



Genetic differentiation and population dynamics of Alpine salamanders (*Salamandra atra*, Laurenti 1768) in Southeastern Alps and Dinarides

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The genetic structure of Alpine salamander (*Salamandra atra*, Laurenti 1768) populations in the Dinarides with respect to continuous populations in the Alps is still poorly understood. To compare Dinaric populations with the nearest Alpine populations, eleven populations were genotyped using seven microsatellite loci. Two major groups were detected: a more diverse Alpine group in the Steiner and Julian Alps, and a less diverse Dinaric group. The Pokljuka population was assigned to the Dinaric group despite its geographical location in the Alps, placing the divide between major groups north of the southern Alpine orographic boundary. Bottlenecks dated at the end of the last glaciation event were suggested for Alpine populations, but not for Dinaric populations. Genetic signatures of migration were detected within Dinaric and Alpine regions, but not between them. Populations from the Prenj Mountain (Bosnia-Herzegovina), where the subspecies *S. atra prenjensis* was described, were not genetically distinct from other Dinaric populations. These results suggest that, if the taxon remains valid, *S. atra prenjensis* should include the entire Dinarides as well as the Pokljuka population located in the Alps.

Key words: Alpine salamander, Alps, Dinarides, genetic diversity, microsatellites, population history

INTRODUCTION

Pliocene and Pleistocene alternations of glacial and interglacial periods and gradual warming after the last glacial maximum have markedly shaped current patterns of temperate biodiversity. The Alpine region has played a major role to shape the geography of post-glacially expanding lineages (Hewitt, 1999; Weigand et al., 2012). The Dinarides, a part of the Alpine Region in the broader sense, are a mostly calcareous mountain range at the western Balkan Peninsula. Their topography is highly complex, with summits reaching into the alpine zone and incised valleys with thermophilous submediterranean vegetation (Surina et al., 2011). Being on the edge of the glaciation zone, the Dinaric mountains have been less affected by Pleistocene glaciations than other southern European mountains (Milivojević et al., 2008). Many phylogeographic studies have revealed that the Balkan Peninsula acted as a major European glacial refugia (Schmitt, 2007). However, only a few studies have focused on cold-adapted mountain species (Krystufek et al., 2007; Weigand et al., 2012; Kutnjak et al., 2014).

During the last ice age, the Dinarides probably included continuous refugial populations of Alpine salamanders (*Salamandra atra*, Laurenti 1768), a cold adapted species that recolonised the rest of the Alps after the retreat of ice (Bolkay, 1924). Today the Alpine salamander inhabits the Alps from western Switzerland to eastern Austria, as well as several isolated areas in the Dinarides as far as Albania (Bruno, 1973; Gasc et al., 1997). In the Prenj Mountain in Bosnia-Herzegovina, a separate subspecies (*S. atra prenjensis*, Mikšič, 1969) was recognised due to its shorter body length and different distribution of the palatal teeth (Mikšič, 1969; Šunje & Lelo, 2010), although its taxonomic status was never fully recognised (Joger, 1986; Klewen, 1988; Grossenbacher, 1994, 1997; Riberon et al., 2001). Findings of other subspecies (*S. atra aurorae*, Trevisan, 1982; *S. atra pasubiensis*, Bonato & Steinfartz, 2005) and previously unrecognised isolated populations in the Dinarides (Kletečki, 1990; Krizmanić, 1997; Krofel, 2005; Jeran et al., 2011; Stanković et al., 2015) suggest that the species' biogeography is not yet fully understood.

