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Genetic diversity and differentiation of alpine salamanders from the Dinarides – an evolutionary perspective with insights for species conservation

Emina Šunje[1,2,3], Ana Zuazu Bermejo[3], Raoul Van Damme[3], Thierry Backeljau[4,5], Naris Pojskić[6], Lada Lukić Bilela[1] & Belma Kalamujić Stroil[6]

[1)] Evolutionary Ecology Group, Faculty of Science, University of Sarajevo, Zmaja od Bosne 33, 71000 Sarajevo, Bosnia and Herzegovina

[2)] Herpetological Association in B&H – ATRA (BH-HU: ATRA), Urijan Dedina 137, 71000 Sarajevo, Bosnia and Herzegovina

[3)] Laboratory of Functional Morphology, University of Antwerp, Universiteitsplein 1, 2610 Antwerp, Belgium

[4)] Evolutionary Ecology Group, University of Antwerp, Universiteitsplein 1, 2610 Antwerp, Belgium

[5)] Royal Belgian Institute of Natural Sciences, Vautierstraat 29, 1000 Brussels, Belgium

[6)] Institute for Genetic Engineering and Biotechnology, University of Sarajevo, Zmaja od Bosne 8, 71000 Sarajevo, Bosnia and Herzegovina

Corresponding author: Emina Šunje, e-mail: sunje.emina@gmail.com

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Abstract. The fragmented population of alpine salamanders from the Dinaric Alps belongs to a distinct evolutionary lineage known as *Salamandra atra prenjensis*. However, the phylogenetic relationships within this lineage are unknown since previous studies did not comprehensively include Dinaric population fragments. In this study, we use six microsatellite loci and two mtDNA markers to examine the evolutionary relationships and genetic structure in several isolated fragments of alpine salamanders that are well spread along the Dinarides. We discovered that during Pleistocene glaciations, *S. atra* persisted in at least two Dinaric refugia: an old one in the Prenj mountain, and a more recent one in Gorski Kotar, whereas there is evidence of colonization for the populations of the Čvrsnica and Prokletije mountains. The results revealed that mountain Prenj was probably the main diversification center of alpine salamanders in the Dinarides. In addition, to provide a state-of-the-art review of the evolutionary history of the species, we also included available sequences of *S. atra* from the entire distribution range; we discuss the results from a conservation perspective.

Key words. Amphibia, Caudata, Salamandridae, Pleistocene, Balkan Peninsula, dispersal routes, refugia, lineage conservation.

Introduction

In the current epoch of the sixth mass extinction, characterized by a widespread and rapid decline of biodiversity (Ripple et al. 2017), phylogenetic, phylogeographic and population genetic studies are becoming increasingly important in the context of conservation biology and species management (Frankham et al. 2002).

The current geographical patterns of genetic variation in many species are a result of climatic and geomorphological changes that happened throughout the Earth's evolution (Lomolino & Heaney 2004). Probably the most famous example of such events are the climatic oscillations in the Pleistocene that caused a series of alternated contractions and expansions of distributional ranges (Hewitt 1999, 2000, 2004). Traditionally, European species were thought to have survived the Pleistocene glaciations in a few large, continuous areas in the extreme south of Europe – an idea largely bolstered by paleoclimatic reconstructions and palynological research (see Olalde et al. 2002). This explains why many studies (e.g. Taberlet et al. 1998, Petit et al. 2003) emphasized the role of glacial refugia in the Iberian, Apennine and Balkan peninsulas on the current distribution patterns of species. More recently, however, phylogeographic analyses have accumulated evidence that many species may have found shelter in multiple small, isolated patches with milder climatic conditions (so-called 'refugia within refugia', Gómez & Lunt 2007). This scenario is more in line with the observed level of genetic variation and population sub-structuring in a wide variety of organisms, including amphibians, from the Apennine (e.g., Canestrelli & Nascetti 2008), the Iberian (e.g., Martinez-Solano et al. 2006) and the Balkan Peninsula (e.g., Sotiropoulos et al. 2007). Refugia are habitats with

space and time dimensions that operate on evolutionary time-scales and have facilitated the survival of species under changing conditions for millennia (Keppel et al. 2012). Consequently, identifying refugia, not only plays an important role in understanding the evolutionary history of the world's biota, but could even contribute to protecting it against future climate change (Keppel et al. 2012). Furthermore, the high genetic diversity of populations that thrive in areas that acted as refugia during environmental instabilities, might represent the overall survival potential of a species, hence these populations may be the key for long-term species persistence (Conner & Hartl 2004).

In this study, we focus on the alpine salamander, *Salamandra atra*, a species endemic to the Alps, and the Dinarides (Fig. 1). Four subspecies have been described: (i) the completely melanistic (black) nominal subspecies, *S. a. atra* Laurenti, 1768 from the Alps; two subspecies inhabiting the Italian Prealps, i.e. (ii) *S. a. aurorae* Trevisian, 1982 and (iii) *S. a. pasubiensis* Bonato & Steinfartz, 2005, that are differentiated by the amount and pattern of yellow patches on the body (see Bonato et al. 2018); and the fourth subspecies, also completely melanistic, (iv) *S. a. prenjensis* Mikšić, 1969, which consists of several isolated population fragments in the Dinarides. However, the validity of *prenjensis* has been debated for a long time (Klewen 1988, Grossenbacher 1994, Guex & Grossenbacher 2003, Bonato & Steinfartz 2005, Dubois & Raffaëlli 2009, Speybroeck et al. 2010), although recent (evolutionary) studies support its separate taxonomic status (Helfer 2010, Razpet et al. 2016, Bonato et al. 2018). Morphometric comparisons between selected populations of alpine salamanders suggest that *prenjensis* could have a larger head and jaws than the nominal subspecies (Šunje et al. 2019), and, allegedly, it has a different arrangement of the palatal teeth (Mikšić 1969, Krizmanić 1997). Although the contact zones between *S. a. atra* and *S. a. prenjensis* have not been defined, both subspecies are found in the Slovenian Alps (Helfer 2010, Razpet et al. 2016). From there, population fragments of alpine salamanders are found across the Dinarides, more specifically at (from north to south): the mountain group of Trnovski gozd (Slovenia: Trnovski gozd, Hrušica, Menešija, Nanos) and Snežnik (Slovenia, Razpet et al. 2016), Gorski Kotar, Žumberak, Istria and Plitvice (Croatia, Jelić et al. 2012), the mountains Prenj and Čvrsnica (Bosnia and Herzegovina, Šunje & Lelo 2010) and in the massive of Prokletije (Montenegro/Kosovo/Albania, Krizmanić 1997). Very recently, a new population fragment was discovered on Mt. Orjen (Montenegro, Cikovac & Ljubisavljević 2020, Fig. 1).

