Accepted: 25 February 2018

DOI: 10.1111/jzs.12224

ORIGINAL ARTICLE

WILEY JOURNAL OF ZOOLOGICAL SYSTEMATICS AND EVOLUTIONARY RESEARCH

Diversity among peripheral populations: genetic and evolutionary differentiation of *Salamandra atra* at the southern edge of the Alps

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Funding information
Regione Lombardia; Department of Ecology and Evolution of the University of Salzburg; MUSE – Museum of Science; Parco Naturale delle Orobie Bergamasche; Rufford Foundation

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Abstract

Separate populations at the edge of a species range are receiving great attention and have been shown to be often different from populations in the core area. However, it has rarely been tested whether neighboring peripheral populations are genetically and evolutionarily similar to each other, as expected for their geographical proximity and similar ecological conditions, or differ due to historical contingency. We investigated isolation and differentiation, within-population genetic diversity and evolutionary relationships among multiple peripheral populations of a cold-adapted terrestrial salamander, *Salamandra atra*, at the southern edge of the species core range. We carried out population genetic, phylogeographic, and phylogenetic analyses on various molecular markers (10 autosomal microsatellite loci, three mitochondrial loci with total length >2,100 bp, two protein-coding nuclear genes) sampled from more than 100 individuals from 13 sites along the southern Prealps. We found at least seven isolated peripheral populations, all highly differentiated from the remaining populations and differentiated from each other at various levels. The within-population genetic diversity was variable in the peripheral populations, but consistently lower than in the remaining populations. All peripheral populations along the southern Prealps belong to an ancient lineage that is also found in the Dinarides but did not contribute to the postglacial recolonization of the inner and northern Alps. All fully melanistic populations from the Orobian mountains to the southern Dinarides represent a single clade, to the exclusion of the two yellow-patched populations inhabiting the Pasubio massif and the Sette Comuni plateau, which are distinguished as *S. atra pasubiensis* and *S. atra aurorae*, respectively. In conclusion, multiple populations of *S. atra* at the southern edge of the species core area have different levels of differentiation, different amount of within-population genetic diversity, and different evolutionary origin. Therefore, they should be regarded as complementary conservation targets to preserve the overall genetic and evolutionary diversity of the species.

KEYWORDS
genetic differentiation, glacial refugia, peripheral populations, *Salamandra atra*, southern Prealps

1 | INTRODUCTION

Genetic differences between peripheral and core populations have frequently been reported in animal and plant species (Hoffmann & Blows, 1994; Eckert, Samis, & Lougheed, 2008), as predicted by theoretical models due to the persistence of relict intraspecific lineages, stochastic demographic dynamics in small populations, and local adaptive divergence (e.g. García-Ramos & Kirkpatrick, 1997; Bridle & Vines, 2007).

On the one hand, adjacent peripheral populations along the edge of a species range are expected to be genetically similar, especially because they have faced similar climatic regimes and ecological conditions. Additionally, their geographical proximity has provided chances of temporary connection and gene flow (e.g. Slatkin, 1987; Hutchison & Templeton, 1999). On the other hand, fine-scale environmental heterogeneity and historical contingency may also account for significant and unpredictable differences between neighboring peripheral populations (e.g. Taylor & McPhail, 2000; Peterman, Connette, Semlitsch, & Eggert, 2014). Some populations may have separated recently and harbor a minor portion of the total genetic diversity of the species, while others may have undergone a longer and complex history of isolation.

Diversity among multiple adjacent peripheral populations has rarely been explored, even among the many terrestrial animal species studied within the well-known Western Palearctic biotas. Most of these species, especially those with narrow ecological tolerance and low dispersal ability, have repeatedly been forced to shift their ranges as a result of climatic oscillations over the last few million years (e.g. Davis & Shaw, 2001; Hampe & Petit, 2005; Haubrich & Schmitt, 2007).

During the glaciations on the Alps, many habitats were unavailable to the species of the current biota. However, various marginal highlands were non-glaciated, allowing the survival of cold-adapted organisms, especially along the southern and the eastern Prealps, from the Orobian mountains to the Styrian Prealps. These refugial areas have been consistently confirmed for many montane and alpine plants (Tribsch & Schönswetter, 2003; Schönswetter, Stehlik, Holderegger, & Tribsch, 2005; Holderegger & Thiel-Egenter, 2009) as well as for numerous terrestrial animals with low dispersal capacity, such as snails (e.g. Nägele & Hausdorf, 2015) and carabids (e.g. Lohse, Nicholls, & Stone, 2011). Even though many studies addressed both peripheral and core populations of different species, they seldom focused on comparisons between multiple adjacent peripheral populations.

We investigated a terrestrial salamander, *Salamandra atra* Laurenti, 1768, which is widely distributed across the northern and inner areas of the Alps, in large and contiguous populations (e.g. Helfer, Broquet, & Fumagalli, 2012; Werner, Lötters, Schmidt, Engler, & Rödder, 2013), but is also occasionally present along the southern Prealps and the Dinarides (Figure 1). Across the southern Prealps, peripheral populations are usually restricted to the upper highlands (usually >1,000 m a.s.l.) separated by deep valleys of unsuitable habitat, from the Orobian mountains in the west, throughout the Dolomites and the Venetian Prealps, to the Carnic Alps and the Slovenian Prealps in the east (Bonato, Fracasso, & Luiselli, 2007). Some of these peripheral populations are distinguished by skin coloration, which includes yellow blotches, unlike all other populations of the species, which are uniformly black (Figure 2). Previous molecular analyses revealed that these and other peripheral populations are genetically differentiated from the core populations (Ribéron, Miaud, Guyetant, & Taberlet, 2004; Bonato & Steinfartz, 2005; Helfer, Gimeno, Balzarini, Schwarzenbacher, & Ferri, 2011).

The aim of our study was to evaluate differences among multiple peripheral populations of *S. atra* across a 250-km-long sector of the southern edge of the species range. In detail, we aimed at (i) evaluating the degree of genetic differentiation among peripheral populations, (ii) estimating the genetic diversity within each peripheral population, (iii) assessing the phylogenetic relationships between peripheral populations and deriving insights into their history. We employed three types of genetic markers with varying mutation rates and expected to provide complementary information at different time scales; that is, 10 microsatellite loci, three segments of the mitochondrial genome (for a total of more than 2,100 bp), and two protein-coding nuclear genes.

Compared to most populations of *S. atra* inhabiting the remaining regions of the Alps and the Dinarides, detecting and sampling the peripheral populations along the southern Prealps has long been hindered by the low detection probability of the salamanders (Grossenbacher, 1994; Romanazzi & Bonato, 2014). However, intensive field surveys and collaborative efforts during the last decade allowed the assemblage of an unprecedented number of samples for molecular analyses: more than 100 individuals from 13 sites.

2 | MATERIALS AND METHODS

2.1 | Occurrence records

All available records of *S. atra* from the southern Prealps were retrieved from the literature, including local publications with limited distribution, and public digital databases. Sources are listed in Table S1. All records were scrutinized for their reliability and georeferenced. The final database included 289 validated records out of a total of 324.

2.2 | Sampling

Between 2008 and 2015, we searched intensively for individuals of *S. atra* in suitable habitats across the southern Prealps, focusing on the following peripheral uplands: west to east, Orobian mountains, Pasubio massif, Sette Comuni plateau, Belluno Dolomites and Cansiglio plateau. Individuals were found while resting concealed in the soil or under shelters, or when active aboveground under favorable weather conditions (i.e. early-morning dew or intense rainfall). Tail clips or buccal swabs were sampled from 104 individuals from 13 sites (Figure 1; Table 1). Clips were preserved in 95% ethanol, and swabs were frozen at −20°C. For comparative purposes, we also

