC80.3.27.98	Central Europe	Balkan	66	57-69
NHMC80.3.27.161	Central Europe	Balkan	67	57-69
NHMC80.3.27.162	Central Europe	Balkan	68	57-69
NHMC80.3.27.99	Central Europe	Balkan	69	57-69
NHMC80.3.27.36	Anatolia	Balkan	70	70-81
NHMC80.3.27.41	Anatolia	Balkan	71	70-81
NHMC80.3.27.46	Anatolia	Balkan	72	70-81
NHMC80.3.27.39	Anatolia	Balkan	73	70-81
NHMC80.3.27.72	Anatolia	Balkan	74	70-81
NHMC80.3.27.75	Anatolia	Balkan	75	70-81
NHMC80.3.27.33	Anatolia	Balkan	76	70-81
NHMC80.3.27.34	Anatolia	Balkan	77	70-81
NHMC80.3.27.35	Anatolia	Balkan	78	70-81
NHMC80.3.27.73	Anatolia	Balkan	79	70-81
NHMC80.3.27.32	Anatolia	Balkan	80	70-81
NHMC80.3.27.30	Anatolia	Balkan	81	70-81

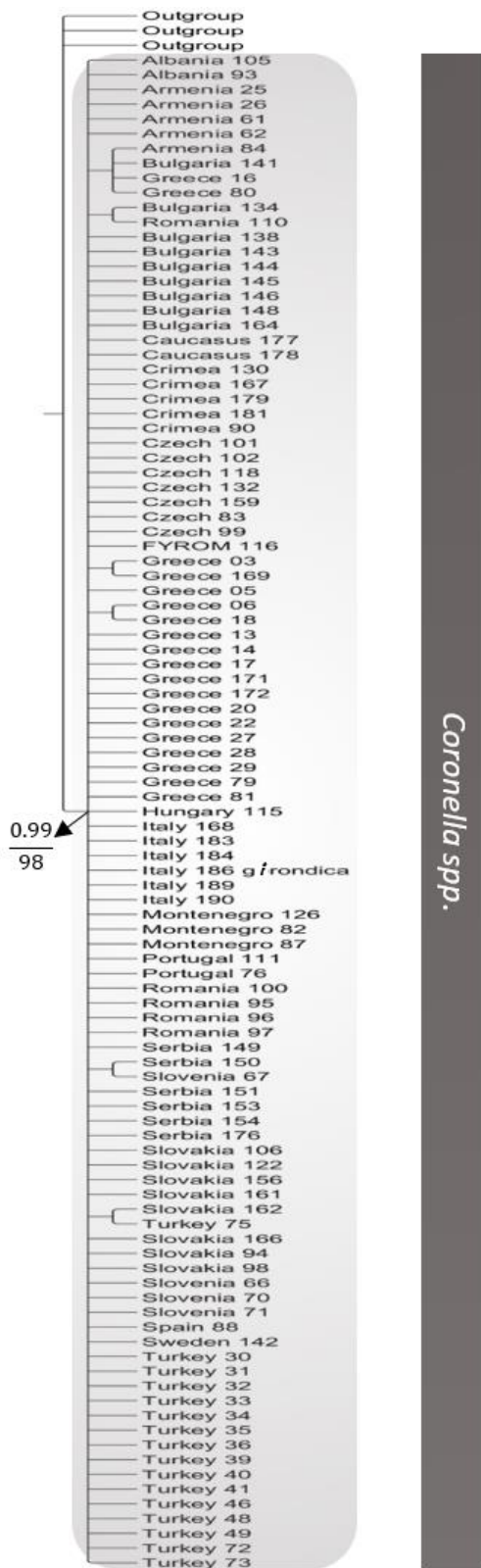|				
NHMC80.3.27.62	Lesser Caucasus	Eastern	82	82-89
NHMC80.3.27.26	Lesser Caucasus	Eastern	83	82-89
NHMC80.3.27.84	Lesser Caucasus	Eastern	84	82-89
NHMC80.3.27.61	Lesser Caucasus	Eastern	85	82-89
NHMC80.3.27.25	Lesser Caucasus	Eastern	86	82-89
NHMC80.3.27.49	Lesser Caucasus	Eastern	87	82-89
NHMC80.3.27.48	Lesser Caucasus	Eastern	88	82-89
NHMC80.3.27.40	Lesser Caucasus	Eastern	89	82-89
NHMC80.3.27.90	East Europe-Caucasus	Eastern	90	90-94
NHMC80.3.27.130	East Europe-Caucasus	Eastern	91	90-94
NHMC80.3.27.181	East Europe-Caucasus	Eastern	92	90-94
NHMC80.3.27.179	East Europe-Caucasus	Eastern	93	90-94
NHMC80.3.27.167	East Europe-Caucasus	Eastern	94	90-94
NHMC80.3.27.178	East Europe-Caucasus	Eastern	95	95,96
NHMC80.3.27.177	East Europe-Caucasus	Eastern	96	95,96
NHMC80.3.27.183	Italy	Apennine	97	97-100
NHMC80.3.27.189	Italy	Apennine	98	97-100
NHMC80.3.27.184	Italy	Apennine	99	97-100
NHMC80.3.27.190	Italy	Apennine	100	97-100
NHMC80.3.27.88	Sicily-North Iberian	North Iberian	101	101,102
NHMC80.3.27.168	Sicily-North Iberian	North Iberian	102	101,102
NHMC80.3.27.76	West Iberian	West Iberian	103	103,104
NHMC80.3.27.111	West Iberian	West Iberian	104	103,104