Dinaric samples of *S. atra* have been included in previous phylogenetic studies on alpine salamanders: from the initial works of Ribéron et al. (2001, 2004) and Bonato & Steinfartz (2005), up to the more recent studies of Helfer (2010) and Bonato et al. (2018). However, the number of sampled specimens was invariably small and sampling was restricted to one or two locations in the Dinarides, hence the phylogenetic and phylogeographic rela-

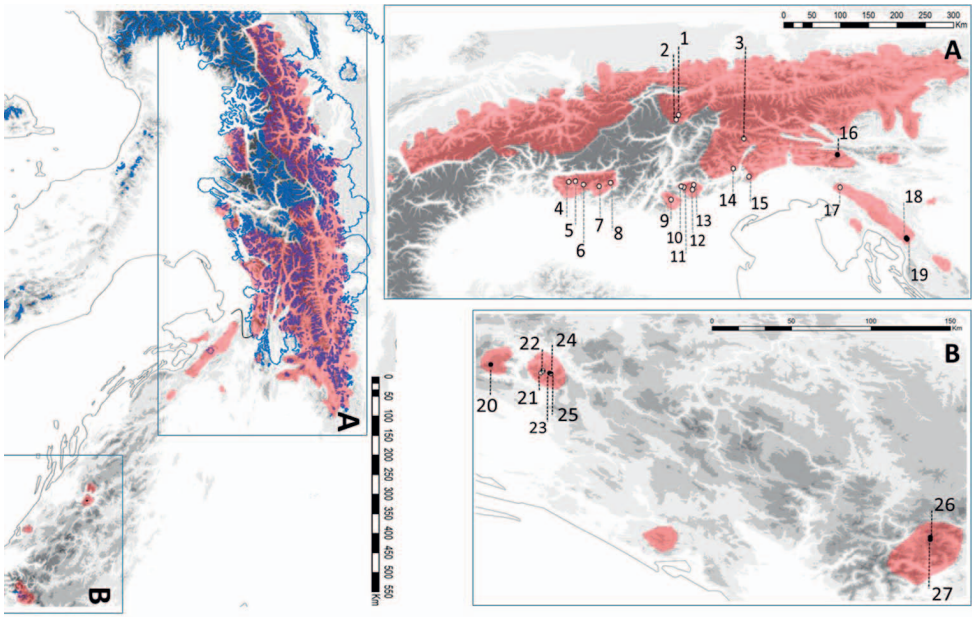

Figure 1. Distribution of *Salamandra atra* with sampling sites (modified from Bonato et al. 2018). Bordeaux polygons represent the known occurrence areas of *S. atra*, and the light blue lines are margins of the glaciers at their maximum extent during the last glacial period (Lgm, in the beggining of the Pleistocene, Ehlers & Gibbard 2004); the grey color variation reflects altitude values, with higher altitudes being darker; the curve represents the border between the Dinarides and the Alps. A – Alpine, Prealpine, and North-Dinaric part of the distribution range. B – Distribution in the Central (Mt. Čvrsnica and Mt. Prenj) and Southern (Mt. Orjen and Prokletije Mts.) Dinarides. Full symbols are sampling sites from this study; empty symbols respresent sequences retrieved from public repositories (see text for details); code 22 (in grey) is both, sampled in this study and in Bonato et al. 2018.

tionships among population fragments remained obscure. Hereby we present the first comprehensive study which includes most of the known population fragments of alpine salamanders in the Dinarides. Using both microsatellite and mitochondrial (mt) DNA markers, we describe the evolutionary relationships, genetic structure and levels of differentiation among and within these isolated population fragments. Further, as many taxa in the Balkans evolved following a 'refugia within refugia' scenario (e.g., *Ichtyosaura alpestris*, Sotiropoulos et al. 2007; *Dinaromys bogdanovi*, Kryštufek et al. 2007; *Vipera ammodytes*, Ursenbacher et al. 2008), and few experienced bottlenecks (e.g., *Pinus nigra*, Naydenov et al. 2017), we explore the possibility that such events occurred in the Dinaric population of *S. atra*. Considering the 'refugia within refugia' scenario, we expect a strong genetic subdivision among population fragments due to their isolation in separate refugia. This study contributes to a better understanding of the effects of past climatic oscillations on species confined to the mountainous habitats in the Dinarides, and provides relevant inputs to identify conservation units within the population of *S. atra* along the Dinaric arc.

Material and methods

Sampling

Samples were collected in July and August 2016. We searched for individuals in suitable habitats focusing on the mountain areas in the Dinarides where *S. atra* has been confirmed: from north to south – Gorski Kotar (Samarske stijene and Vihoraški put), Mt. Čvrsnica (Pločno), Mt. Prenj (Podotiš, Kopilice and Zakantar) and Mt. Prokletije (Bogićevica and Gorazdevac). The sampling of individuals in Kredarica (Julian Alps, sampling site: Aljažev dom – see Table 1) was done in 2010 and is included in this study for comparative purposes (unpublished data). Individuals were found on the soil or within shelters during favorable weather conditions (late night, early-morning or after intense rainfall). Small tail clips were taken from a total of 95 individuals (Table 1). Clips were preserved in 96% ethanol.

DNA extraction

Total genomic DNA was extracted using the NucleoSpin® Tissue kit (Macherey-Nagel, Düren, Germany). DNA extraction from the samples of Kredarica was done in 2010 using the protocol from Taggart et al. (1992). All samples were brought to working concentrations by making 10-fold dilutions.

mtDNA markers amplification and sequencing

Two mitochondrial (mt) DNA markers were analyzed: the control region (*D-loop*) and cytochrome b gene (*cob*). Both regions were amplified and sequenced in two parts, using the Saat-H339-dloop/Saat-LPro-short (340 bp amplicon size) and L-Pro-ML/H-12S1-ML (820 bp) primers for *D-loop*, and the Sa_Cytb_12F/ Saat-H550 (560 bp) and Saat-L129/SaatCytb1046R (920 bp) primers for *cob*. All primers were designed by Bonato et al. (2018), except for L-Pro-ML/H-12S1-ML primer pair which was designed by Steinfartz et al. (2000). PCRs were performed in 25 µl volumes, following the protocols of Bonato et al. (2018). The PCR products were sent to Macrogen Europe (Amsterdam, The Netherlands) for Sanger sequencing (using the forward PCR primers).

The sequences were inspected in Jalview 2.9.0b2 (Waterhouse et al. 2009) and manually corrected when needed; sequences were concatenated in Notepad (Microsoft), aligned using ClustalW (Thomson et al. 1994) and trimmed in Bioedit v5.09 (Hall 1999) to harmonize their length across the alignment. The protein-coding *cob* fragment was translated using the Translate tool on the ExPASy server (Gasteiger et al. 2003) to check for open reading frames. The obtained sequences were submitted to GenBank under the accession numbers (Acc. No.) MN255339 – MN255350 and MN255326 – MN255337 (*cob* and *D-loop* respectively). Haplotype combinations (concatenated *cob* and *D-loop*) are given in Supplementary Table S1.

Microsatellites genotyping

Six nuclear microsatellite loci were amplified: SalE6, SalE7, SalE8, SalE12, SalE14 and SalE23 (Steinfartz et al. 2004) in two multiplex and one single reactions (Supplementary Table S2). Each PCR reaction (total volume 10 µl) contained optimized concentrations of each primer pair (Supplementary Table S2), 1 × PCR buffer (20 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% (v/v) Nonidet P40, 0.5% (v/v) Tween 20, 50% (v/v) glycerol), 2 mM $MgCl_2$, 0.25 mM dNTPs, 1 U TaqNovaHS Polymerase (Blirt, DNA Gdansk, Poland) and 1 µl of DNA. All PCR reactions were done in Veriti Thermocycler (Applied Biosystems) following the thermal protocols given in Supplementary Table S2. Genotyping was performed on the Genetic Analyzer 3500 Series (Applied Biosystems). PCR products from Mix 2 and the single reaction (SalE14) were pooled (1 µl each) and jointly genotyped. Allele scoring was done in GeneMapper v.5 using Liz 500 Size Standard (Applied Biosystems).