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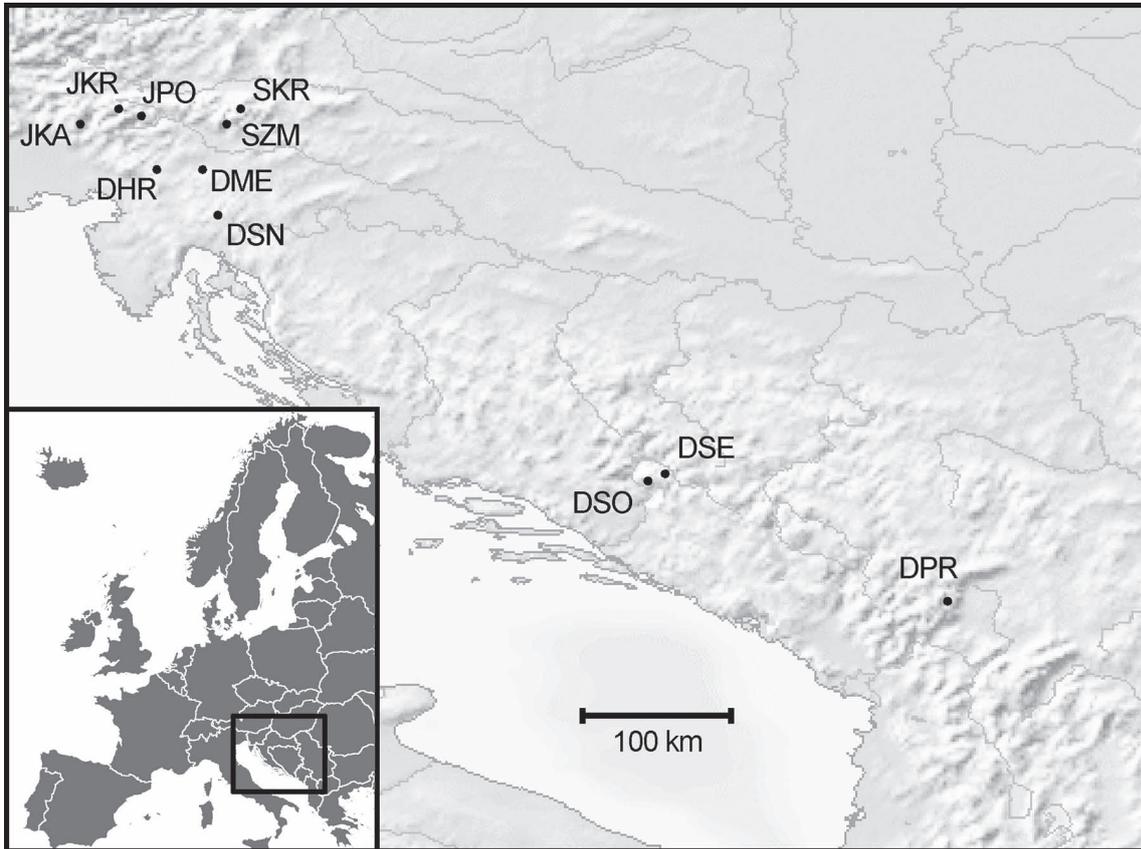


Fig. 1. Sampling locations in the Dinarides (location names starting with D), Julian (J) and Steiner Alps (S).

While *S. atra prenzensis* is morphologically and genetically closer to the nominal species than *S. atra aurorae* and *S. atrapasubiensis*, specimens from the entire Dinaric range do belong to a distinct phylogeographic lineage identified from mtDNA sequences which diverged in pre-Pleistocene time 3.1 ± 0.6 million years ago (mya) (Riberon et al., 2001), or 5.2 mya (confidence interval 1.6–10.5; Helfer, 2010). Initial mtDNA studies suggested low genetic differentiation among Alpine and Dinaric populations (Riberon et al., 2001; Steinfartz et al., 2000). A subsequent study revealed intrapopulation diversity of mitochondrial haplotypes (Helfer, 2010), with the Southeastern Alps representing a crossroad of phylogeographic lineages that diverged before the Pleistocene. Helfer (2010) described four lineages of *S. atra*: Dinaric, Northern Alps, NE Italy and S Austria lineages.

Differences between mtDNA and nuclear DNA based phylogenies were observed in other vertebrate species in the region (Pustovrh et al., 2014). Due to the specific inheritance of mtDNA, nuclear markers (Amplified Fragment Length Polymorphisms) were previously applied to Alpine salamanders but were identified as uninformative because of low differentiation between populations (Riberon et al., 2004). Microsatellite markers were developed for fire salamanders (Steinfartz et al., 2004), and some of them were successfully applied in Alpine salamanders (Hendrix et al., 2010). Here we report the results of the first study on the genetic structure of Alpine salamander populations from Southeastern Alpine and Dinaric locations based on polymorphic microsatellite loci. The aims of this study were to (i)

assess the genetic structures, (ii) determine genetic relationships among populations, (iii) detect historical events that influenced genetic differentiation.