31

32

33   (a)

34

35   (b)

36
37   (c)
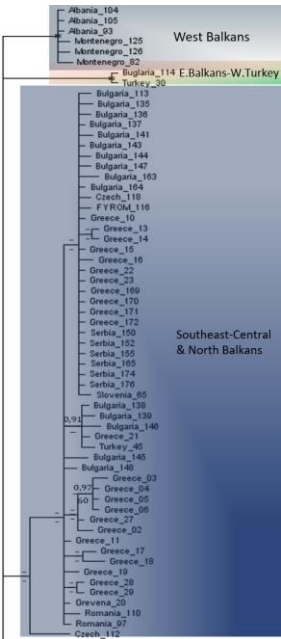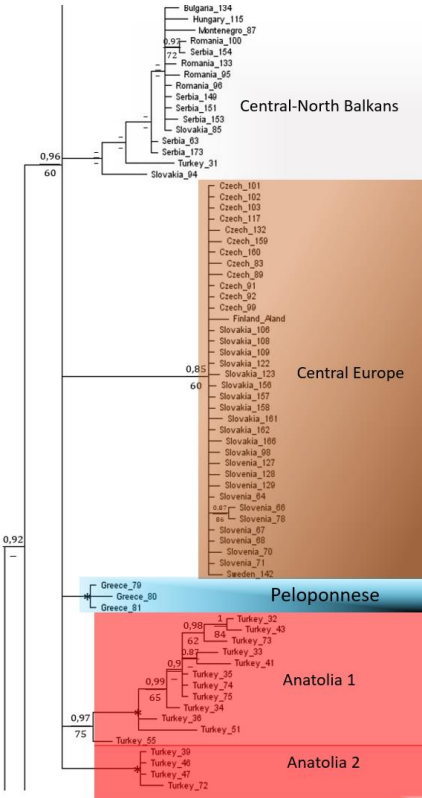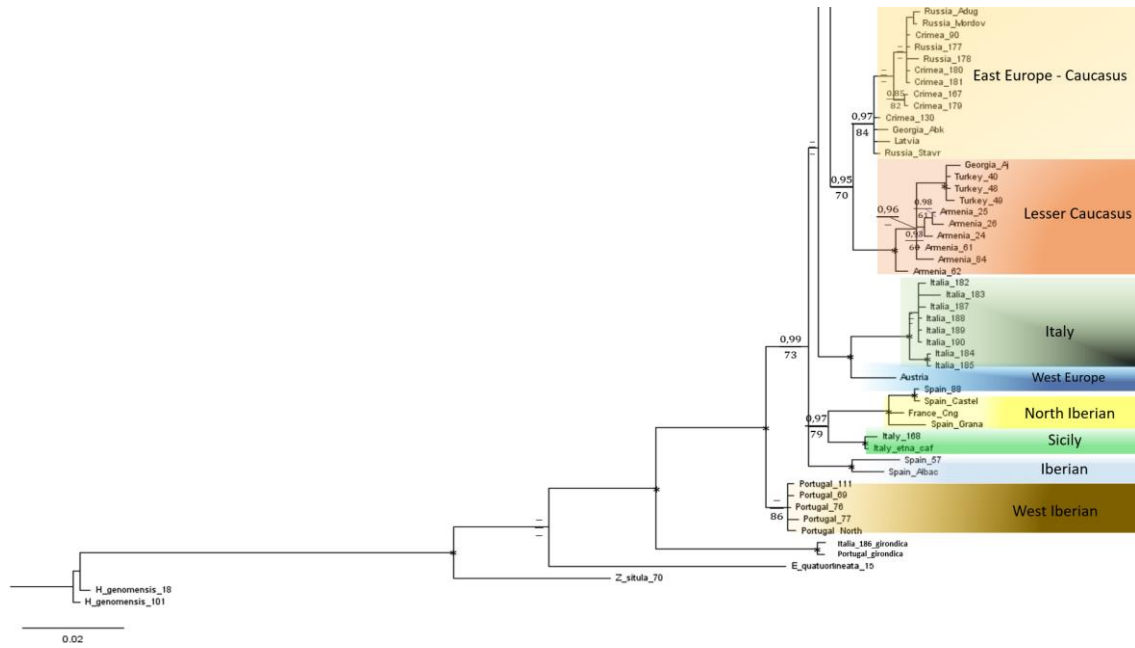
38   **Figure S1** (a), (b), (c): The BI concatenated tree of mitochondrial genes (16S, cytb).
39   Posterior probabilities (pp) and bootstraps support values (bsv) are shown by the
40   numbers left to the nodes as pp/bsv. The symbol '–' indicates that there is no significant
41   support for this node (<0.95 posterior probability, <60 bootstraps). The asterisk means
42   pp > 0.95 and bsv >85. The name of every specimen corresponds to its origin and the
43   last two numbers of its NHMC code.