Phylogenetic and phylogeographic analysis

DnaSP 6.0 (Rozas et al. 2017) was used to access the concatenated mitochondrial sequence haplotypes (*cob* and *D-loop*). These haplotypes were analyzed together with 24 other haplotypes (concatenated *cob* + *D-loop*) retrieved from published sequences (mainly Bonato et al. 2018, but also Vences et al. 2014, and Steinfartz et al. 2000 for some *cob* sequences; see Supplementary Table S1) from 118 individuals distributed over 19 sites across the species

Table 1. Dataset details and detected haplotypes (Hap) – Codes are as in Figure 1; codes from 17 to 27 are located in the Dinarides; bold codes are samples from this study, others are retrieved from public repositories (see text for details); code 22 is both: * – sampled in this study, and also ° – taken from Bonato et al. (2018). The number of *S. atra* individuals included in the analyses of microsatellites (μs) and concatenated – *cob* + *D-loop* – dataset (mt) is given in the respective columns; "/" – no data. Hap codes are as in Figure 2. At code 23, heteroplasmy (H6 + H12) was detected in one sample, hence four sequences were generated although only three individuals were sequenced. B&H – Bosnia and Herzegovina.

Code	Country/ Sampling area	Sampling site	μs	mt	Hap (number of individuals)	Lineage
1	Italy / Breonie	Pflerschtal	/	6	H18 (1), H19 (1), H20 (4)	*S.a.atra*
2	Italy / Breonie	Ridnauntal	/	6	H19 (5), H20 (1)	*S.a.atra*
3	Italy / Dolomites	Valle Fiscalina	/	1	H25 (1)	H25
4	Italy / Orobie	Val Tronella	/	1	H29 (1)	Orobie
5	Italy / Orobie	Val Terzera	/	8	H30 (4), H31 (4)	Orobie
6	Italy / Orobie	Laghi Gemelli	/	8	H27 (6), H28 (1), H32 (1)	Orobie
7	Italy / Orobie	Pizzo della Presolana	/	1	H27 (1)	Orobie
8	Italy / Orobie	Val di Scalve	/	9	H26 (1), H27 (8)	Orobie
9	Italy / Pasubio	Val Fontana d'Oro	/	13	H14 (9), H15 (4)	*S.a.pasubiensis*
10	Italy / Sette Comuni	Bosco del Dosso	/	16	H21 (15), H23 (1)	*S.a.aurorae*
11	Italy / Sette Comuni	Monte Fossetta	/	1	H21 (1)	*S.a.aurorae*
12	Italy / Sette Comuni	Val di Nos	/	13	H21 (11), H22 (2)	*S.a.aurorae*
13	Italy / Sette Comuni	Val Postesina	/	1	H21 (1)	*S.a.aurorae*
14	Italy / Belluno	Schiara-Val dell'Ardo	/	15	H24 (15)	H24
15	Italy / Cansiglio	Pian di Landro-Baldassare	/	3	H24 (3)	H24
16	Slovenia / Kredarica	Aljažev dom	8	8	H16 (1), H17 (7)	*S.a.atra*
17	Slovenia / Trnovski Gozd	Trnovski Gozd	/	1	H12 (1)	*S.a.prenjensis*
18	Croatia / Gorski Kotar	Samarske stijene	7	4	H1 (1), H2 (1), H3 (1), H4 (1)	*S.a.prenjensis*
19	Croatia / Gorski Kotar	Vihoraški put	10	5	H4 (4), H5 (1)	S.a.prenjensis
20	B&H / Čvrsnica	Pločno	20	9	H8 (8), H12 (1)	*S.a.prenjensis*
21	B&H / Prenj	Soplje	/	7	H11 (2), H12 (4), H13 (1)	*S.a.prenjensis*
22	B&H / Prenj	Zakantar	7	3*+ 3°	H9 (1°), H10 (1°) H12 (2*), H13 (1°+1*)	*S.a.prenjensis*
23	B&H / Prenj	Kopilice	7	3	H6 (1), H12 (3)	*S.a.prenjensis*
24	B&H / Prenj	Podotiš	11	4	H11 (1), H13 (3)	*S.a.prenjensis*
25	B&H / Prenj	Sedlo	/	5	H11 (1), H12 (3), H13 (1)	*S.a.prenjensis*
26	Montenegro / Prokletije	Bogićevica	22	9	H7 (9)	*S.a.prenjensis*
27	Montenegro / Prokletije	Gorazdevac	3	1	H7 (1)	*S.a.prenjensis*

distribution range (Fig. 1, Table 1; the haplotypes of these individuals were provided by B. Crestanello – Table S1). In total, 164 individuals were included in the phylogenetic and phylogeographic analysis (Table 1).

To test for phylogenetic congruence, we performed a partition homogeneity test (Farris et al. 1995) in Paup 4.0a (Swofford 2002) with 1,000 replicates and heuristic search using the TBR branch swapping algorithm. Partitioning of the *cob* gene fragment (on 1st, 2nd and 3rd codon position) was done in DAMBE (Xia & Xie 2001), whereas the non-protein coding *D-loop* fragment was not partitioned. DAMBE was also used to conduct a substitution saturation test (Xia et al. 2003) on the two mtDNA gene fragments.

To build phylogenetic trees, sequences of *D-loop* and *cob* from *Salamandra corsica* Savi, 1838 (Acc. No.: MF043388) and *S. lanzai* Nascetti, Andreone, Capula & Bullini, 1988 (Acc. No.: MF043391) were used as outgroups (following Bonato et al. 2018). Phylogenetic relationships were estimated using maximum likelihood (ML) and Bayesian inference (BI). ML analyses were conducted in RAxML v. 8.2 (Stamatakis 2014) accessed through the CIPRES Science Getaway (Miller et al. 2010) using the GTRGAMMA model with 1,000 bootstraps. BI analyses were done in MrBayes v. 3.1.2 (Ronquist & Huelsenbeck 2003) using the best-fit nucleotide substitution model that was selected on the partitioned datasets and *D-loop* by the corrected Akaike Information Criterion (AICc) implemented in JModeltest 2.1.7 (Darriba et al. 2012) as suggested by Burnham & Anderson (2004). The analysis was run twice for 2 x 10^6 generations, with four parallel chains and sampled every 500 generations.

The MCMC convergence was checked every 1,000 generations by the output of standard deviation of split frequencies. The resulting tree was constructed from 6,000 trees sampled from the posterior distribution, once the first 2000 trees were excluded as burn-in (25%). Trees were visualized and edited with FigTree v. 1.3.1 (Rambaut 2009).

Sequence divergence and geographical distribution of the concatenated haplotypes (invariable sites removed) were analyzed using unrooted median-joining networks (Bandelt et al. 1999) constructed with Popart (http://popart.otago.ac.nz).