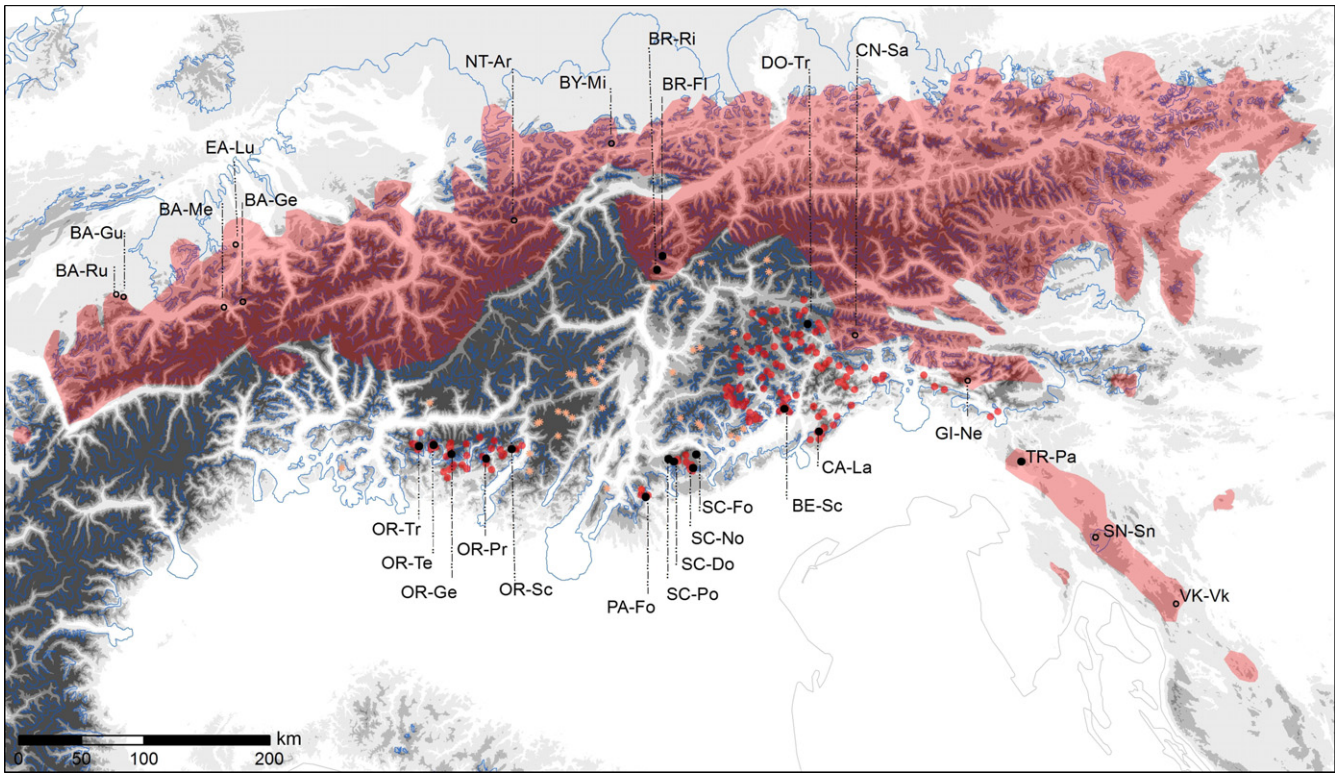

FIGURE 1 Distribution of *Salamandra atra* in the Alps and sampled sites. Pale red areas: core range of the species and fragmented areas along the Dinarides, drawn approximately from major sources (mainly GBIF and IUCN). Red dots: reliable records of peripheral populations along the southern Prealps (see Materials and Methods). Pink asterisks: dubious records. Black dots: sampled sites (codes as in Table 1). Empty dots: sites sampled for previous genetic analyses (codes as in Table S4). The grayscale map is drawn from the NASA elevation model SRTMGL1 (Farr & Kobrick, 2000). The thin blue line indicates the margins of the glaciers at their maximum extent during the last glacial period, ca. 30–18 kya (Ehlers & Gibbard, 2004)

FIGURE 2 Photographs of *Salamandra atra* from some sampled populations: (a) Val Fontana d'Oro-Vajo del Ponte, 1 July 2016, *S. a. pasubiensis*; (b) Bosco del Dosso-Val d'Anime, 14 July 2012, *S. a. aurorae*; (c) Pich, 2 May 2017, *S. a. atra*; (d) Val Ridanna/Ridnauntal, 23 June 2015, *S. a. atra*; (e) Zakantar, 12 September 2010, *S. a. prenjensis*. Photos (a–d) by L. Bonato, (e) by E. Šunje

sampled 16 individuals from four sites in two different areas of the Dinarides (Trnovski gozd and Prenj mountains) and 12 individuals from two sites in an area of the inner Alps (Breonie mountains), which belongs to the core range of the species. Our sampling strategy also included the "type localities" for all described subspecies (Table 1).

TABLE 1 Sampled sites and number of individuals of *Salamandra atra* analyzed for different molecular markers

			Number of individuals								
Site	Code	Subspecies	All	Microsatellites	*16S*	*cytb*	*D-loop*	mt (concatenated)	*CXCR4*	*RAG1*	nu (concatenated)
Southern Prealps											
Orobie: Val Tronella	OR-Tr	*atra*	1	1	1	1	1	1	1	1	1
Orobie: Val Terzera	OR-Te	*atra*	9	8	9	8	8	8	9	9	9
Orobie: Laghi Gemelli	OR-Ge	*atra*	9	8	8	8	8	8	8	8	8
Orobie: Pizzo della Presolana	OR-Pr	*atra*	1	1	1	1	1	1	1	1	1
Orobie: Val di Scalve	OR-Sc	*atra*	9	9	9	9	9	9	9	9	9
Pasubio: Val Fontana d'Oro-Vajo del Ponte[a]	PA-Fo	*pasubiensis*	14	9	14	14	13	13	7	8	7
Sette Comuni: Val Postesina	SC-Po	*aurorae*	1	1	1	1	1	1	1	1	1
Sette Comuni: Bosco del Dosso-Val d'Anime[a]	SC-Do	*aurorae*	20	12	16	19	17	15	10	8	6
Sette Comuni: Val di Nos-Fiaretta-Ghelpach	SC-No	*aurorae*	18	14	17	17	13	13	12	13	12
Sette Comuni: Monte Fossetta	SC-Fo	*aurorae*	2	2	2	2	1	1	2	2	2
Dolomiti Bellunesi: Schiara-Val dell'Ardo	BE-Sc	*atra*	16	16	16	16	15	15	16	16	16
Cansiglio: Pian di Landro-Baldassare	CA-La	*atra*	3	3	3	3	3	3	2	2	1
Northern Dolomites: Tre Cime/Drei Zinnen	DO-Tr	*atra*	1	1	1	1	1	1	1	1	1
Dinarides											
Trnovski gozd: Velika ledena jama v Paradani	TR-Pa	*prenjensis*	1	1	1	1	1	1	0	1	0
Prenj: Soplje[a]	PR-So	*prenjensis*	7	7	7	7	7	7	7	7	7
Prenj: Zakantar[a]	PR-Za	*prenjensis*	3	3	3	3	3	3	3	3	3
Prenj: Sedlo[a]	PR-Se	*prenjensis*	5	5	5	5	5	5	5	5	5
Inner Alps											
Breonie: Val Ridanna/Ridnauntal[a]	BR-Ri	*atra*	6	6	6	6	6	6	6	6	6
Breonie: Val di Fleres/Pflerschtal[a]	BR-Fl	*atra*	6	6	6	6	6	6	6	6	6
Total			132	113	126	128	119	117	106	107	101

Sites are listed approximately west to east. Current subspecies classification is reported. Accession numbers of sequences: see par. 2.4.2. Data on other sequences already available and included in our analyses are given in Table S4.

[a]Sites corresponding to type localities (see Table S5).

2.3 | DNA extraction

Total genomic DNA was extracted manually from clips using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) and from swabs using the QIAamp DNA Investigator Kit (Qiagen), following the manufacturer's instructions for "Animal Tissues" and "Surface and Buccal Swab," respectively. The NucleoMag 96 Trace Kit (Macherey-Nagel, Düren, Germany), coupled with the magnetic particle processor KingFisher Flex (Thermo Fisher Scientific, Waltham, MA, USA), was also used for the DNA extraction from buccal swabs.