MATERIALS AND METHODS

Tissue samples were collected from 162 individuals. Locations (Table 1 and Fig. 1) were visited several times in various weather conditions. The majority of samples were taken from adult individuals, and also included juveniles. Two specimens from Prokletije were intrauterine larvae, which were included due to the scarcity of samples from that area. Small clips of tissue ($<5 \text{ mm}^3$) from the tail or toes were sampled and stored in 96% ethanol. The samples from Prokletije were provided by the Regional Institute for Nature Conservation (Krizmanić, 1997). Total DNA was isolated using the extraction method of Taggart et al. (1992) or by Wizard Genomic DNA Purification Kit (Promega), following the supplier's instructions.

Seven microsatellite loci (*SalE2*, *SalE6*, *SalE7*, *SalE8*, *SalE12*, *SalE14* and *Sal29*, Steinfartz et al., 2004) were amplified using fluorescently labelled forward primers. Each PCR reaction (total volume 10 μl) contained 6.1 μl H_2O , 1 μl 10 \times PCR buffer, 0.9 μl 25 mM MgCl_2 , 0.5 μl 0.2 mM dNTPs, 0.25 μl 10 mM of each primer, 0.05 μl Fermentas Taq polymerase (5U/ μl) and 1 μl of template ($\sim 100 \text{ ng}$ DNA). The PCR parameters were initial denaturation (95 $^\circ\text{C}$, 5 min) followed by 35 cycles of strand denaturation (95 $^\circ\text{C}$, 45 s), primer annealing (59 $^\circ\text{C}$, 45 s; 53 $^\circ\text{C}$, 45 s for *SalE6* only) and DNA extension (72 $^\circ\text{C}$, 45 s). Aliquots of amplified DNA, mixed with formamide and GeneScan-500 ROX Size Standard (Applied Biosystems)

Table 1. Sample locations with coordinates and approximate altitudes, mountain range, number of individuals analysed (N) and genetic diversity indices of microsatellite marker data. A , average alleles per locus; H_E , expected heterozygosity in the population; H_O , observed heterozygosity; p -values, proportion of randomisations that gave a larger F_{IS} than the observed; deviations of F_{IS} values from HWE were not significant ($p < 0.001$).

Code	Location	Range	Coordinates	Altitude	n	A	H_E	H_O	P -values	F_{IS}
JKR	Krnica	Central Julian Alps	N46°25'35" E13°47'28"	1290 m	13	6.4	0.674	0.637	0.0491	0.094
JPO	Pokljuka	Eastern Julian Alps	N46°21'01" E13°55'20"	1380 m	12	5.6	0.546	0.543	0.2073	0.05
JKA	Kanin	Western Julian Alps	N46°21'47" E13°29'14"	2005 m	33	5.6	0.542	0.513	0.0665	0.078
SZM	Žrnovnica	Southern Steiner Alps	N46°21'15" E14°34'18"	1555 m	12	4.9	0.640	0.665	0.4764	0.006
SKR	Krofička	Northern Steiner Alps	N46°22'34" E14°38'19"	1450 m	7	5.4	0.625	0.650	0.2890	0.041
DHR	Hrušica	Dinarides	N45°53'43" E14°08'57"	540 m	15	4.3	0.494	0.515	0.5408	-0.007
DME	Menišija	Dinarides	N45°53'13" E14°18'57"	690 m	11	3	0.416	0.443	0.6153	-0.016
DSN	Snežnik	Dinarides	N45°36'21" E14°28'28"	1010 m	21	4.3	0.466	0.461	0.2919	0.036
DSE	Sedlo	Dinarides	N43°33'05" E17°54'53"	1885 m	15	3.1	0.368	0.362	0.3245	0.05
DSO	Soplje	Dinarides	N43°32'31" E17°51'48"	1770 m	15	2.7	0.283	0.267	0.1999	0.09
DPR	Prokletije	Dinarides	N42°39'53" E20°11'56"	1070 m	8	1.9	0.157	0.166	0.5848	0.01
	Overall				162	11				

were run on an ABI Prism 310 and 3130xl Genetic Analysers. A subset of samples was genotyped on both genetic analysers to ensure consistent allele reading.