Genetic distances among lineages

Arlequin 3.5 (Excoffier & Lischer 2010) was used to calculate the uncorrected pairwise genetic distances (pi; simple p distances) for the concatenated mtDNA sequences among the lineages of *S. atra* inferred from the phylogenetic analysis (see previously). The analysis was done under 10,000 permutations and a significance level of 0.05.

Inferring the number of populations

Using STRUCTURE 2.3.4 (Pritchard et al. 2010), the microsatellites were analyzed to group the sampled individuals of *S. atra* in clusters (*K*) which we define as populations. Following Bonato et al. (2018), exploratory analyses, with *K* values between two and nine (the total number of sampling sites) were conducted with five simulations (100,000 iterations, 25,000 burn-in) implementing different combinations of ancestry models (admixture vs. no admixture) and allele frequency models (correlated vs. independent). The final analysis was performed under the admixture model and assuming correlated allele frequencies among populations (following Razpet et al. 2016). The admixture model was chosen due to its flexibility (Pritchard et al. 2010), while the correlated allele frequency model was chosen because previous results showed genetic similarity between Dinaric population fragments (Helfer 2010, Razpet et al. 2016, Bonato et al. 2018). The analyses were run without using the information on sampling sites, and for *K* values from 2 to 10, running 20 simulations (500,000 iterations, 125,000 burn-in). The most likely number of clusters was estimated by means of likelihood ratios (Evanno et al. 2005) in STRUCTURE Harvester (Earl & von Holdt 2012).

Using Genetix 4.05 (Belkhir et al. 1996–2004), we performed a factorial correspondence analysis (FCA) on sampled individuals also to reveal potential population clusters and their genetic variation.

Genetic variation within populations

Microsatellite data were were checked for genotyping errors with Micro-checker 2.2.3 (Van Oosterhout et al. 2004). Further analyses were performed in Arlequin. Deviation from Hardy-Weinberg equilibrium (HWE) was tested using the exact test (Guo & Thompson 1992). A linkage disequilibrium test was run between pairs of microsatellite loci for each population with a likelihood-ratio test (Excoffier & Slatkin, 1998; with 10,000 permutations). Levels of significance (alpha = 0.01, see Wigginton et al. 2005), were adjusted using the standard Bonferroni correction. For each population we estimated the observed and expected heterozygosity, the mean number of alleles per locus and the inbreeding coefficient (Fis; tested using 10,000 permutations of alleles between individuals within each population). The PopGenReport R package (Adamack & Gruber 2014) was used to assess the mean allelic richness of each population adjusted for sample size (using a rarefaction procedure). The number of private alleles per population was obtained using the StrataG R package (Archer et al. 2016).

mtDNA data: For the concatenated mtDNA sequences, the number of (private) haplotypes, haplotype diversity, number of polymorphic sites, and nucleotide diversity were computed in Arlequin.

Genetic differentiation among populations

The overall genetic variation was partitioned into within and among population components with an analysis of molecular variance (AMOVA, Excoffier et al. 1992) using Arlequin 3.5 (Excoffier & Lischer 2010); populations (as after STRUCTURE) were treated as one single group of data (one species, i.e. *S. atra*). The degree of among population divergence was estimated by pairwise Fst (microsatellite data, Weir & Cockerhiam 1984). Differentiation among mtDNA sequences was estimated by correcting for multiple hits with the method of Tamura & Nei (1993) because this is the alternative for the HKY + I best fit evolutionary model (unavailable in Arlequin) that is detected within the *cob* gene (also following Bonato et al. 2018). The significance of the differentiation values was assessed after 10,000 random permutations (Excoffier et al. 1992) in Arlequin.

A Mantel test was conducted using ade4 R package (Dray & Dufour 2007) with 9,999 permutations to evaluate whether the level of genetic differentiation among populations is correlated with their geographic distance.

Demographic tests and bottleneck

DnaSP 6.0 (Rozas et al. 2017) was used to compute Strobeck's S, Fu and Li's statistics and Tajima D test, for which we specified the coding (*cob*) and non-coding (*D-loop*) part of the sequence. The Tajima D test, combined with Fu and Li's statistics, tests the hypothesis of range expansions, while Strobeck's statistics is relevant for the estimation of the number of existing haplotypes in relation to the number of recorded ones.

The bottleneck hypothesis was tested for the inferred populations, using the one-tailed Wilcoxon test for heterozygosity excess implemented in Bottleneck 1.2.02 (Piry et al. 1999) under different mutation models (infinite allele – IAM, two-phased – TPM, stepwise mutation – SMM). The TPM model was tested with a variance of 14 and a proportion of SMM between 70 and 95% (Piry et al. 1999).

Results

Phylogenetic and phylogeographic relationships

To obtain a final consensus sequence including outgroups, we had to trim our concatenated sequences by 10 bp (the first two of *cob* and the last eight from the *D-loop*). The final sequence included 1,657 bp (*cob* = 962 bp, *D-loop* = 695 bp). To achieve the same length, sequences of Bonato et al. (2018) had to be also trimmed, which caused several of their haplotypes to collapse with ours (Supplementary Table S1). Finally, 32 distinct haplotypes were generated (Supplementary Table S1). The partition homogeneity test revealed no significant incongruence between *cob* and *D-loop* (p = 1). The substitution saturation test showed that *cob* partitions and *D-loop* are suitable for phylogenetic analysis. jModelTest revealed that *cob* evolution is best described under the models TPM2, HKY, HKY+I (1st, 2nd and 3rd position respectively), while the *D-loop* evolution is under the TPM2UF + G model. The standard deviation of the split frequencies after 2×10^6 generations was 0.009 which is a good indication of convergence (Ronquist et al. 2011).

The clusters in the phylogenetic trees follow the ones in the haplotype network (Fig. 2) corresponding to those previously revealed by Bonato et al. (2018). The BI and ML revealed the same tree topology, suggesting a basal split between the Orobie population and all the others, and an unresolved polytomy between individuals from the Dolomites (H25), the populations in the Venetian Prealps (H24 – Belluno and Cansiglio) and the clade uniting the populations assigned to the four subspecies (Fig. 2). The latter clade separated populations from the Pasubio and Dinarides on one side, and the populations of Sette Comuni and the Alps on the other, although the monophyly of the first group is not well supported (branch values 0.84/49 – Fig. 2). In conclusion, the phylogenetic analysis revealed seven lineages, two of them being represented by single haplotypes (H24 and H25 respectively; Table 1, Fig. 2).