2.4 | Amplification and sequencing

2.4.1 | Microsatellites

Ten nuclear tetranucleotide autosomal microsatellite loci were amplified: SalE6, SalE7, SalE8, SalE12, SalE14, Sal23 (Steinfartz, Küsters, & Tautz, 2004); SST-C3, SST-E11, SST-G6, SST-G9 (Hendrix, Hauswaldt, Veith, & Steinfartz, 2010). All loci were originally designed for the related species *Salamandra salamandra* (Linnaeus, 1758) and tested for amplification in *S. atra* (Hendrix et al., 2010). Five of them

were already used to analyze Dinaric populations of the latter species (Razpet et al., 2016). For each locus, the forward primer was fluorescently labeled with G5 matrix (6FAM, VIC, PET, NED; Applied Biosystems, Thermo Fisher Scientific). Three multiplex assays were developed (Table S2). Amplification reactions were performed in a total volume of 10 μl, with a different concentration of each primer (Table S2), 0.25 mM of each dNTP, 1× Taq Buffer (25 mM Tris-HCl pH 8.0, 35 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% Tween-20, 0.5% Igepal CA-630 and stabilizers), 1 U HotMaster Taq DNA polymerase (5-Prime), and 2 μl template DNA. Negative controls were used to exclude contamination. PCR amplifications were carried out in Veriti Thermal Cyclers (Applied Biosystems) using the temperature profiles reported in Table S2. Genotyping was carried out on an AB3730XL Genetic Analyzer (Applied Biosystems). Allele sizes were scored in GeneMapper 3.7 (Applied Biosystems) using 500 LIZ size standard and checked by comparison with previously sized control samples.

2.4.2 | Mitochondrial loci and nuclear coding genes

Partial sequences of three mitochondrial marker sequences (16S ribosomal RNA gene, *16S*; cytochrome *b* gene, *cytb*; control region, *D-loop*) and two protein-coding nuclear genes (chemokine C-X-C motif receptor 4 gene, *CXCR4*; recombination activating gene 1 gene, *RAG1*) were amplified by PCR. Primers, sources, and temperature profiles are reported in Table S3. The *16S* was amplified in a total volume of 20 μl, with 0.5 μM of primer, 0.25 mM of each dNTP, 1× Taq Buffer (25 mM Tris-HCl pH 8.0, 35 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% Tween-20, 0.5% Igepal CA-630, and stabilizers), 1.25 U of HotMaster Taq DNA polymerase (5-Prime), and 1 μl template DNA. For *cytb* and *D-loop*, several *S. atra* sequences were initially obtained using universal primers and then aligned to homologous sequences of Salamandrinae to design specific primers. The two *cytb* fragments and one of the two *D-loop* fragments (Saat-LPro-short/Saat-H339-dloop primers) were amplified in a total volume of 20 μl, with 0.5 μM of primer, 0.125 mM of each dNTP, 1× Colorless GoTaq Flexi Buffer (Part # M890A), 2 mM $MgCl_2$, 1 U GoTaq Hot Start polymerase (Promega, WI, USA) and 1.5 μl (*cytb*) or 1 μl (*D-loop*) template DNA. The second *D-loop* fragment (L-Pro-ML/H-12S1-ML primers) was amplified in a total volume of 20 μl, with 0.5 μM of primer, 0.25 mM of each dNTP, 1× Taq Buffer (25 mM Tris-HCl pH 8.0, 35 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% Tween-20, 0.5% Igepal CA-630 and stabilizers), 1.25 U of HotMaster Taq DNA polymerase (5-Prime), and 2 μl template DNA. *CXCR4* and *RAG1* were amplified in a total volume of 40 μl, with 0.63 μM of primer, 0.5 μl of a 0.5 mg/ml BSA solution, 0.2 mM of each dNTP, 1× Colorless GoTaq Flexi Buffer (Part # M890A), 0.8 mM $MgCl_2$, 1.25 U of GoTaq Hot Start polymerase (Promega), and 4 μl template DNA. For all amplifications, contamination was checked using blank extractions and PCR-negative controls. Before sequencing, the excess primers and dNTPs were removed using ExoSAP-IT (USB, Cleveland, OH, USA), following the manufacturer's protocol. DNA sequences of both strands were obtained for all PCR products following the ABI Prism Big-Dye Terminator Kit 3.1 (Applied Biosystems) standard protocol. The sequencing reaction products were run on an AB3730XL Genetic Analyzer (Applied Biosystems). The resulting sequences were edited with Sequencher 4.7 (Gene Codes, Ann Arbor, MI, USA), aligned using Clustal X 2.0 (Larkin et al., 2007), and checked by eye. Protein-coding sequences (*cytb*, *CXCR4*, *RAG1*) were translated into amino acids for authentication, with MEGA 5 (Tamura et al., 2011). Sequences were deposited in the EMBL database (accession numbers: *16S* MG968380-9, *cytb* MG968402-19, *D-loop* MG968390-401, *CXCR4* MG968420-1, *RAG1* MG968422-6).

2.5 | Analysis of genetic diversity

2.5.1 | Inference of populations

Microsatellite data were checked for genotyping errors with Microchecker 2.2.3 (Van Oosterhout, Hutchinson, Wills, & Shipley, 2004) and then analyzed to group all individuals of *S. atra* (Table 1) in homogenous genetic clusters with Structure 2.3.4 (Pritchard, Stephens, & Donnelly, 2000). As exploratory analysis, all *K* values between 1 and 19 (i.e. the total number of sampling sites) were tested with five independent simulations (100,000 iterations, burn-in 25,000) following different combinations of ancestry and allele frequency models. The final analysis was performed for *K* values from 2 to 16, running 20 independent simulations (500,000 iterations, burn-in 125,000) under the admixture model and the assumption of independent allele frequencies among populations, without using information on sampling sites. Genetic substructure within the inferred clusters was analyzed by independently re-running Structure on the individuals of the single clusters, with model parameters as described above, using *K* from 1 to 6 and both independent and correlated allele frequency models. The most likely number of clusters was inferred by comparing data likelihood by means of likelihood ratios (Evanno, Regnaut, & Goudet, 2005) in Structure harvester (Earl & vonHoldt, 2012).

As the analysis with Structure is known to be sensitive to uneven samples (Puechmaille, 2016), the resulting clusters were confirmed through a factorial correspondence analysis, with Genetix 4.02 (Belkhir, Borsa, Goudet, Chikhi, & Bonhomme, 1999).

2.5.2 | Differentiation between populations

Considering the populations inferred from the clustering analyses, the overall genetic variation was partitioned into within- and between-populations components with an analysis of molecular variance (AMOVA; Excoffier, Smouse, & Quattro, 1992) with Arlequin 3.1 (Excoffier, Laval, & Schneider, 2005), both for the microsatellite data and the mtDNA sequences. The differentiation between populations was evaluated from the microsatellite data by estimating both the classical F_{ST} (Weir & Cockerham, 1984) and its molecular equivalent R_{ST} (Slatkin, 1995) with Arlequin. It was evaluated from the mtDNA sequences by correcting for multiple hits with the method

of Tamura and Nei (1993), alternative model to the best-fit estimated HKY + I model, which is unavailable in ARLEQUIN. The significance of the different variance and ϕ_{ST} components (molecular equivalents of Wright's *F* statistics) was evaluated by 10,000 random permutations (Excoffier et al., 1992).

2.5.3 | Diversity within populations

Deviation from the Hardy-Weinberg equilibrium was tested for each microsatellite locus, in every inferred population, with the exact test (Guo & Thompson, 1992). Linkage disequilibrium between pairs of microsatellite loci was tested with a likelihood-ratio test (Excoffier & Slatkin, 1998). Both tests were performed with ARLEQUIN.

Diversity within inferred populations was evaluated both from the microsatellite data and the concatenated mtDNA sequences, only for samples with $n \geq 8$ individuals. For microsatellite data, observed and expected heterozygosity, mean number of alleles per locus and inbreeding coefficient F_{IS} were calculated with GENETIX, whereas allelic richness was estimated with FSTAT 2.9.3 (Goudet, 2001). For mtDNA sequences, number of haplotypes, haplotype diversity, number of polymorphic sites, and nucleotide diversity were computed with ARLEQUIN.

The bottleneck hypothesis was tested in the inferred populations with $n \geq 10$ individuals, from the microsatellite data, using the Wilcoxon sign-rank test implemented in BOTTLENECK 1.2.02 (Piry, Luikart, & Cornuet, 1999) under different mutation models (infinite allele, two-phased, stepwise mutation).

For all analyses, statistical significance level was adjusted using a sequential Bonferroni correction in the case of multiple tests.