The program Micro-Checker v. 2.2.3 (Van Oosterhout et al., 2004) was used to check for the presence of null alleles. Number of alleles per locus (A), expected (H_E) and observed (H_O) heterozygosity were calculated with GENETIX 4.04 (Belkhir et al., 2003). FSTAT v. 2.9.3.2 (Goudet, 2002) was used to calculate deviations from Hardy–Weinberg equilibrium (HWE) and pair-wise F_{ST} values. In all tests, p -values were adjusted using Bonferroni correction (Rice, 1989). Genetic relationships between populations were estimated as a proportion of shared alleles at each locus, i.e. allele sharing distances (D_{AS}) (Bowcock et al., 1994). A matrix of D_{AS} was used to construct a Neighbor-Joining tree using POPULATIONS software (Langella et al., 2002).

Genetic population structure was inferred using STRUCTURE v.2.3.2.1 program that runs Markov chain Monte Carlo (MCMC) simulations to segregate individuals into K clusters (Pritchard et al., 2000). STRUCTURE analysis assuming correlated allele frequencies between populations and the admixture model was run over a range of K values (1–10) and contained seven repetitions of 10^6 iterations following a burn-in period of 10^5 iterations. $\ln(K)$ values that stood out were omitted, however at least six repetitions were considered for each model. The most probable K was estimated by ΔK method (Evanno et al., 2005) and by the largest change in $\ln(K)$ between two consecutive K . Output files were compiled using CLUMPP v. 1.1.2 (Jakobsson & Rosenberg, 2007) and visualised using Distruct v. 1.1 (Rosenberg, 2004). In addition, CLUMPP's H' values were used to assess convergence

of repeats. The method of hierarchical STRUCTURE partitioning as suggested by Vähä et al. (2007) was also applied. The most differentiated cluster was excluded from the analysis, allowing for more precise clustering of the remaining individuals. Also, each excluded cluster was investigated for possible hidden substructures by choosing K values according to each specific setting. The amount of population genetic structure was evaluated by AMOVA in Arlequin v. 3.1 (Excoffier et al., 2005). Variance components were estimated for three hierarchical levels: among groups, between sampling sites within groups and within sampling sites.

Historical population declines were estimated using the coalescent analysis approach implemented in MSVAR v. 1.3 (Beaumont, 1999). A Stepwise Mutation Model (SMM), linear decline and eight-year generation time were assumed. At least three independent chains with 2×10^8 iterations were run for each population. Additional and/or longer chains were run when there was no convergence or highest probability density (HPD) intervals were wide. The strength of evidence of population decline was evaluated by Bayes factor (Kass & Raftery, 1995), as suggested by Beaumont (1999) and Storz & Beaumont (2002). A gene flow-drift equilibrium model was tested against a pure drift model using coalescent-based MCMC simulations implemented in 2MOD (Ciofi et al., 1999). Simulations were run in several independent runs with 10^5 – 10^8 . Rates of contemporary migrations over the most recent generations were estimated using BayesAss v.1.1 (Wilson & Rannala, 2003), 5×10^6 iterations were run, with a sampling frequency of 1000. The initial 10% of all MCMC iterations were discarded. HPD intervals for all chains were determined in Tracer