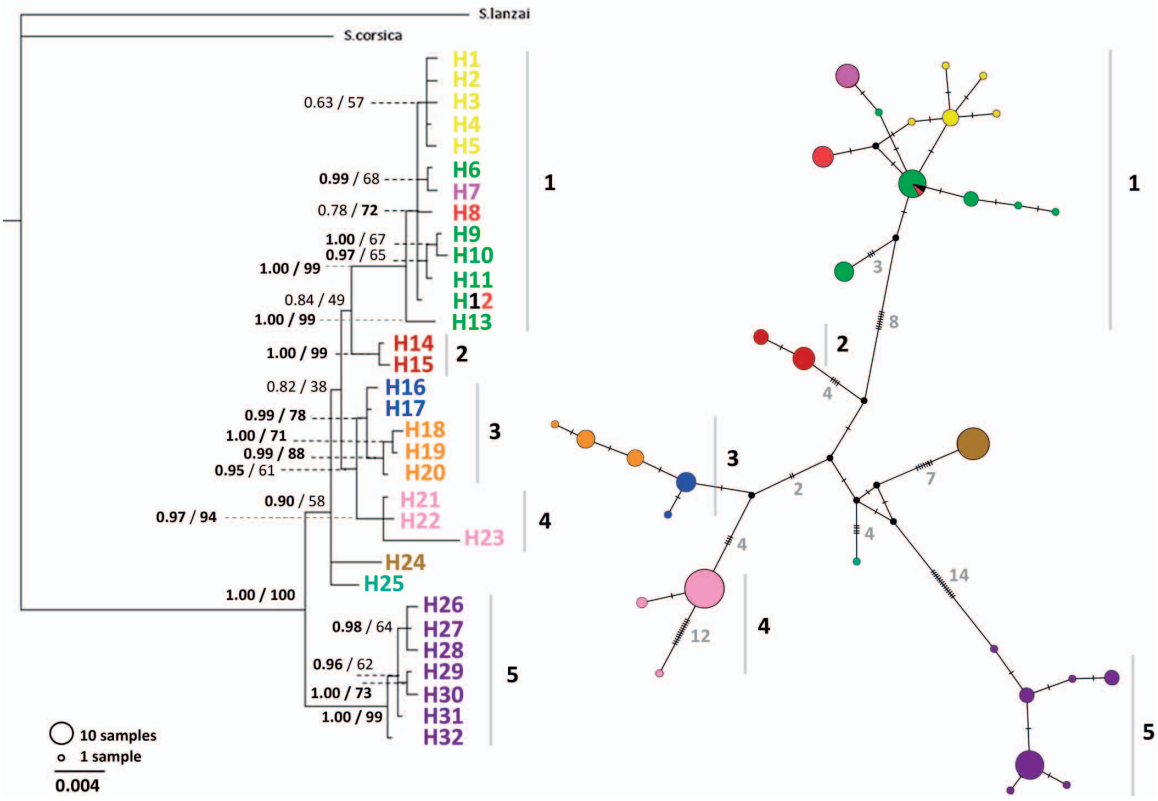

Figure 2. Phylogenetic tree and Median-joining (MJ) network of the 32 haplotypes (H) based on the concatenated *cob* and *D-loop* markers in 164 individuals of *Salamandra atra* (46 samples from this study, and 118 from public repositories) from 27 sampling sites across the species range. Bayesian posterior probabilities (PP) and ML bootstrap values (in percentages from 1000 replications) are shown on the tree nodes separated by a slash ("/"); PP ≥ 0.90 and ML values ≥ 0.70 are bolded (considered high support values). In the MJ, the size of the haplotype (pie chart) is proportional to the number of samples in which it was found (as in the figure legend). The colors of haplotypes represent the sampling areas as in Table 1. The analysis revealed seven lineages : 1 – *S. a. prenjensis* (Dinaric clade, from north to south: black – Trnovski Gozd, yellow – Gorski Kotar, red – Čvrsnica, green – Prenj, pink – Prokletije); 2 – *S. a. pasubiensis* (Pasubio clade); 3 - *S. a. atra* (Alpine clade); 4 – *S. a. aurorae* (Sette Comuni clade); 5 – the Orobie clade potentially belonging to a new, yet undescribed subspecies; two lineages are represented by a single haplotype (H24 – brown, H25 – turquoise). The dashes along the branches of the MJ network represent the minimum number of substitutions between haplotypes that is also indicated by a (grey) number if different from one. Black circles in the MJ network represent missing haplotypes.

Table 2. Uncorrected p distances (pi) among *Salamandra atra* lineages; H24 – Venetian Prealps (Belluno and Cansiglio), H25 – Dolomites, as in Table 1. Above diagonal: average pi between lineages; diagonal elements: average pi within lineages; below the diagonal are the p – values for estimates between the lineages (significant ones are in bold).

	Orobie	*S. atra prenjensis*	*S. atra atra*	*S. atra aurorae*	*S. atra pasubiensis*	H25	H24
Orobie	1.28	27.29	23.05	20.45	22.20	22.00	25.00
S. atra prenjensis	**0.00**	3.03	15.53	16.19	14.53	16.22	16.44
S. atra atra	**0.00**	**0.00**	1.18	6.50	9.36	9.05	13.05
S. atra aurorae	**0.00**	**0.00**	**0.00**	0.90	11.76	11.45	15.45
S. atra pasubiensis	**0.00**	**0.00**	**0.00**	**0.00**	0.46	10.31	14.31
H25	**0.03**	1.00	1.00	0.06	0.08	0.00	12.00
H24	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	1.00	0.00

Genetic distances among lineages

According to the detected lineages from the previous analysis, we merged the sequences from the Dinarides (Trnovski Gozd, Gorski Kotar, Čvrsnica, Prenj and Prokletije – sampling areas as in Table 1) in a single group named *S. atra prenjensis*; the sequences from Kredarica (Julian Alps, code 16 in Fig. 1) were merged with the sequences from Breonie (codes 1 and 2 in Fig. 1) as these are part of the same lineage (*S. a. atra,* Table 1). Following the same principle we defined the other groups representing the inferred lineages (*S. a. aurorae*, *S. a. pasubiensis*, Venetian Prealps – H24, Dolomites – H25, Orobie, Table 1). The analysis of p-distances showed that all lineages are significantly distant among each other (Table 2) except H25 which differs significantly only from the Orobian lineage (p = 0.03, Table 2). However, taking into consideration that only one individual represents lineage H25 in the Dolomites (code 3, Table 1) the estimated distance between this and other lineages (Table 2) must be taken with precaution.

Summary statistics for samples originating from this study

A total of 95 individuals of *S. atra* were typed for six microsatellite loci (Table 1). All loci were polymorphic, with seven to 12 alleles per locus (mean = 8.7, Supplementary Table S2). No null alleles, allelic dropout, or stuttering were detected for any locus. Raw microsatellite data are given in Supplementary Table S3.

The alignment of the concatenated (consensus) mtDNA sequences had a length of 1667 bp (964 bp *cob* + 703 bp D–loop) and comprised 46 individuals from nine sampling sites (Table 1). Within the *D-loop*, heteroplasmy was observed (506 bp, C-T) in one individual from Prenj (sampled in Kopilice), therefore the two alternative copies of this sequence were kept (Accession numbers: MN255332 and MN255334). The Dinaric samples involved 13 haplotypes (Fig. 2), with 28 polymorphic nucleotide positions (1.6%; including nine transitions, four transversions, one indel and 14 substitutions [0.8%]).

Inferring the number of populations

All exploratory model combinations performed in STRUCTURE proposed five clusters (Fig. 3, Supplementary Fig. S1). Although each cluster (population) mainly corresponds to a separate mountain area (from north to south: Kredarica (Julian Alps), Gorski Kotar, Mt. Čvrsnica, Mt. Prenj, Mt. Prokletije), the clusters are not completely differentiated because some individuals in each population, resemble individuals from others with a high probability (especially noted in Gorski Kotar, Čvrsnica [1st sample] and Prokletije, Fig. 3).