2.6 | Analysis of evolutionary relationships between populations

2.6.1 | Phylogeographic analysis

The haplotypes of the mitochondrial sequences obtained from our sample (Table 1) were analyzed together with all other homologous sequences previously obtained for *S. atra* and available in public repositories (Table S4), including *cytb* and *D-loop* haplotypes sampled from different sites in the core range of the species (Ribéron, Miaud, Grossenbacher, & Taberlet, 2001; Bonato & Steinfartz, 2005). Divergence and geographical distribution of the haplotypes were analyzed using unrooted median-joining networks (Bandelt, Forster, & Röhl, 1999) with POPART (http://popart.otago.ac.nz), both for the single loci and for their concatenated sequences.

2.6.2 | Phylogenetic analysis

The haplotypes of the nuclear genes *CXCR4* and *RAG1* obtained from our samples (Table 1) were analyzed together with homologous sequences previously obtained from *S. atra*, as ingroup, and from other species of *Salamandra*, as outgroup (Table S4). We included both *Salamandra corsica* Savi, 1838 and *Salamandra lanzai* Nascetti, Andreone, Capula & Bullini, 1988, since all recent analyses on different molecular datasets indicate either *S. corsica* or *S. lanzai* or a putative lineage comprising both species as the sister group of *S. atra* (Weisrock et al., 2006; Pyron & Wiens, 2011; Arntzen, Beukema, Galis, & Ivanović, 2015; Vences et al., 2014; Rodríguez et al., 2017).

Phylogenetic relationships were estimated using maximum likelihood (ML) and Bayesian inference (BI). Congruence between the nuclear genes in the phylogenetic signal was evaluated with a partition homogeneity test (Farris, Källersjö, Kluge, & Bult, 1995) in PAUP 4.0a147 (Swofford, 2002), with 1,000 replicates and heuristic search using the TBR branch swapping algorithm. Both ML and BI analyses were conducted using the best-fit nucleotide substitution model that was selected on the complete dataset by the Bayesian information criterion in JMODELTEST 2.1.7 (Darriba, Taboada, Doallo, & Posada, 2012). ML analyses were performed in PHYML 3.0 (Guindon & Gascuel, 2003) under the "BEST" branch swapping method. The statistical support was assessed using 1,000 bootstrap pseudo-replicates. BI was conducted using MRBAYES 3.1.2 (Huelsenbeck & Ronquist, 2001). The analysis was run twice for 2,000,000 generations, with four parallel chains and sampling every 500 generations. The resulting tree was constructed from 6,000 trees sampled from the posterior distribution, once the first 2,000 trees were excluded as burn-in.

3 | RESULTS

3.1 | Inferred populations

A total of 113 individuals of *S. atra* from 19 sites were typed for ten autosomal microsatellite loci (Table 1). All loci were found to be polymorphic, with 3–14 alleles per locus (mean 9.3). No null alleles, allelic dropout, or stuttering were detected for any locus.

A first analysis of the genetic structure separated the individuals unambiguously into six highly homogeneous groups (Figure 3a). Each group corresponds to a cluster of neighboring sites or to a single site, and different clusters were geographically separated from each other (Figure 1). In detail, four groups correspond to separate areas along the southern Prealps, that is, Orobian mountains (27 individuals from five sites), Pasubio massif (nine individuals from a single site), Sette Comuni plateau (29 individuals from four sites), and an area comprising the Belluno Dolomites and the Cansiglio plateau (19 individuals from two sites). The remaining two groups include all the samples from the Dinarides (16 individuals from four sites) and those from the inner Alps (12 individuals from two sites), respectively. Finally, a single individual from the northern Dolomites was separated from all groups.

A second-level analysis only revealed substantial genetic substructure within two of the primary clusters (Figure 3b): (i) three highly homogeneous groups were identified within the Orobian mountains, corresponding to the three main sampling sites (west to east: Val Terzera, Laghi Gemelli, Val di Scalve); (ii) the Belluno Dolomites were separated from the Cansiglio plateau. Similar results were obtained with both independent and correlated allele frequencies.

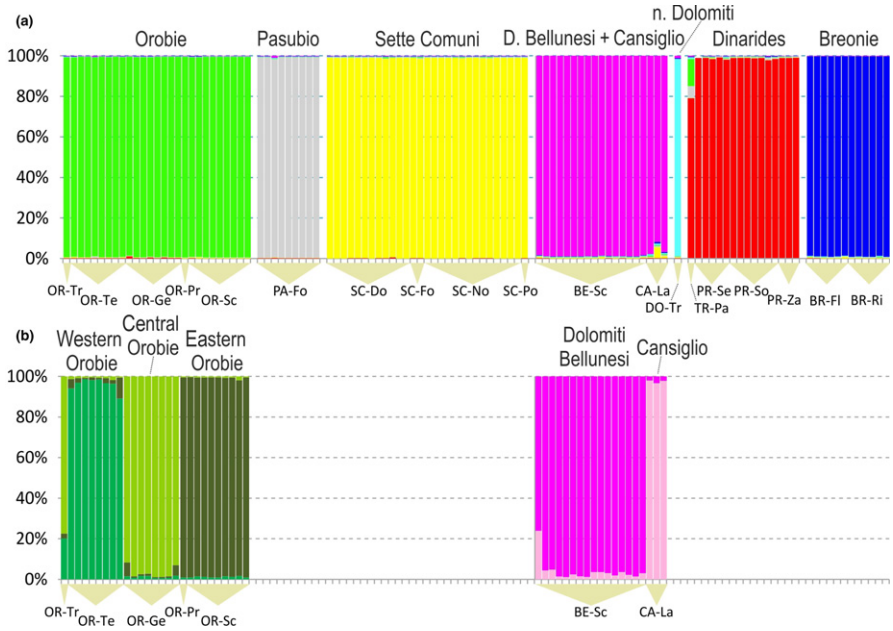

FIGURE 3 Partition of 113 individuals of *Salamandra atra* from 19 sites in genetically homogeneous clusters, based on ten microsatellite loci, as inferred from STRUCTURE after two consecutive steps (a,b). Labels as in Table 2

The genetically homogeneous populations inferred across the southern Prealps (Table 2) were confirmed by a factorial correspondence analysis of the individual genotypes (Figure 4). The combination of the first three axes (accounting for 32.1% of the total variation) distinguished Orobian mountains, Pasubio massif, Sette Comuni plateau, Belluno Dolomites, and Cansiglio plateau. Additionally, all samples from southern Prealps were clearly separated from Dinarides and inner Alps by the first and the third axes, respectively. A second-step analysis on the individuals from the Orobian mountains confirmed the identification of the three contiguous populations (Figure S1).

3.2 | Genetic differentiation between populations

The divergence between the inferred populations was large and statistically significant, accounting for 64%–87% of the total microsatellite genetic variation, depending on the F_{ST} or R_{ST} indices (AMOVA: $p \leq .0001$ in both cases). All pairwise values of F_{ST} and R_{ST} between populations were high and significant (F_{ST} = 0.54–0.77; R_{ST} = 0.45–0.99; Table 3). The lowest values of both indices were found between the three populations of the Orobian mountains.

Considering the mitochondrial loci, a concatenated sequence of 2,157 bp (526 bp *16S* + 927 bp *cytb* + 704 bp *D-loop*), was obtained after alignment and trimming from 117 individuals representing all 19 sites (Table 1). A total of 23 haplotypes were identified, with 72 polymorphic nucleotide positions (3.3%, including 48 transitions, 20 transversions, and six indels). The haplotypes differed for up to 35 substitutions (1.6%). The median-joining network (Figure 5) identified seven principal groups, separated by more than five substitutions, corresponding exactly to the genetic homogeneous clusters inferred from microsatellites in the first-level Bayesian clustering analysis (Figure 3). Shared haplotypes were found only between populations separated in the second-level Bayesian clustering analyses: a single haplotype is shared by Belluno Dolomites and Cansiglio plateau, and another haplotype is common to central and eastern Orobian mountains.

The mitochondrial divergence between the populations inferred from the microsatellites accounted for 96% of the total variation (AMOVA; $p < .0001$). Most pairwise ϕ_{ST} values between populations were high and statistically significant (≥0.90; Table 3), except for the much less differentiated populations of the Orobian mountains (≤0.70).