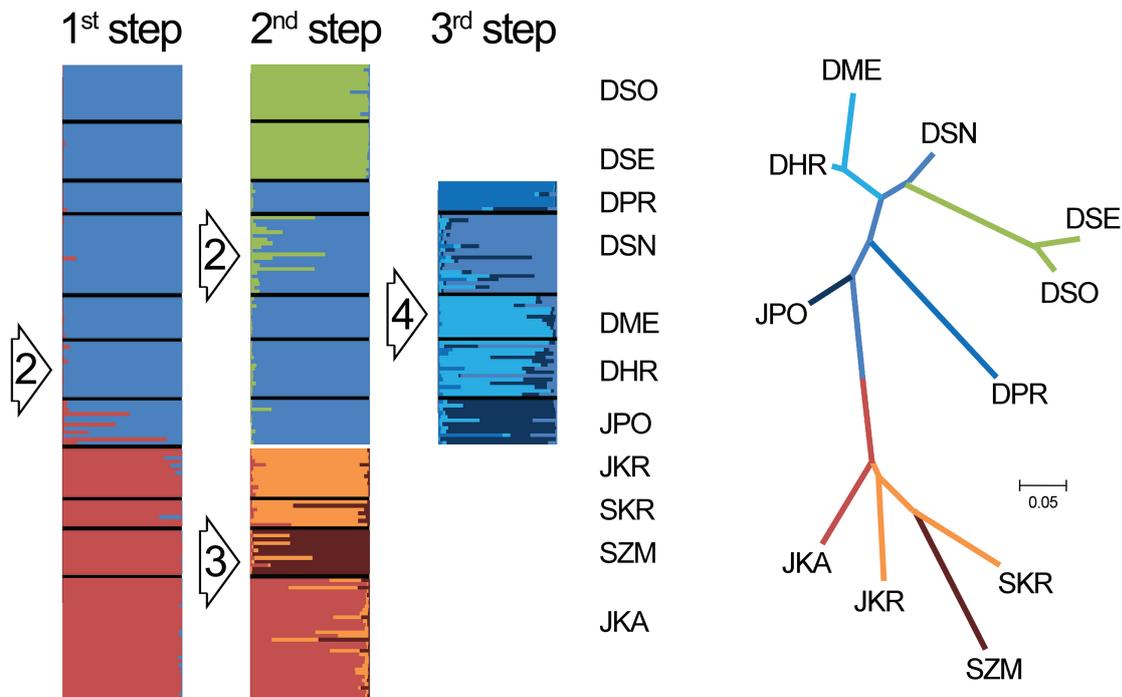


Fig. 2. Estimated population structure as inferred by hierarchical STRUCTURE analysis (left) and tree of populations based on DAS genetic distances (right). Black lines separate sampling sites. After three steps eight clusters were identified. The most probable K for analysed samples given in arrows is based on the ΔK method; no further structures were detected in subsequent rounds and within sampling locations ($K=1$).

v. 1.5.0 (Rambaut & Drummond, 2009). Brooks, Gelman & Rubin convergence tests between independent chains were performed in BOA (Smith, 2007) as implemented in *R* (Ihaka & Gentleman, 1996). The PGDSpider program (Lischer & Excoffier, 2012) for conversions between different genotype formats was used during the analyses.

RESULTS

A total of 162 individuals of *S. atra* from 11 sampling locations were scored for seven microsatellite loci. Of these individuals only three failed to be genotyped at more than one loci. As revealed by Microchecker, none of the loci at any sampling site exhibited a null allele.

All seven microsatellite loci were polymorphic in at least one alpine salamander population. Populations from the Julian and Steiner Alps exhibited higher levels of heterozygosity and allelic richness than Dinaric populations (Table 1). No population deviated from Hardy-Weinberg equilibrium. The delta K metric of Evanno et al. (2005) revealed a high value for $K=2$ (Appendix, Table A1, Online Appendix) in the first step of the hierarchical clustering using STRUCTURE, which separated Dinaric and Alpine populations (Fig. 2). A notable exception was the Pokljuka population in the Eastern Julian Alps, which clustered with Dinaric populations. A further two steps identified relatively homogeneous clusters that mostly corresponded to individual locations. In total, STRUCTURE identified eight genetic clusters. Analyses of individual populations revealed no intrapopulation structure which would indicate hybrid populations. Similar relations among populations were also visible in the D_{AS} based tree (Fig. 2). Pair-wise F_{ST} values ranged between 0.05 and 0.57 with only four pairs where values

were not statistically significant (Table 2). Different hierarchical AMOVA produced similar results; 72.0% ($p<0.05$) of variation was detected within sampling sites, 18.7% ($p<0.001$) between sampling sites within groups and 9.3% ($p<0.001$) among groups when groups were defined as three mountain ranges and as clusters in step 2 of structure analysis (Fig. 2). When groups were defined as clusters in step 1 (Alpine vs. Dinaric populations and JPO), percentages of variation were 72.8, 22.8 and 4.4 respectively.