44

Outgroup
Outgroup
Outgroup
Albania 105
Albania 93
Armenia 25
Armenia 26
Armenia 61
Armenia 62
Armenia 84
Bulgaria 141
Greece 16
Greece 80
Bulgaria 134
Romania 110
Bulgaria 138
Bulgaria 143
Bulgaria 144
Bulgaria 145
Bulgaria 146
Bulgaria 148
Bulgaria 164
Caucasus 177
Caucasus 178
Crimea 130
Crimea 167
Crimea 179
Crimea 181
Crimea 90
Czech 101
Czech 102
Czech 118
Czech 132
Czech 159
Czech 83
Czech 99
FYROM 116
Greece 03
Greece 169
Greece 05
Greece 06
Greece 18
Greece 13
Greece 14
Greece 17
Greece 171
Greece 172
Greece 20
Greece 22
Greece 27
Greece 28
Greece 29
Greece 79
Greece 81
Hungary 115
Italy 168
Italy 183
Italy 184
Italy 186 g*i*rondica
Italy 189
Italy 190
Montenegro 126
Montenegro 82
Montenegro 87
Portugal 111
Portugal 76
Romania 100
Romania 95
Romania 96
Romania 97
Serbia 149
Serbia 150
Slovenia 67
Serbia 151
Serbia 153
Serbia 154
Serbia 176
Slovakia 106
Slovakia 122
Slovakia 156
Slovakia 161
Slovakia 162
Turkey 75
Slovakia 166
Slovakia 94
Slovakia 98
Slovenia 66
Slovenia 70
Slovenia 71
Spain 88
Sweden 142
Turkey 30
Turkey 31
Turkey 32
Turkey 33
Turkey 34
Turkey 35
Turkey 36
Turkey 39
Turkey 40
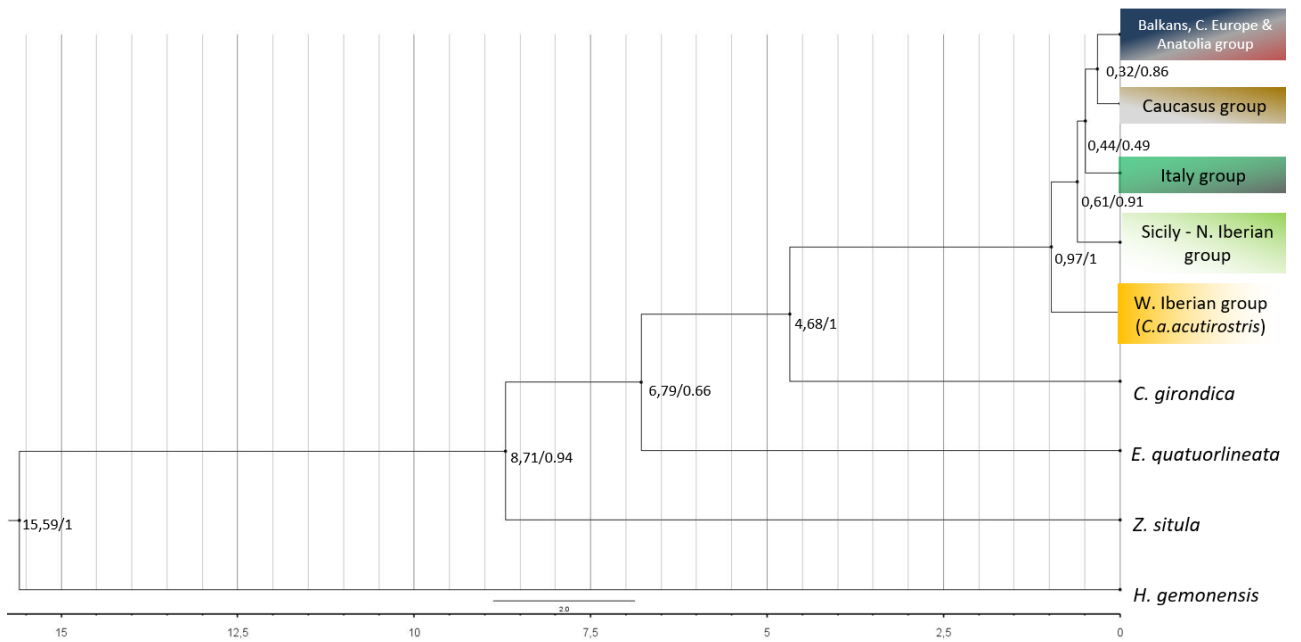Turkey 41
Turkey 46
Turkey 48
Turkey 49
Turkey 72
Turkey 73