The first FCA axis revealed two main groups: the first is more variable and contains only the samples from the Julian Alps (Kredarica), and the second groups all the Dinaric samples. Within the second group, four fairly distinct clusters can be recognized, each corresponding to a separate mountain area (Fig. 4). The combination of the three FCA axes accounted for 24.02% of the total variation. No clear substructure was detected among individuals of the same mountain area (clustering of individuals from different sampling sites within the same mountain area, Supplementary Fig. S2).

Genetic variation within populations

Estimated diversity indices for both microsatellites and mtDNA are shown in Table 3. Only the population of Prokletije deviated from HWE for the locus SalE7 (p = 0.0001, after Bonferroni correction). No deviations from linkage equilibrium were detected.

The observed microsatellite heterozygosity for Dinaric specimens varied between 0.31 and 0.39, but was nearly twice as high in the Alpine population (Kredarica; Table 3). The highest and only significant inbreeding coefficient (Fis) was recorded in Gorski Kotar (0.19, p = 0.009, Table 3).

The mtDNA diversity (pS, Hap, pHap, Hd in Table 3) was higher in Prenj and Gorski Kotar, and lower in Prokletije, Čvrsnica and Kredarica. Only one (private) haplotype was found in the population of Prokletije. The highest number of haplotypes (N = 5, all private) was observed in Gorski Kotar, but the highest nucleotide diversity – in Prenj (Nd = 0.18, Table 3).

Table 3. Genetic diversity from microsatellite data (μs – in grey) and mtDNA haplotypes (concatenated *cob* and *D-loop* – in white) in the populations (Pop) of *Salamandra atra* from this study; N – number of individuals (total microsatellites – N = 95, total mtDNA – N = 46), Ho – observed heterozygosity, He – expected heterozygosity, mA – mean number of alleles, R – allelic richness, pA – number of private alleles, Fis – inbreeding coefficient (bold if significant); pS – number of polymorphic sites, Hap – number of haplotypes, pHap – number of private haplotypes, Hd – haplotype diversity, Nd – nucleotide diversity (averaged over the two gene fragments). Numbers in brackets are standard deviations.

Pop	N	Ho	He	mA	R	pA	Fis	N	pS	Hap	pHap	Hd	Nd (%)
Kredarica	8	0.58 (0.25)	0.58 (0.19)	3.83 (0.98)	3.60	10	-0.002	8	1	2	2	0.25 (0.18)	0.02 (0.02)
Gorski Kotar	17	0.31 (0.21)	0.39 (0.26)	3.83 (1.47)	2.93	7	**0.19**	9	5	5	5	0.72 (0.16)	0.05 (0.05)
Prenj	25	0.39 (0.28)	0.36 (0.28)	3.50 (2.81)	2.78	4	-0.07	10	7	4	3	0.71 (0.10)	0.18 (0.11)
Čvrsnica	20	0.31 (0.32)	0.30 (0.27)	3.33 (1.75)	2.40	3	-0.03	9	2	2	1	0.22 (0.17)	0.03 (0.03)
Prokletije	25	0.33 (0.35)	0.37 (0.28)	3.83 (1.72)	2.72	5	0.09	10	0	1	1	0	0

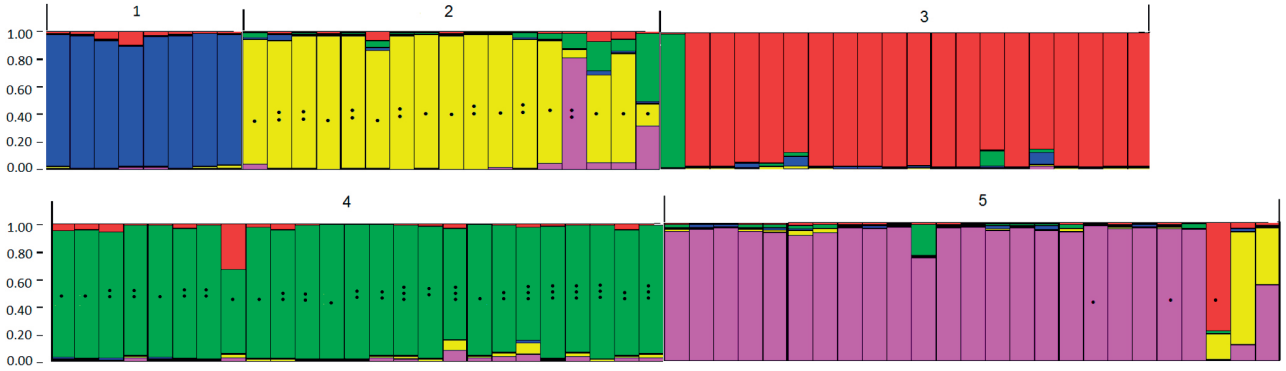

Figure 3. Results of the STRUCTURE assignment of 95 individuals of *Salamandra atra* (from this study) based on six microsatellite loci; clusters (populations) are divided by tick black lines as follows: 1 – Kredarica, 2 – Gorski Kotar (**.** – Vihoraški put, **..** – Samarske stijene), 3 – Čvrsnica, 4 – Prenj (**.** – Kopilice, **..** – Podotiš, **...** – Zakantar), 5 – Prokletije (no dot – Bogićevica, **.** – Gorazdevac,). Compared to the Kredarica population (population 1 – in the Julian Alps), the Dinaric ones (populations – 2–5) are less differentiated, as some individuals in each population resemble individuals from others.

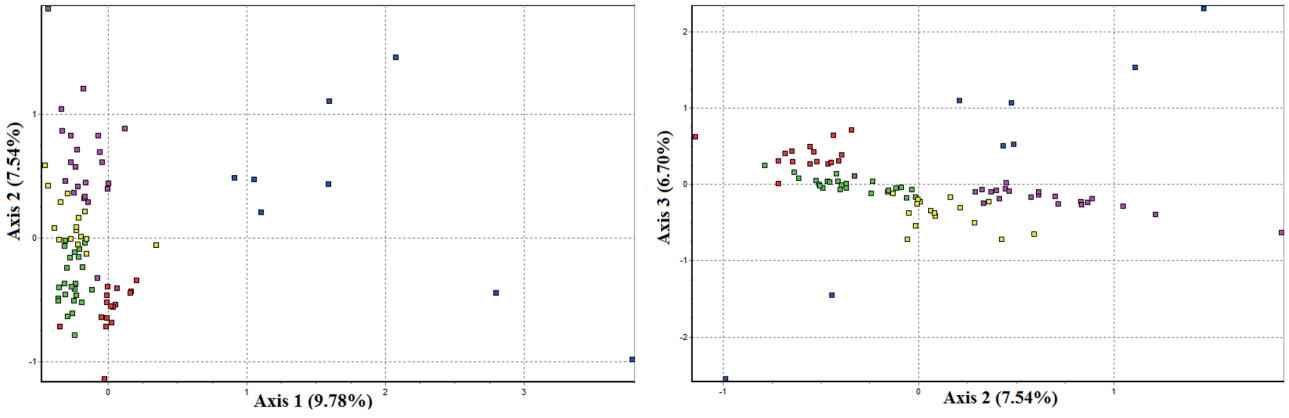

Figure 4. Factorial Correspondence Analysis (FCA) ordination along the first three axes of 95 individuals of *Salamandra atra* from four sampling areas in the Dinarides (following Table 1) and one in the Julian Alps (Kredarica), based on six microsatellite loci. Each label corresponds to a sampling area (from north to south): blue – Kredarica, yellow – Gorski Kotar, red – Čvrsnica, green – Prenj, pink – Prokletije. Axis 1 separates the Dinaric individuals on one side, and the ones from Kredarica on the other; Axis 3 separates Gorski Kotar (Northern Dinarides) from the other sampling areas in the Dinarides (Central and Southern – see also Supplementary Fig. S2).