3.3 | Genetic diversity within populations

No deviation from the Hardy-Weinberg equilibrium was found for any microsatellite locus in any inferred population, and no evidence of linkage disequilibrium was found between loci. The within-population genetic diversity estimated from the microsatellites (Table 2) was lower in all analyzed populations from the southern Prealps (western, central, and eastern Orobian mountains; Pasubio massif; Sette Comuni plateau; Belluno Dolomites) than in the populations from the Dinarides and the inner Alps (Breonie), as indicated by observed heterozygosity (0.11–0.35 vs. 0.37–0.47), expected heterozygosity (0.10–0.30 vs. 0.35–0.45), mean number of alleles (1.5–2.5 vs. 3.3–3.5), allelic richness (1.46–2.25 vs. 2.83–3.20), and number of private alleles (0–5 vs. 11–13).

Also the within-population mitochondrial haplotype diversity (Table 2) was found to be lower in the populations from the southern Prealps than in the populations from Dinarides and inner Alps (0–0.58 vs. 0.71–0.77). The lowest values were estimated for the Sette Comuni plateau (0.13; only two haplotypes, differing by a single nucleotide, out of 30 individuals) and the Belluno Dolomites (15 individuals with the same haplotype). Instead, the mitochondrial nucleotide diversity in the populations of the southern Prealps was lower than that of the

TABLE 2 Genetic diversity from microsatellite and mitochondrial loci for the inferred populations of *Salamandra atra*

		Microsatellites							mtDNA (concatenated: *16S*, *cytb*, *D-loop*)					
Inferred population	Sites	*N*	H_o (*SD*)	H_e (*SD*)	Mean number of alleles	Allelic richness	Private alleles	F_{is}	*N*	Haplotypes	Private haplotypes	Haplotype diversity (*SD*)	Polymorphic sites	% nucleotide diversity (*SD*)
Southern Prealps														
Western Orobie	OR-Te	8	0.20 (0.26)	0.20 (0.28)	1.8	1.80	0	−0.03	8	2	2	0.57 (0.09)	4	0.05 (0.04)
Central Orobie	OR-Tr OR-Ge	9	0.35 (0.23)	0.23 (0.20)	2.3	2.24	1	0.34	9	4	3	0.58 (0.18)	8	0.07 (0.05)
Eastern Orobie	OR-Pr OR-Sc	10	0.11 (0.20)	0.10 (0.16)	1.5	1.46	0	0.12	10	2	1	0.20 (0.15)	1	0.01 (0.01)
Pasubio	PA-Fo	9	0.22 (0.19)	0.22 (0.23)	2.1	2.07	5	−0.04	13	2	2	0.38 (0.11)	1	0.02 (0.02)
Sette Comuni	SC-Po SC-Do SC-No SC-Fo	29	0.27 (0.24)	0.28 (0.26)	2.1	1.83	1	−0.07	30	2	2	0.13 (0.08)	1	0.01 (0.01)
Dolomiti Bellunesi	BE-Sc	16	0.30 (0.31)	0.30 (0.32)	2.5	2.25	1	0.00	15	1	0	0	0	0
Cansiglio	CA-La	3	–	–	–	–	2	–	3	1	0	–	–	–
Northern Dolomites	DO-Tr	1	–	–	–	–	3	–	1	1	1	–	–	–
Dinarides														
Dinarides	TR-Pa PR-So PR-Za PR-Se	16	0.37 (0.30)	0.35 (0.33)	3.3	2.83	13	0.06	16	6	6	0.77 (0.08)	10	0.14 (0.08)
Inner Alps														
Breonie	BR-Fl BR-Ri	12	0.47 (0.29)	0.45 (0.27)	3.5	3.20	11	0.05	12	4	4	0.71 (0.11)	3	0.03 (0.03)

Inferred populations are defined in Figure 3. Sites are coded as in Table 1.

F_{is}, heterozygote deficiency; H_e, expected heterozygosity; H_o, observed heterozygosity; *N*, number of individuals; *SD*, standard deviation.

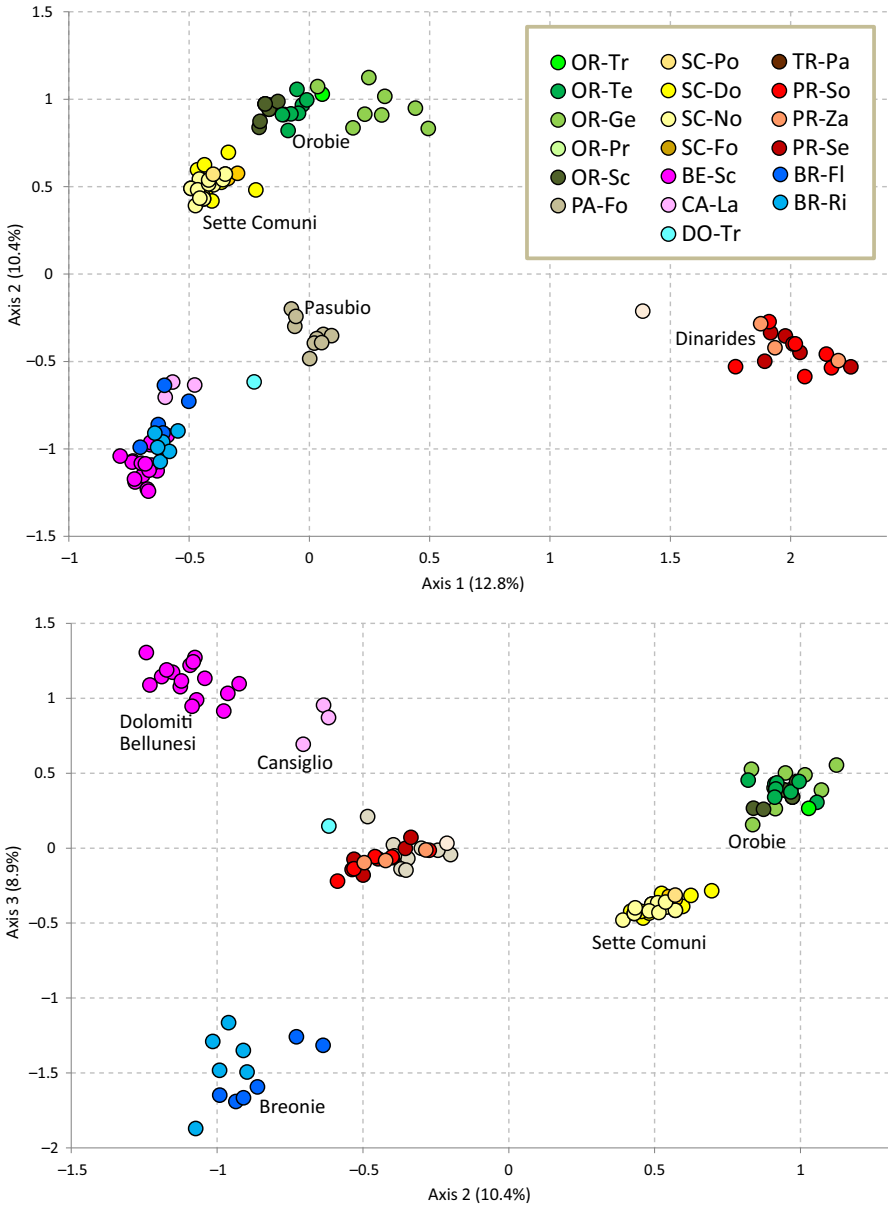

FIGURE 4 Distribution of 113 individuals of *Salamandra atra* from 19 sampling sites along the first three axes obtained by a factorial correspondence analysis of the microsatellite data. Labels as in Table 2

population in the Dinarides (0%–0.07% vs. 0.14%), but comparable to that estimated in the population of the inner Alps (0.03%).

No significant bottleneck signal was found from the microsatellite data in any of the analyzed populations (Wilcoxon signed-rank test: $p > .05$).

3.4 | Evolutionary relationships between populations

We compared the mitochondrial sequences of our sample with all other homologous sequences previously obtained (*cytb* from 54 individuals from 13 sites, *D-loop* from 20 individuals from seven sites; Figure 1 and Table S4). Most of the sequences previously obtained from the southern Prealps (Orobian mountains, Pasubio massif, Sette Comuni plateau) and the Dinarides are either identical or highly similar to the haplotypes identified in our study from the same or neighboring sites (Figures S2 and S3). Some remarkable exceptions are hard to explain based on the available information on some deposited sequences. A *cytb* haplotype referred by Ribéron et al. (2001) to the Sette Comuni plateau was found also in our study but only on the Pasubio massif, whereas all 30 individuals hereby sampled from the Sette Comuni plateau harbor a different, yet similar haplotype. Two *D-loop* haplotypes referred by Steinfartz, Veith, and Tautz (2000) to the Cansiglio plateau and the Prenj mountains appear far apart from the haplotypes found in our study in the same areas. On the other hand, all sequences previously obtained from the northern and inner Alps (5 *cytb* haplotypes from 22 individuals from eight sites, and 3 *D-loop* haplotypes from five individuals from two sites; Figure 1) are very similar to our haplotypes from the Breonie (Figures S2 and S3).