0.99
98

Coronella spp.

45 **Figure S2** The BI concatenated tree of nuclear genes. Posterior probabilities (pp) and bootstraps
46 support values (bp) are shown by the numbers right to the nodes as pp/bsv and only for those
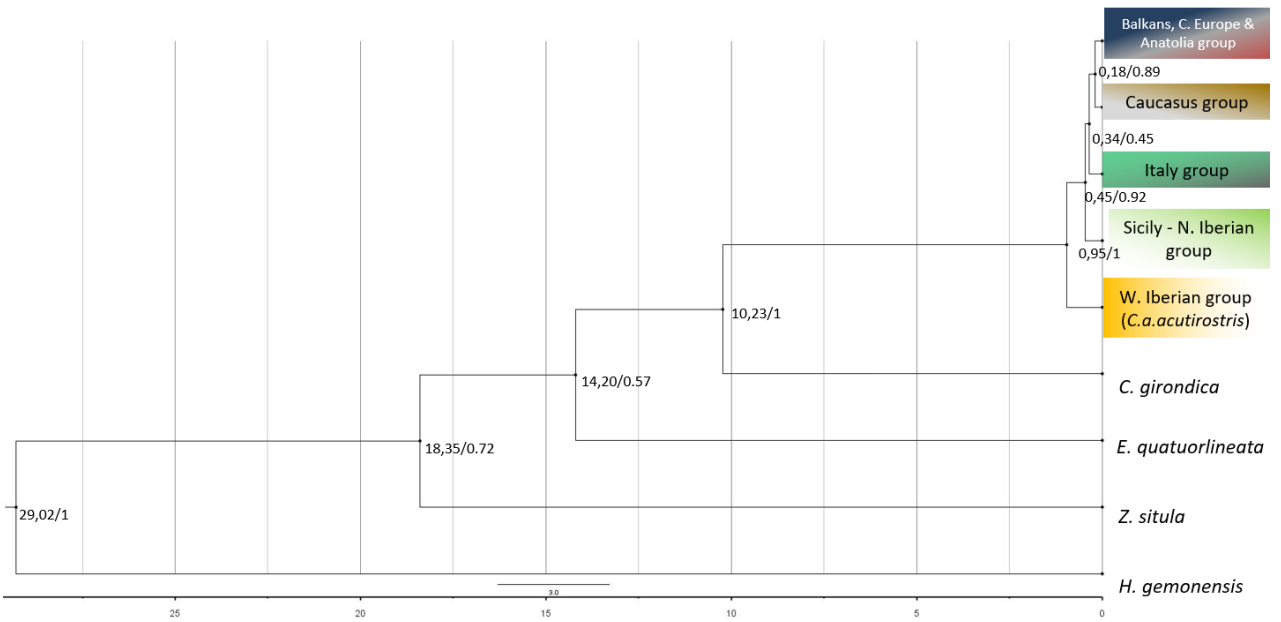47 which have significant support (<0.95 posterior probability, <60 bootstraps).