Genetic differentiation among populations

With the microsatellite data, the among-population differentiation accounted for a significant 35.09% of the total variation (Amova, $p < 0.001$). Pairwise Fst values ranged from 0.28 (between Gorski Kotar and Prenj) to 0.46 (between Prokletije and Čvrsnica) and were all significant ($p < 0.001$, Table 4).

With the mtDNA data, the among-population differentiation accounted for a significant 87% of the total variation (Amova, $p < 0.001$). Pairwise mtDNA values (φ values – Tamura & Nei 1993) between populations ranged from 0.43 to 0.99, and were all significant ($p < 0.001$, Table 4). The strongest differentiation was observed between Kredarica and all other populations; within the Dinarides, mtDNA showed the highest differentiation between Čvrsnica and Prokletije (0.94) and the lowest between Prenj and Gorski Kotar (0.43; Table 4) which is concordant to previously reported pairwise Fst values.

The Mantel tests revealed no correlation between genetic differentiations and geographic distances ($p_{microsatellites} = 0.9$, $p_{mtDNA} = 0.6$).

Demographic tests and bottleneck

The Tajima D test could not reject neutral mtDNA sequence evolution (coding region: $D = -0.65$, $p > 0.10$; synonymous sites: $D = -0.16$, $p > 0.10$; nonsynonymous sites: $D = -0.24$, $p > 0.10$; silent sites: $D = -0.24$, $p > 0.10$), and thus did not provide evidence of range expansions. The results of the Tajima D test were in line with those of Fu and Li's statistic, which neither could reject neutrality ($D = -1.27$, $p > 0.10$; $F = -1.24$, $p > 0.10$). Strobeck's S statistic predicted a higher number of haplotypes than it has been observed ($S = 1$, $p = 0.00$).

The bottleneck tests were unable to demonstrate that such events occurred in our populations (IAM: $p \geq 0.50$, Smm: $p \geq 0.96$, Tpm: $p \geq 0.44$).

Table 4. Pairwise divergence between populations of *S. atra* from this study; pairwise Fst for microsatellite loci is above the diagonal, and differentiation among mtDNA loci (*cob* + *D-loop*) is below the diagonal (φ values – Tamura & Nei 1993, see text for details); all values (for both microsatellite and mtDNA) were significant ($p < 0.001$).

	Kredarica	Gorski Kotar	Prenj	Čvrsnica	Prokletije
Kredarica	/	0.30	0.36	0.33	0.33
Gorski Kotar	0.96	/	0.28	0.41	0.30
Prenj	0.88	0.43	/	0.35	0.35
Čvrsnica	0.98	0.78	0.53	/	0.46
Prokletije	0.99	0.88	0.61	0.94	/

Discussion

Phylogenetic and phylogeographic relationships – taxonomic implications

All studied populations in the Dinarides belong to a unique evolutionary lineage (Fig. 2). Individuals from the Julian Alps (Kredarica) belong to *S. a. atra* (Table 1, Fig. 2) as it could have been anticipated from the high differentiation values between this and all the Dinaric populations (mtDNA, Table 4), and its high heterozygosity (Table 3) that are characteristic to this lineage when compared to all the others (Helfer 2010, Razpet et al. 2016, Bonato et al. 2018). This study confirmed already observed trends considering the evolutionary separation of the Dinaric population and the delineation of the commonly accepted subspecies: *S. a. aurorae, S. a. pasubiensis* and *S. a. atra* (Helfer 2010, Bonato et al, 2018). Moreover, the study also confirmed the evolutionary separation of the Orobian population of alpine salamanders and the populations from the Venetian Prealps and Dolomites (H24 and H25 respectively, Table 1, see also Helfer 2010, Helfer et al. 2011 and Bonato et al. 2018). However, as our phylogenetic results were inferred from mtDNA data, conclusions need further corroboration, since in *S. atra*, nuclear markers may generate different tree topologies (see Bonato et al. 2018, Ribéron et al. 2004 vs. Helfer 2010). By reconfirming the separate evolutionary history of our Dinaric specimens and by presenting their significant genetic distance from other lineages (Table 2), we justify the subspecific taxon *S. a. prenjensis* Mikšić, 1969 as already proposed by previous authors (Helfer 2010, Razpet et al. 2016, Bonato et al. 2018, Šunje et al. 2019).

In order to fill the remaining gaps in our knowledge about the complex evolutionary history of *S. atra,* we highlight the importance to study other population fragments that are not included in this study; primarily the ones in the Prealps and Dinarides (Fig. 1, see also Helfer 2010).

Inference of populations

Both STRUCTURE and FCA showed that the specimens from this study belong to five populations that mainly correspond to the sampled mountain areas. Populations are well differentiated but not completely, which is especially visible within the Dinaric clusters where in nearly all populations some individuals are genetically more similar to individuals from other populations than to members of their own population (Fig. 3). Considering the large geographic distance among the population fragments and the strong geographic barriers between them (Fig. 1), it is improbable that the incomplete population differentiation is due to current gene flow. Indeed, a lack of gene flow is confirmed by the high and significant differentiation values (Table 4 and Amova). Instead, the incomplete differentiation among the Dinaric specimens may be due to a past connection between the populations and/or a common origin.

The population of Kredarica was the most homogenous (Fig. 3) and most distantly related (Fig. 4) compared to all

Dinaric specimens (*S. a. prenjensis* lineage – Table 1, Fig. 2) which is explained by the fact that it belongs to another lineage (*S. a. atra* – Table 1, Fig. 2).

Distribution of genetic diversity

The observed heterozygosity of the Prokletije population (Ho = 0.33) is twice as high as the Ho reported by Razpet et al. (2016; Ho = 0.17) probably because the latter study involved a smaller number of individuals from this location, two of which were intrauterine larvae related to a third sample (mother). The observed heterozygosity in Prenj was in line with previous observations (Razpet et al. 2016, Bonato et al. 2018: Ho = 0.36 and 0.37 vs. Ho = 0.39 in Table 3). The microsatellite genetic diversity indices were similar in all the Dinaric populations, whereas for mtDNA, the levels of genetic diversity are higher in Gorski Kotar and Prenj, and lower in Čvrsnica and Prokletije (Table 2). On the other hand, the heterozygosity indices for Kredarica, were almost twice as high as in the Dinaric populations (despite a considerably smaller sample size) but, the mtDNA diversity was lower than in Čvrsnica and Prokletije (probably due to a considerable smaller sample size – Tables 1 and 3). A high heterozygosity in Kredarica (both observed and expected) suggests gene flow that may be a consequence of a "leading edge" range expansion characteristic for other populations of *S. atra atra* across the northern and central Alps (Helfer 2010). Just like Razpet et al. (2016) we were unable to detect any signs of bottlenecks.