Sequences of the nuclear genes *CXCR4* (454 bp) and *RAG1* (692 bp) were obtained for 106 individuals from 18 sampling sites and 107 individuals from all 19 sites, respectively (Table 1). A total of 4 haplotypes were identified for *CXCR4*, with five polymorphic nucleotide positions (one transition and four transversions), whereas five haplotypes were identified for *RAG1*, with four polymorphic

TABLE 3 Pairwise divergence between the inferred populations of *Salamandra atra*

	Western Orobie	Central Orobie	Eastern Orobie	Pasubio	Sette Comuni	Dolomiti Bellunesi	Dinarides	Breonie
Western Orobie	–	**.69/.45**	**.57/.52**	**.70/.88**	**.64/.96**	**.69/.83**	**.65/.82**	**.64/.79**
Central Orobie	**.40**	–	**.54/.81**	**.61/.89**	**.62/.91**	**.64/.89**	**.56/.78**	**.57/.77**
Eastern Orobie	**.70**	.04	–	**.77/.99**	**.69/.99**	**.73/.92**	**.70/.92**	**.68/.87**
Pasubio	**.97**	**.97**	**.99**	–	**.70/.98**	**.67/.83**	**.61/.92**	**.57/.78**
Sette Comuni	**.98**	**.98**	**.99**	**.98**	–	**.64/.94**	**.65/.90**	**.60/.72**
Dolomiti Bellunesi	**.99**	**.98**	**1.00**	**.99**	**.99**	–	**.63/.90**	**.56/.75**
Dinarides	**.92**	**.92**	**.94**	**.92**	**.94**	**.93**	–	**.57/.77**
Breonie	**.97**	**.96**	**.98**	**.96**	**.94**	**.98**	**.90**	–

Above the diagonal: F_{ST}/R_{ST} calculated on the microsatellites. Below the diagonal: ϕ_{ST} calculated on the concatenated mitochondrial loci (*16S*, *cytb*, *D-loop*). Only populations with ≥8 sampled individuals were compared. Significant values ($p < .05$) are in bold.

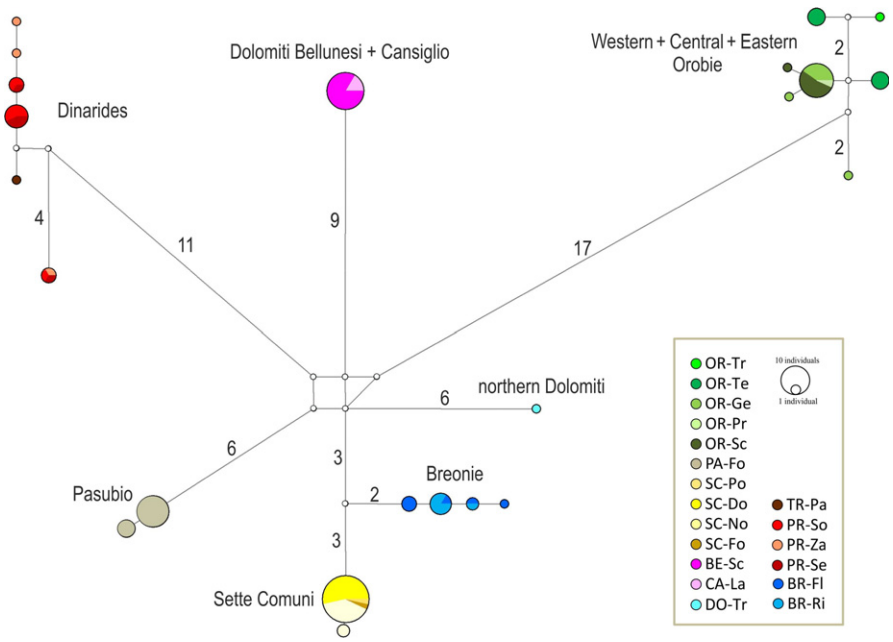

FIGURE 5 Median-joining network of the 23 haplotypes of the concatenated mitochondrial loci (*16S*, *cytb*, *D-loop*) from 117 individuals of *Salamandra atra* from 19 sampling sites. Each haplotype is represented by a pie chart indicating the number of individuals from different sites. Branch lengths are proportional to the minimum number of mutations between haplotypes, which is indicated when >1. Populations inferred from the microsatellite data are labeled as in Table 2

positions (three transitions and one transversion). For both genes, all previously available sequences for *S. atra* (Table S4) were obtained from four of our sampling sites (Pasubio massif, two sites in the Sette Comuni plateau, Cansiglio plateau) and resulted identical to the haplotypes found by us in the respective sites.

Almost all individuals were found homozygous for both genes, except for three individuals from the western Orobian mountains (heterozygous for either *CXCR4* or *RAG1*) and a single individual from the Dinarides (heterozygous for *RAG1*). A partition homogeneity test indicated no significantly incongruent phylogenetic signal between the two nuclear markers. After concatenation and trimming to 924 bp to allow alignment with outgroups, a total of five different haplotypes were identified, with eight polymorphic positions (0.8%), including three parsimony informative positions (Figure 6a). Pairwise differences between haplotypes comprised up to seven substitutions (0.8%). One haplotype was found exclusively in the single population sampled from the inner Alps (Breonie) and was common to all those individuals. Two very similar haplotypes were exclusive of the Pasubio and the Sette Comuni populations: One was found in a single individual from the Pasubio by Vences et al. (2014), while the other one was common to all remaining individuals. Other two very similar haplotypes were exclusive of all other populations of the southern Prealps and the Dinarides: One was found in a heterozygous individual from the western Orobian mountains, whereas the other one was common to all remaining individuals.

Both ML and BI phylogenetic trees of the haplotypes were consistent in topology (Figure 6b). They confirmed *S. atra* as monophyletic with respect to other *Salamandra* species and suggested (i) a basal split between a lineage represented by the Breonie population (belonging to the core range of the species) and another comprising all populations surveyed across the southern Prealps and the Dinarides, and (ii) a basal position of the Pasubio and Sette Comuni populations with respect to all other populations of the southern Prealps and the Dinarides.

4 | DISCUSSION

4.1 | Sampling peripheral populations of *S. atra*

Mapping and sampling *S. atra* along the southern Prealps have long been hindered by the very small extent of the populations and the unpredictability of their occurrence across a highly heterogeneous landscape. Moreover, salamanders inhabiting the warmer and drier karstic Prealps engage in epigean activity less frequently than those living in the cooler conditions commonly found in the Alps (e.g. Grossenbacher, 1994; Romanazzi & Bonato, 2014). In addition, the species has a remarkably low detectability in comparison with most other amphibians because it has a fully terrestrial life cycle, lacks aggregative behaviors, and overwinters for months underground.

Over the last decade, however, intensive search efforts have been undertaken in different areas of the southern Prealps (Corbetta & Giovine, 2010; Romanazzi & Bonato, 2014), so that the extent of previously recorded populations has been determined more accurately, and new populations have been detected (Figure 1; Table S1). At the same time, an unprecedented number of tissue samples has been assembled for molecular analyses.

4.2 | Differences among peripheral populations

4.2.1 | Levels of differentiation between populations

In general, all peripheral populations of *S. atra* sampled across the southern Prealps are genetically distinct from those in the core range of the species in the northern and inner Alps. This pattern of genetic differentiation between southern periphery and northern core range is particularly clear in our dataset and confirms previous analyses on the geographic variation of *S. atra*, including an AFLP analysis of few samples from the Orobian mountains and the Sette Comuni plateau (Ribéron et al., 2004), and a preliminary analysis of the mitochondrial variation in samples from the Orobian mountains (Helfer et al., 2011).