Balkans, C. Europe & Anatolia group

0,35/0.86

Caucasus group

0,66/0.92

Italy group

0,45/0.42

Sicily - N. Iberian group

W. Iberian group (*C.a.acutirostris*)

1,13/1

3,99/1

C. girondica

14,49/0.76

E. quatuorlineata

27,14/1

Z. situla

37,65/1

H. gemonensis

4.0

35   30   25   20   15   10   5   0

48   (a)

49

Balkans, C. Europe & Anatolia group

0,32/0.86

Caucasus group

0,44/0.49

Italy group

0,61/0.91

Sicily - N. Iberian group

W. Iberian group (*C.a.acutirostris*)

0,97/1

4,68/1

C. girondica

6,79/0.66

E. quatuorlineata

8,71/0.94

Z. situla

15,59/1

H. gemonensis

2.0

15   12,5   10   7,5   5   2,5   0

50

51   (b)

52

(c)

Figure S3 Species trees of the *Coronella austriaca* with the time axis in million years; (a)
Species tree based on calibration points according to the study of Burbring and Lawson (2007)
(C.p 1), (b) and (c) species tree based on calibration points (C.p 2 and C.p 3, respectively)
according to the study of Salvi et al. (2018); the values on nodes show the divergence time
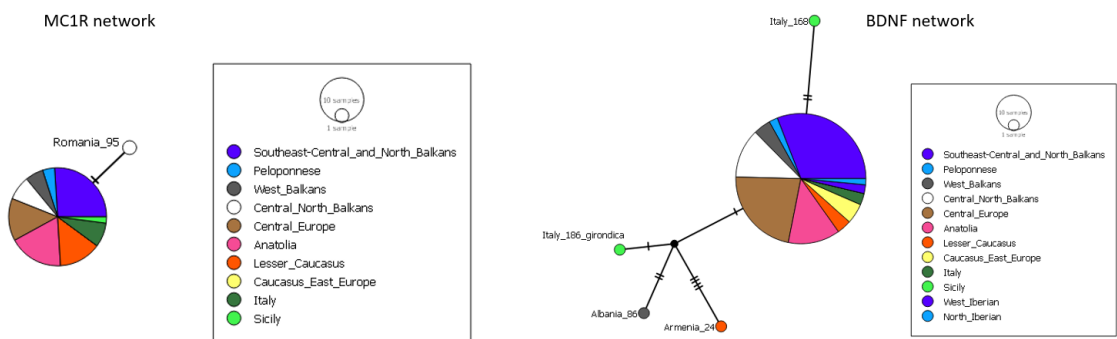estimation in millions years and the posterior probability (my/pp).