'Refugia within refugia': a plausible historical scenario for the *prenjensis* lineage

The 'refugia within refugia' hypothesis describes survival patterns in multiple glacial refugia, each within a larger refugial area (Gómez & Lunt 2007). The Balkan refugial area was mostly free of ice during the last glacial maxima (LGM, Fig. 1) but many glaciers persisted during other phases of the Pleistocene at higher altitudes (generally above 1,300 m a.s.l., Cvijić 1899, Hughes et al. 2011, Lepirica 2013, Žebre & Stepišnik 2015), which explains why multiple refugia must have characterized the Balkans. Our results suggest that during the Pleistocene, Dinaric populations of alpine salamanders thrived in at least two refugia: an ancestral in Mt. Prenj and, a more recent, in Gorski Kotar. The diversity pattern in Prenj (the highest Nd, high Hd with many pHap, Table 3) and its central position in the MJ network (Fig. 2), suggest that it represented the main diversification center of alpine salamanders (following Avise 2000). The fact that the lowest mtDNA differentiation is found between Prenj and all other populations (Table 4) supports this idea. Moreover, Razpet et al. (2016) showed that Mt. Prenj is home to the oldest populations of alpine salamanders in the Dinarides (according the estimated time since decline). Gorski Kotar was another diversification center of alpine salamanders in the Dinarides, as is suggested by its high mtDNA diversity, which resemblances the pattern found at Prenj (Table 3). However, the diversification at Gorski Kotar appears to have a more recent origin given its low nucleotide diversity and its star-like network of very similar haplotypes (Fig. 2, following Avise 2000). Hence, Prenj and Gorski Kotar, interpreted as alpine salamander refugia, might not have co-existed. Furthermore, the central haplotype in Prenj (H12) is the most frequent in the '*prenjensis*' clade (Fig. 2, Table 1), suggesting that it is a very old haplotype that was probably distributed over the entire Dinaric range and persisted in big populations but was lost in others during fragmentations. This ancestral haplotype is separated by only one single mutational step from the central haplotype in Gorski Kotar (H4, Fig. 2), suggesting that alpine salamanders at Gorski Kotar originate from Prenj. Nevertheless, the many private haplotypes found in Prenj and Gorski Kotar are also evidence of a long-term population establishment (see Haydar 2011), which supports our conclusion that these areas acted as refugia for *S. atra*.

In contrast, the lower mtDNA diversity and fewer private haplotypes in Čvrsnica and Prokletije (Table 3), suggest that these populations were established more recently (following Austerlitz et al. 1997 and Excoffier & Petit 2009). Considering Čvrsnica, these results are rather unexpected, as this mountain is neighboring Prenj. Although several areas of both mountains were glaciated in the middle Pleistocene (after the LGM; Mt. Čvrsnica – above 1,200 m a.s.l. [Stepišnik et al. 2016], Mt. Prenj – above 1,500 m [Lepirica 2008]) the population of Prenj has a considerably higher mtDNA diversity than Čvrsnica. We suspect that the relatively larger glacier on Čvrsnica (ca. 200 km², see also Miličević 2013) hindered the population stability more than did the glacier on Mt. Prenj. This is especially plausible given that the survival of individuals on Mt. Čvrsnica was thwarted by several barriers: on the east, by the deep canyon of the river Neretva (at the foothill of Mt. Čvrsnica), on the west – by the glaciers Svinjača and Dugo polje (also on Mt. Čvrsnica, Stepišnik et al. 2016), and on the south by the glacier of Mt. Čabulja (south border of Mt. Čvrsnica, see Prskalo 2008). Moreover, the highest differentiation (both Fst and mtDNA) found between Čvrsnica and Prokletije (the southernmost population; Table 4) confirms that there is no dispersal from Mt. Čvrsnica southwards. The missing haplotype link between Čvrsnica and both, Gorski Kotar and Prenj (separated by a single mutational step, Fig. 4) suggests that the stabilization of Čvrsnica occurred from another population (not sampled/not existing) from which dispersal was probably bidirectional, i.e from north (Gorski Kotar) to south (Prenj) and vice versa (Fig. 2). Although speculative, the HWE deviation at SalE7 may be a hint that this area was colonized, as founding events are likely to create persistent non-equilibrium structures (Boileau & Hebert 1991). Individuals that were part of this founding event likely originated from Prenj since the only haplotype registered in Prokletije (H7, Fig. 4) is separated from the Prenj haplotype by only one mutational step (H6, Fig. 2 and Table 1). We conclude that

our results are in line with the 'refugia within refugia' hypothesis that was suggested for several other species in the Balkans (Kryštufek et al. 2007, Sotiropoulos et al. 2007, Ursenbacher et al. 2008, Surina et al. 2011).

Conservation insights for the *prenjensis* lineage

A major conservation task is to define the proper distribution range of *prenjensis*. Strobeck's S statistic and the newly discovered population on Mt. Orjen (Cikovac & Ljubisavljević 2020) suggest that there are populations of alpine salamanders in the Dinarides that are yet to be discovered (see also Klewen 1988). Therefore, field investigations of potential occurrence areas (see in Cikovac & Ljubisavljević 2020) are needed to clarify the distribution range of *prenjensis*. The northernmost locations where *prenjensis* is reported are in the Southern Prealps (Kobarid – Krn, Helfer 2010 and Loibl Pass, Šunje et al. 2019). Since these two areas are relatively close to our sampling site of Kredarica (ca. 20 and 30 km of air distance respectively), several contact zones between *S. a. atra* and *S. a. prenjensis* may be present in the wider areas connecting the three sites. The identification of contact zones is, not only important from an evolutionary perspective and the study of speciation processes (Wollenberg Valero et al. 2019), but it is relevant in a conservation context (Allendorf et al. 2001), since conservation efforts should be preferably oriented towards populations where there is no risk of genetic swamping with other lineages. Genetic swamping may cause outbreeding depression (Frankham et al. 2011), that, at it's extreme form, could be detrimental for the population (Rhymer & Simberloff 1996). The levels of genetic diversity of the Dinaric populations in this study suggest that these are not of immediate conservation concern, although the significant level of inbreeding in the population of Gorski Kotar (Fis, Table 3), signals a potential threat to its health and must be monitored. The significant differentiation among populations, indicates that each should be treated as a separate conservation unit. As the population of Prenj harbors substantial genetic diversity and no signs of inbreeding (Table 3), we believe that it is in conservation interest to focus the efforts on its preservation on the long term. Another reason to focus conservation efforts on the Prenj population is that Prenj was a refugium for *S. atra* during past environmental perturbances, so that it might also offer the best chances for survival under upcoming climatic change (for the rationale see Keppel et al. 2012).

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Supplementary data

The following data are available online:

Supplementary Figure S1. STRUCTURE exploratory analysis using different combinations of ancestry and allele frequency models

Supplementary Figure S2. Factorial Correspondence Analysis (FCA) ordination along the first three axes of 67 individuals of *Salamandra atra* from the Dinarides based on six microsatellite loci.

Supplementary Table S1. Accession numbers (Acc. No) of the sequences (*cob* and *D-loop*) from public repositories (GenBank) used in this study.

Supplementary Table S2. PCR conditions and characteristics of the microsatellite loci.