The genetic differentiation among populations of *S. atra* within the core range is still largely unknown. Nevertheless, the level of divergence measured between peripheral populations in the southern Prealps (pairwise $F_{st} > 0.54$ from microsatellites) is much higher than that estimated, with a similar set of microsatellite loci, between populations separated by a maximum of 25 km of suitable territory in the north-western Alps, within the core range of the species ($F_{st} = 0.02$; Helfer et al., 2012). The peripheral populations of the southern Prealps are also more differentiated than the fragmented populations of the Dinarides ($F_{st} < 0.57$; Razpet et al., 2016). Moreover, the among-population differentiation of *S. atra* across the southern Prealps is higher than that found in the related species *S. salamandra* among populations inhabiting the foothills of the southern Prealps and fragmented during recent human landscape modifications ($F_{st} < 0.08$; Pisa et al., 2015), and between insular and mainland Iberian populations separated about 9 kya ($F_{st} < 0.27$; Velo-Antón, Zamudio, & Cordero-Rivera, 2012). In general, in terrestrial organisms with narrow climatic tolerance and low dispersal ability, adjacent isolated populations are expected to accumulate genetic divergence even in the absence of eco-morphological adaptive differentiation. This explanation has often been invoked for the genetic differentiation commonly found among neighboring populations in

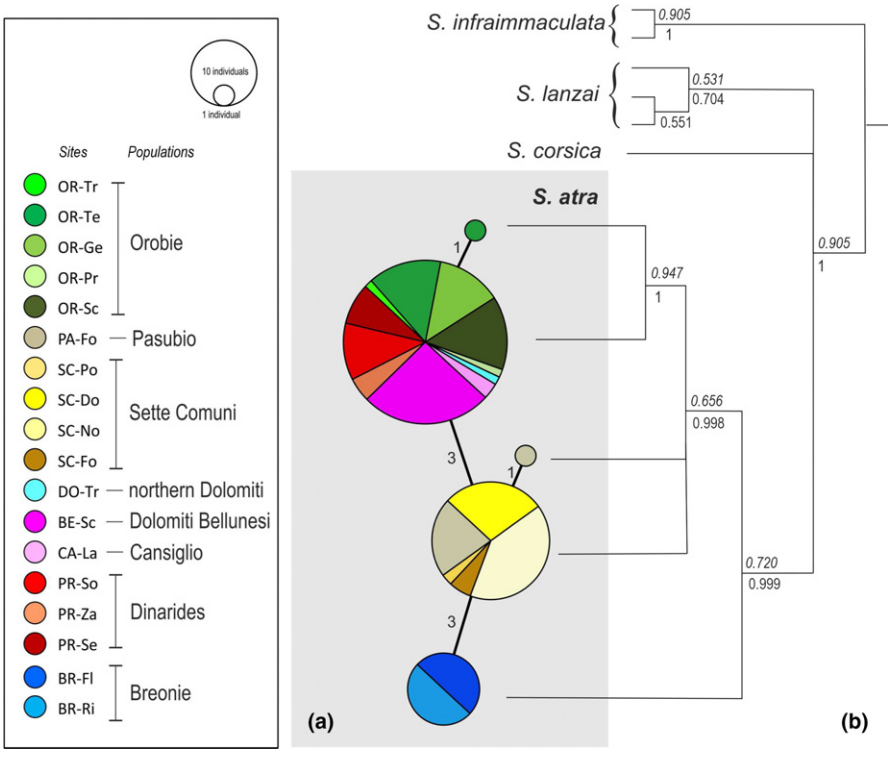

FIGURE 6 Median-joining network (a) and phylogenetic relationships (b) of the five haplotypes of the concatenated nuclear genes (*CXCR4*, *RAG1*) in 105 individuals of *Salamandra atra* from 18 sampling sites, including 101 individuals sampled by us from 18 sites (Table 1) and four individuals previously sequenced from four of the same sites (Table S4). Each haplotype is represented by a pie chart indicating the number of individuals from different sites. The minimum number of substitutions between haplotypes is indicated along the branches. Labels as in Table 2. Maximum likelihood bootstrap values from 1,000 replications and Bayesian inference posterior probabilities are indicated, respectively, above and below the nodes when >0.5, otherwise the nodes are collapsed

different species of *Salamandra* (Blank et al., 2013; Bani et al., 2015; Pereira, Martínez-Solano, & Buckley, 2016; Vörös et al., 2017).

However, levels of genetic differentiation between peripheral populations of *S. atra* are not homogeneous. The differentiation is higher between single populations, or clusters of neighboring populations, inhabiting highlands at least 20 kilometers apart and separated by deep valleys and unsuitable habitat, such as Orobian mountains, Pasubio massif, Sette Comuni plateau, and the area comprising the Belluno Dolomites and the Cansiglio plateau. Some differentiation is also found within the Orobie mountains, where at least three distinct populations are present along the main ridge, at most 5–10 km apart (Corbetta & Giovine, 2010), separated by mountain passes no lower than 1,820 m and without obviously inhospitable stretches in between. Conversely, the Sette Comuni plateau appears to be inhabited by a single, genetically panmictic population. Lower divergence is also found between the Belluno Dolomites and the Cansiglio plateau, despite a wide, deep, and currently unsuitable valley in between.

4.2.2 | Levels of genetic diversity within populations

All investigated populations of *S. atra* across the southern Prealps show a lower genetic diversity than is usually found in the remaining range of the species. In particular, the heterozygosity estimated from microsatellite data in the Prealpine peripheral populations ($H_o < 0.35$, $H_e < 0.30$) is lower than that found in populations from the Breonie mountains ($H_o = 0.47$, $H_e = 0.45$), north-western Alps ($H_e > 0.45$; Helfer et al., 2012), Julian and Carinthian-Slovenian Alps ($H_o > 0.51$, $H_e > 0.54$; Razpet et al., 2016), and northern Dinarides ($H_o > 0.44$, $H_e > 0.42$; Razpet et al., 2016), although similar to that found in well-separated populations in the southern Dinarides (Razpet et al., 2016). Similar contrasts of genetic diversity (isolated southern populations vs. northern interconnected populations) have been found in other mountain regions for other species of *Salamandra* (Blank et al., 2013; Vörös et al., 2017).

However, levels of genetic diversity are not homogeneous among the peripheral populations of the southern Prealps. For instance, within the Orobian mountains, a distinctly higher diversity is found in the central population compared to the eastern and western ones. An especially low diversity is noted in the populations of the Pasubio massif and the Sette Comuni plateau. Nevertheless, no signal of demographical bottleneck was found for any of the peripheral populations, contrary to the expectation for terrestrial organisms with narrow climatic tolerance and low dispersal ability such as *S. atra*.

4.2.3 | Evolutionary relationships between populations

Our phylogenetic analyses corroborate the traditional view of *S. atra* as a monophyletic species (e.g. Vences et al., 2014), despite the remarkable genetic and phenotypic differentiation of some of the southern populations. Monophyly is also confirmed by derived anatomical features, including smaller body size and reduced number of skin glands on the flanks compared to other *Salamandra* species (Grossenbacher, 1994).

On the other hand, we found evidence of two ancient lineages within the species, one currently represented by the widespread core populations in the northern and inner Alps, and another comprising the fragmented populations of the Dinarides and the peripheral populations of the southern Prealps. However, different peripheral populations are representative of at least two deeply diverging sublineages. The two populations of the Pasubio massif and the Sette Comuni plateau, which are distinguished for their yellow-blotched skin, comprise one or two sublineages that are presently confined to a small sector of the Venetian Prealps. Instead, all other uniformly black populations scattered along the remaining southern Prealps (both east and west of the Pasubio massif and the Sette Comuni plateau) comprise a different sublineage that extends also to the Dinarides.

4.3 | A plausible history for the peripheral populations

The timing of the intraspecific divergence of *S. atra* cannot be estimated reliably, as fossil records are lacking. Nevertheless, considering that previous phylogenetic analyses have estimated the split between *S. atra* and its putative sister species (either *S. corsica* or *S. lanzai*) at 7.5–2.3 Mya (Vences et al., 2014), and that we have found higher levels of molecular variation within *S. atra* than within any other species of *Salamandra*, it is reasonable to hypothesize that the present geographical diversity within *S. atra* originated and was shaped during multiple cycles of climatic oscillations during the last few million years. The Quaternary environmental dynamics have been thoroughly investigated for the Alps and the surrounding regions, and the expansions and contractions of glaciated and non-glaciated areas have been precisely mapped (Ehlers & Gibbard, 2004; Hughes, Woodward, & Gibbard, 2006; Ivy-Ochs et al., 2008; Figure 1).