59
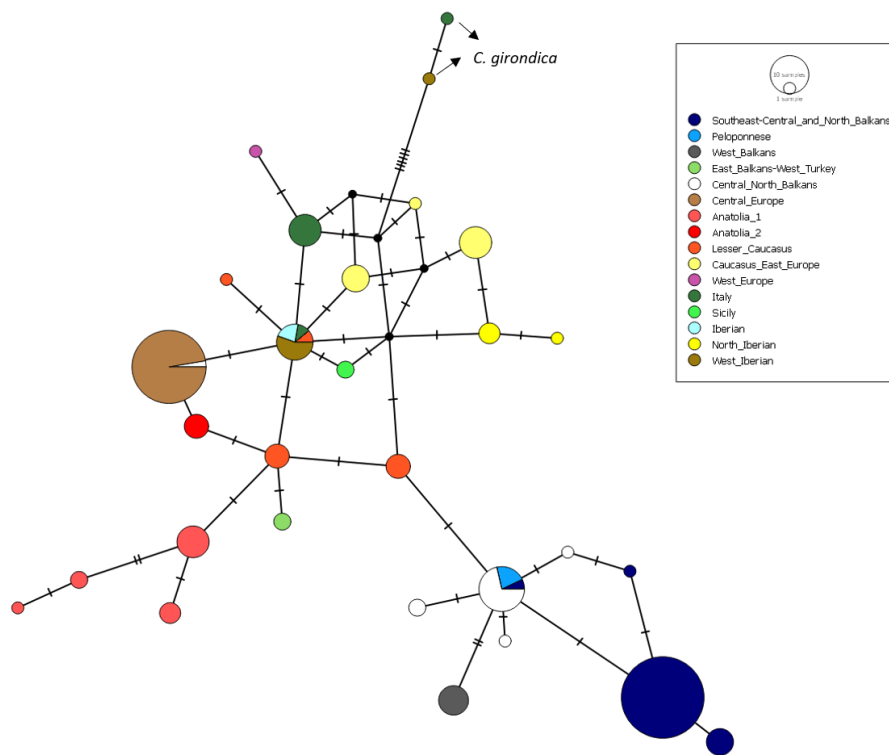
Figure S4 Median-joining network inferred from 16S sequences of *Coronella austriaca*. Circle sizes are proportional to the number of sequences. Each color represents a clade from mtDNA phylogenetic tree. Black nodes represent inferred (unsampled) sequences by iteratively adding 'median' sequence vectors when these nodes shorten the total length of the network (Leigh and Bryant, 2015).