Heterozygosity excess that is characteristic for recent bottlenecks could not be detected with certainty, because sample size and number of loci did not meet the criteria (results not shown). On the other hand, ancient bottlenecks were detected by coalescent analyses implemented in MSVAR v.1.3. Posterior distribution for most populations consistently indicates a strong decline that occurred 16,000-40,000 years ago (Table 3). Exceptions are DSE, DSO and DPR populations where Bayes factors for population decline were well below 100 (3.19, 27.45, 0.76), meaning that the evidence for decline is not as conclusive as in other populations (Kass & Raftery, 1995). Only the JPO population failed to converge even after additional and longer chains were included; the value for the multivariate Brooks, Gelman and Rubin's diagnostic was 1.22, while all other populations had values below 1.1. Individual non-converging parameters (0.975 quantile greater than 1.20) appeared in three populations (SKR, JPO and JKA).

Coalescent modelling implemented in 2MOD consistently selected a model that assumed equilibrium between drift and gene flow over a pure drift model in all steps after burn-in. However, most migration rates estimated by BayesAss were very low (mean values

Table 2. F_{ST} pairwise values above the diagonal and their significance below the diagonal. *** $p > 0.999$, ** $p > 0.99$, * $p > 0.95$, NS - non significant.

	JKR	JPO	JKA	SZM	SKR	DHR	DME	DSN	DSE	DSO	DPR
JKR		0.1762	0.1206	0.1338	0.0499	0.2426	0.2589	0.2839	0.3386	0.4038	0.4154
JPO	***		0.2125	0.2754	0.2234	0.0945	0.1372	0.1381	0.2582	0.2749	0.2486
JKA	***	***		0.1778	0.1264	0.3030	0.3549	0.3219	0.3901	0.4348	0.4104
SZM	***	***	**		0.0965	0.3189	0.3231	0.3580	0.4034	0.4648	0.4967
SKR	NS	*	NS	*		0.2502	0.2848	0.3143	0.3962	0.4739	0.4946
DHR	***	***	**	***	*		0.0811	0.1201	0.2355	0.2525	0.3218
DME	***	**	**	**	*	NS		0.2162	0.3293	0.3795	0.4802
DSN	***	***	***	***	**	***	***		0.1977	0.1663	0.3480
DSE	***	***	***	***	**	***	***	***		0.1225	0.5587
DSO	***	***	***	***	**	***	***	***	***		0.5657
DPR	**	*	**	**	NS	***	**	***	***	***	

between 0.01-0.02) with L-shaped posterior distribution. An arbitrarily set value of 0.1 for the upper 95% HPD was used for identifying migration rates greater than zero. However, independent runs did not always converge to completely identical non-zero migration routes. After several preliminary tests, ten independent runs with identical initial parameters were performed. Four migration routes (migration rate > 0.1) were identified in all ten independent runs. An additional set of three routes was detected among Dinaric populations in five independent runs, while the other half of the runs supported a set of two other routes among Dinaric populations. Another route from the Steiner to Julian Alps was supported by only three of the ten runs, but was not tied to any of previously mentioned sets (Table 4). No routes between Alpine and Dinaric locations were detected in either run with the exception of the JPO population; the only bidirectional migration was detected between DHR and DME populations. Migration rates and population sizes were also estimated in MIGRATE-n v. 3.2.16 (Beerli & Felsenstein, 2001; Beerli, 2006). Even after several attempts under different models and scenarios, convergence was not reached and migration rates were approaching zero (results not shown).

The reason for non-convergence could be an ancient bottleneck, as MIGRATE assumes constant population sizes and migration rates over the coalescence period.

DISCUSSION

The observed populations of Alpine salamanders can be divided into two major groups (Fig. 2): the more diverse group of Steiner and Julian Alps populations and a less diverse Dinaric group with lower levels of observed heterozygosity and mean numbers of alleles per loci. In general, genetic diversity decreased with increase in distance from the Alps. It has to be stressed that while sampling of adults was random, two samples from Prokletije were intrauterine larvae, therefore related to the third sample (mother) from that location (Krizmanić, 1997). Because of lack of other samples from the southernmost range of Alpine salamander, these three related individuals were included in all analyses.