How *S. atra* responded to the repeated climatic pulsations was arguably conditioned by specific biological traits such as the narrow climatic tolerance and the low dispersal potential. The species is physiologically adapted to the montane and subalpine climate, while it is intolerant to moderately warm and dry conditions (Klewen, 1988; Guex & Grossenbacher, 2004; Araújo, Thuiller, & Pearson, 2006). Unlike most other amphibians, the life cycle of *S. atra* is fully terrestrial, so that water bodies are not required for reproduction. Adults are usually sedentary within home ranges extending only a few hundred square meters (Klewen, 1988; Bonato & Fracasso, 2003), although there is indirect genetic evidence for more extensive male dispersal (Helfer et al., 2012). In addition, biotic interactions may have also played a role: *S. atra* rarely coexists with the related *S. salamandra* (Werner, Lötters, & Schmidt, 2014), which inhabits the broadleaf forests at lower elevations, surrounding all the higher elevation habitats inhabited by *S. atra* (Bani et al., 2015). However, it is still unclear if the relative distribution of the two species is

constrained by competition (Werner et al., 2014). Finally, it should be noticed that the reproductive potential of *S. atra* is very low in comparison with most other terrestrial vertebrates living in the same region, including *S. salamandra*. The average generation time is moderately short, but the female fecundity is very low: 1 or 2 offspring are usually born to an adult female after a gestation period of 3–4 years (Häfeli, 1976; Luiselli, Andreone, Capizzi, & Anibaldi, 2001).

Considering the past climatic and environmental dynamics and the peculiar biological traits of *S. atra* summarized above, we argue that populations underwent repeated alternating phases of contraction, extinction and recolonization in the Alpine region. During glacial periods, multiple populations most probably remained isolated and differentiated in non-glaciated areas along the southern Prealps. Conversely, during the relatively shorter interglacial periods, some populations may have expanded but others may have remained trapped into narrow uplands and further contracted. The effects of such dynamics have been probably accumulating through multiple glacial cycles. During the last glacial period (coldest climate and broadest extent of glaciers about 30–18 kya; Figure 1), *S. atra* was probably more broadly distributed along the Dinarides than today, as indicated by the shallow genetic divergence across this entire mountain chain (Razpet et al., 2016). The species was, instead, arguably absent from the inner Alps while multiple populations — some of them already separated and differentiated previously — survived along the southern Prealps in suitable non-glaciated areas (Figure 1). Based on the current distribution and genetic differentiation, one or more populations probably survived in the Bergamasque Prealps, adjacent to the Orobian mountains, between the Adda and the Oglio glacier tongues, and two populations remained in the areas between the Adige and the Brenta rivers, separated by the Astico glacier tongue between the Pasubio massif and the Sette Comuni plateau. A population probably survived in the area between the Piave glacier, descending from the Fadalto saddle, and the Tagliamento glacier, comprising the Cansiglio plateau. Other populations survived in other suitable areas along the Julian and the Slovene Prealps. Perhaps additional populations could have persisted in other intermediate non-glaciated areas along the southern Prealps, that is, between the Oglio and the Adige glacier tongues, between the Brenta river and the Fadalto saddle, but there is no evidence for their survival today. We cannot exclude that other populations may have persisted in inner non-glaciated areas within the Alps (so-called nunatak refugia; Holderegger & Thiel-Egenter, 2009), as proposed for some alpine plants and invertebrates such as spiders (e.g. Stehlik, Blattner, Holderegger, & Bachmann, 2002; Schönswetter et al., 2005; Wachter et al., 2016); however, this hypothesis is not necessary to explain the current distribution of the evolutionary and genetic diversity of *S. atra*.

With climate warming and glacial retreat, populations of *S. atra* would have undergone a shift to higher, cooler altitudes, and newly suitable areas in the Alps were colonized. However, most of the populations already established in the southern Prealps remained confined to small areas, realizing at most minor shifts and rarely re-establishing connections. The populations surviving between the Adda and the Oglio shifted northwards to colonize the major ridge of the Orobian mountains, like inferred for wingless carabid beetles and land snails (Lohse et al., 2011; Scheel & Hausdorf, 2012). It is also possible that the population surviving between the Fadalto saddle and the Tagliamento river expanded northwards to some extent and contributed to the colonization of the Belluno Dolomites. Yet, other populations underwent further contraction and fragmentation, like those in the Dinarides. Additionally, it is possible that some relic populations could have gone extinct, as suggested by the fact that *S. atra* is absent today from some highlands that were suitable refugial areas during the last glacial period (Figure 1).

4.4 | Insights for conservation

The current taxonomy of *S. atra* only recognizes the distinctiveness of the few peripheral populations with yellow-blotched individuals: *S. atra pasubiensis* Bonato & Steinfartz, 2005 on the Pasubio massif and *S. atra aurorae* Trevisan, 1982 on the Sette Comuni plateau. Even a species-level distinction has been proposed for *S. atra aurorae* (Joger, 1986; Dubois & Raffaëlli, 2009). Additionally, some or all the populations along the Dinarides are often referred to as *S. atra prenjensis* Mikšić, 1970, although this classification is debated (e.g. Dubois & Raffaëlli, 2009; vs. Speybroeck, Beukema, & Crochet, 2010). All other peripheral populations in the southern Prealps have invariably been united with those in the northern and inner Alps as a single subspecies *S. atra atra*. Even though the current intraspecific taxonomy fails to acknowledge the evolutionary distinctness and genetic differentiation of most southern peripheral populations, a satisfactory taxonomic classification will be possible only after further investigations, to corroborate the evolutionary hypothesis emerging from our analysis and to settle some nomenclatural issues (Table S5).

Conservation biologists have debated and proposed various conceptual bases and operational criteria to distinguish within-species units that should be primary targets for conservation and management (e.g. Moritz, 1994; Crandall, Bininda-Emonds, Mace, & Wayne, 2000; Fraser & Bernatchez, 2001; Palsbøll, Bérubé, & Allendorf, 2007). Even when applying the most stringent criteria (substantial genetic differentiation and no gene flow), our analyses indicate that at least six units should be recognized within *S. atra*: (i) the core populations of the inner Alps; (ii) the fragmented populations along the Dinarides; (iii) all adjacent populations on the Orobian mountains; (iv) the single population confined to the Pasubio massif; (v) the population found on the Sette Comuni plateau; (vi) the populations on the Belluno Dolomites and the Cansiglio plateau. It is possible that we have underestimated the number of distinct significant units, especially across the southern Prealps, as some populations may not have been sampled. In addition, our analyses have highlighted that the vulnerability of most of these populations, especially those of *S. atra pasubiensis* and *S. atra aurorae*, is exacerbated by low within-population genetic diversity. Consequently, the special attention conveyed to *S. atra pasubiensis* and *S. atra aurorae* (e.g. Andreone et al., 2009; Romanazzi & Bonato, 2014) should be

reinforced. On the other hand, other populations such as those in the Orobian mountains should be primary targets of monitoring and preservation, as they harbor exclusive and substantial portions of the overall genetic diversity of the species (Figures 4 and 5).

ACKNOWLEDGEMENTS

Many people helped us with information and support in the field, including L. Balzarini, G. Comotti, A. R. Di Cerbo, I. Plasinger, K. Tabarelli de Fatis, and members of the mountaineering club Željezničar. Fieldwork was supported by: Regione Lombardia, Parco Naturale delle Orobie Bergamasche, MUSE—Museum of Science, Department of Ecology and Evolution of the University of Salzburg, Department of Ecology of the University of Sarajevo, Rufford Foundation. Permits for sampling were obtained from the Italian "Ministero dell'Ambiente e della Tutela del Territorio e del Mare" (protocol numbers 14198 13/07/2012, 1758 29/01/2014, 3128 18/02/2014) and the Region of Lombardy (protocol number 3978 4/03/2010). We also thank all reviewers, who help us to improve the manuscript, and H. C. Hauffe, who kindly improved the language. The manuscript is dedicated to the memory of Enrico Romanazzi, who recently passed away.

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SUPPORTING INFORMATION

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How to cite this article: Bonato L, Corbetta A, Giovine G, et al. Diversity among peripheral populations: genetic and evolutionary differentiation of *Salamandra atra* at the southern edge of the Alps. *J Zool Syst Evol Res*. 2018;00:1–16. https://doi.org/10.1111/jzs.12224