65   (a)
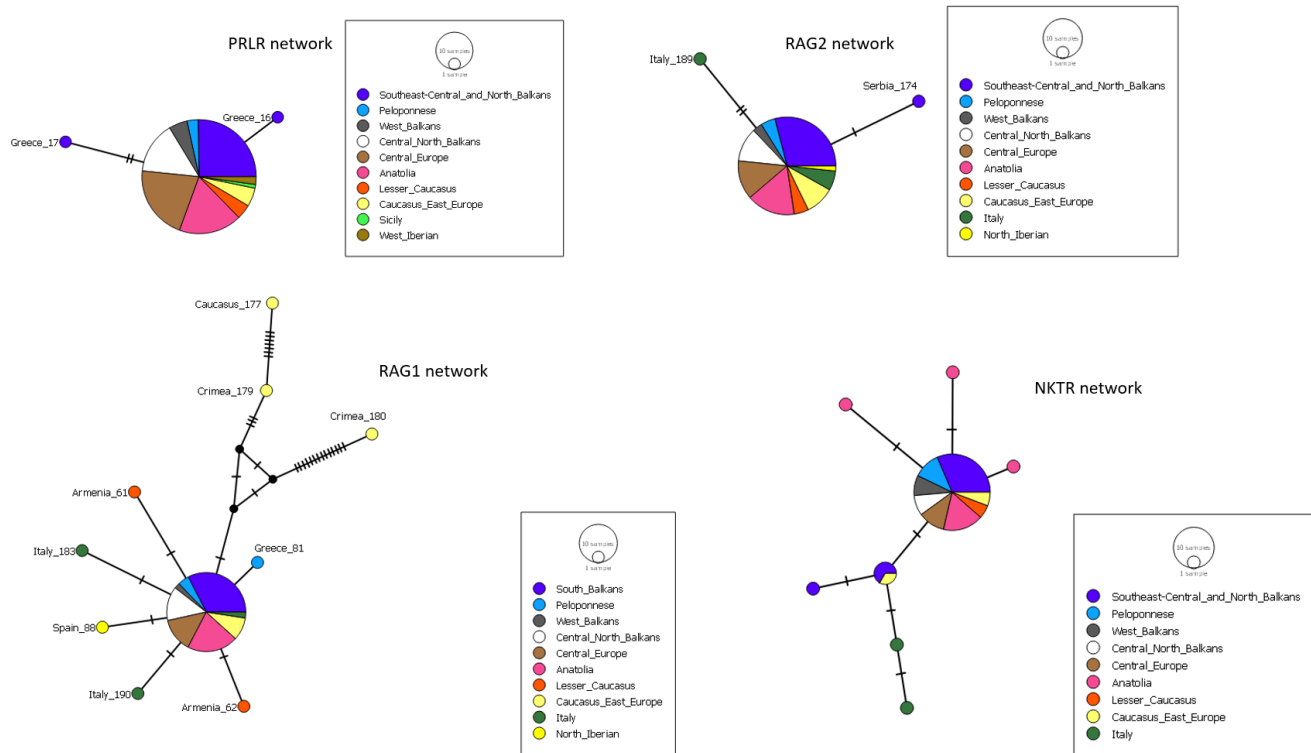
66

67   (b)

68

69   **Figure S5** (a), (b) Median-joining network inferred from nuclear sequences of *Coronella*
70   *austriaca*. Circle sizes are proportional to the number of sequences. Each color represents a
71   clade from the concatenated tree. Black nodes represent inferred (unsampled) sequences by
72   iteratively adding 'median' sequence vectors when these nodes shorten the total length of the
73   network (Leigh and Bryant, 2015).

74
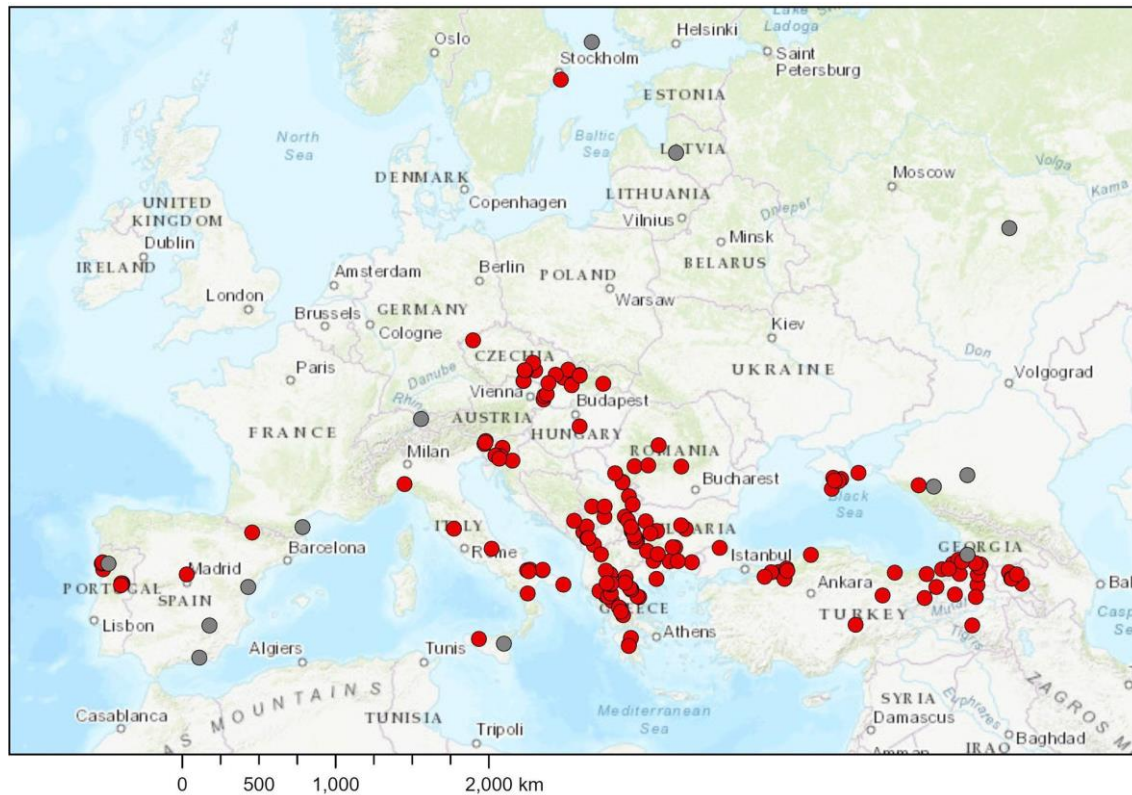
Figure S6 Geographic distribution of specimens of *Coronella austriaca* used for the mt dataset
of the present study. Red dots represent sequences produced in the current study. Gray dots
represent the sequences from Genbank that were used in mtDNA dataset.