Current levels of genetic diversity are most likely consequences of an ancient bottleneck dated to the last ice age 16,000–40,000 years ago (Table 3). A bottleneck for the central and south Dinaric populations has weaker support from the coalescent analysis implemented in

Table 3. Mean values (95% HPD interval) on a log₁₀ scale from single run MSVAR output, mean values in years and Bayes factor (BF) for population decline. * - indication of non-converging parameters after at least three independent runs.

Population	Time since decline (log ₁₀)	Time since decline (yrs)	BF
JKR	4.160 (3.155–5.116)	14.454	∞
JPO*	4.588 (3.853–5.371)	38.726	∞
JKA	4.444 (3.647–5.291)	27.797	∞
SZM	4.309 (3.325–5.236)	20.370	642.36
SKR	4.270 (3.348–5.228)	18.621	309.84
DHR	4.327 (3.431–5.245)	21.232	∞
DME	4.680 (3.800–5.585)	47.863	∞
DSN	4.231 (3.252–5.124)	17.021	∞
DSE	5.141 (1.495–9.065)	138.357	3.19
DSO	5.495 (3.238–8.239)	312.608	27.45
DPR	4.455 (0.100–8.993)	28.510	0.76

MSVAR v. 1.3. A first assumption for the bottleneck analysis was a linear decline. An exponential model was tested, but the convergence of several parameters were consistently poor (results not shown). An exponential model is appropriate for fluctuations of population size on a short timescale (Beaumont, 1999). A second assumption was a generation time of eight years. Fecundity and gestation of Alpine salamanders depend on altitude, and juveniles mature at an age of 2-3 years before reproducing at the age of 3–5 years (Steward, 1964; Tortonese & Lanza, 1968). During their roughly 11–16 years lifespan (Duellmann & Trueb, 1986; Kalezić et al., 2000; Guex & Grossenbacher, 2004), females give birth to two juveniles per pregnancy every 2-4 years (Vilter & Vilter, 1960). Gestation duration of up to four years was observed at altitudes above 1700 m and can rise to five or more years above 2500 m (Guex & Grossenbacher, 2004); the majority of our sampling localities were from altitudes below 1700 m. In contrast to the often-reported two juveniles per pregnancy, Luiselli et al. (2001) found single births in all the observed pregnant females (location Sella Nevea, Italy, altitude 1100 m). Additional MSVAR runs with generation times up to 16 years were made, but this change did not affect the results considerably. MSVAR is a very sensitive tool for detecting bottlenecks (Girod et al., 2011), although it can generate false signals under certain conditions (Chikhi et al., 2010). F_{ST} values and number of loci were appropriate for the analysis and no intrapopulation structure was detected in any location using STRUCTURE. We conclude that a historical bottleneck coinciding with the last ice age is highly plausible, while population decline in the central and south Dinarides either did not occur (lower Bayes Factor) or could not be detected with the sample size and markers used.

According to Bolkay (1924), the population of *S. atra* on Prenj Mountain represents a glacial relict. During the glacial period, *S. atra* inhabited the far south-eastern parts of the Dinarides and probably survived on plains located at lower altitudes. As the climate became warmer after

the retreat of ice, *S. atra* migrated to higher mountains, where populations survived until the present day, while the surrounding lower regions of Dinarides are no longer inhabited by *S. atra*.

Even geographically close sampling sites are inhabited by genetically distinct populations of Alpine salamanders. Low mobility of Alpine salamanders (Bonato & Fracasso, 2003) is often assumed, but gene flow among populations (most within 6 km) driven by male migration was detected by Helfer et al. (2012). While most of the low F_{ST} values were observed between neighbouring populations, the lowest value (0.05) and the same cluster in the 2nd step of STRUCTURE analysis for populations SKR and JKR is difficult to explain. The locations belong to different Alpine ranges and migration between them is unlikely; random genetic drift might have caused genetic similarity of two distant populations. Between genetic drift and drift/gene flow equilibrium, the 2MOD program consistently favoured the latter. BayesAss was used to estimate the direction and rate of contemporary migrations (Wilson & Rannala, 2003). Data were run several times, and some significant migration routes, arbitrarily defined as upper 95% HPD > 0.1, were obtained by all 10 independent runs. In addition, half of the runs favoured one migration scenario (several significant migration routes) and the other half a different migration scenario among Dinaric populations (Table 4). The present study meets the criteria assumed by the BayesAss software (Wilson & Rannala, 2003; Faubet et al., 2007), as populations exhibit high differentiation rates and low migration rates. Furthermore, based on observation of high densities of *S. atra* during favourable conditions at some sampling sites (Societas Herpetologica Slovenica and Centre for Cartography of Fauna and Flora database on amphibian distribution in Slovenia), we can assume that populations are large enough that there was negligible genetic drift during the past few generations.

There is a lack of studies that address the question of how small migration rates should be to ensure that subpopulations have independent dynamics.

Table 4. Significant mean migration rates (95% HPD intervals), calculated from combined converging runs. Non-significant migrations and some populations are omitted for clarity. ¹combined from all ten runs, ²five runs, ³five runs, ⁴three runs.

		From population				
		SKR	JPO	DHR	DME	DSE
To population	JKR	0.065 ⁴ (0.000–0.147)				
	JKA	0.049 ³ (0.000–0.111)				
	SZM	0.052 ¹ (0.000–0.123)				
	DHR		0.103 ² (0.000–0.203)		0.179 ² (0.111–0.245)	
	DME		0.113 ³ (0.000–0.207)	0.183 ³ (0.119–0.245)		
	DSN			0.060 ² (0.003–0.127)		
	DSO					0.203 ¹ (0.143–0.263)
	DPR		0.046 ¹ (0.000–0.131)			

Hastings (1993) suggests that two populations become demographically independent when migrations fall below about 0.1. While low migration rates within Alpine subpopulations support their isolation and independence, higher migration rates in detected routes among Dinaric populations support the notion of Krofel (2005) that they should not be regarded as isolated one from another. The JPO population, which belongs to the Dinaric group, but is geographically in the Julian Alps, followed the migration scenario of other populations belonging to the Dinaric group. A better understanding of migration routes within the Dinaric populations could be gained by increasing sampling coverage, as there are no samples from the distribution area in western Croatia. No significant migration was observed between Alpine and Dinaric (including JPO) groups. However, 2MOD still strongly favoured migrations over drift even when analysing pairs of Alpine-Dinaric populations, for example JKA-DSN and JKR-JPO.

The divergence of phylogeographic lineages of Alpine salamander is dated before the last glaciation event, and it is assumed that the Alps were recolonised from glacial refugia located in the Balkans and in the Italian prealpine area (Riberson et al., 2001). The presence of a Dinaric population in the Julian Alps (JPO) can be explained as a recolonisation event after the retreat of glaciers, confirmed by finding Dinaric mtDNA even in other locations in the Julian and Steiner Alps (Helfer, personal communication). No indications of postglacial migration from the Alps to the Dinarides were found. Dinaric populations with low genetic diversity, particularly in the central and southern Dinarides can be explained by reduced habitat and competition with the fire salamander (*S. salamandra*). While the exact nature of fire and alpine salamander interaction is not known, overlapping ranges are rare although the local presence of one species has no apparent effect on the occupancy probability of the other (Werner et al., 2013). Alternatively, high genetic diversity in the Alps can be explained by recolonisation from several glacial refugia.

Conclusions

A historical bottleneck event at the end of the last glaciation period resulted in two genetically distinct extant groups of Alpine salamanders, the Alpine group, and a genetically less diverse Dinaric group. The observed genetic structure, lack of migrations between the Alps and the Dinarides, migration rates within the Dinarides and indications of different population history in the central and southern Dinarides suggest that the recognition of *S. atra prenzensis* as a separate subspecies should be considered for the Dinarides as well as Pokljuka. This suggestion requires further support by morphological and ethological findings. Migration rates within Dinaric populations that are more than 25 km apart and some recent field discoveries suggest the possible existence of other undiscovered populations of Alpine salamanders in the region between the Alps and Prokletije (Kletečki, 1990; Krizmanić, 1997; Krofel, 2005; Jeran et al., 2011).

ACKNOWLEDGEMENTS

We thank members of Alpine Club “Željezničar” who participated in field work in Bosnia and Herzegovina. This work was financed by the Federal Ministry of Environment and Tourism of Bosnia and Herzegovina (grant no. 06/1-22-1564/10) and Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant No. 173045).

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Accepted: 19 June 2015

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