



# Small-scale spatial genetic structure of Alpine chamois (*Rupicapra rupicapra*) in Northern Dinarides

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## Abstract

Chamois (*Rupicapra rupicapra*) is a mountain-dwelling ungulate inhabiting predominantly rocky habitat with steep slopes. Since it mostly inhabits boreal habitats at high altitudes, low valleys tend to separate populations, thereby limiting gene flow. In the present study, we genotyped 54 georeferenced chamois using 20 SSR loci to test the influence of the Kupa River on the spatial genetic structure of the population in the bordering area between Croatia and Slovenia in the northern Dinaric Mountains. Both GENELAND and STRUCTURE assigned all individuals to one spatial cluster, indicating that the Kupa River does not represent a barrier to gene flow for chamois. Unfortunately, in 2015, a razor wire fence was constructed along the Croatian-Slovenian border at the Kupa River. This fence represents a major threat to the chamois population as it may cause mortality, obstruct seasonal dispersal, and reduce the effective population size. If the fence remains as it is, changes in the genetic structure and genetic diversity of the population due to the effect of drift and reduced effective population size can be predicted over the next generations.

**Keywords** Mountain ungulate · Genetic structure · Microsatellites · Habitat fragmentation · Northern Dinarides

## Introduction

Chamois (*Rupicapra rupicapra*) is a mountain-dwelling ungulate with habitat distribution over Central and Southern Europe and adjacent Western Asia (Corlatti et al. 2011). It

inhabits predominantly rocky habitats with steep slopes (Ramanzin et al. 2002) at altitudes from around 1000 to 2500 m, though it occasionally descends to areas at lower altitudes characterized by rocky ground, ecological heterogeneity, and steep slopes (Tosi and Perco 1981). Since the species inhabits mostly boreal habitats at high altitudes, rivers and low valleys can present obstacles (Loison et al. 1999), which may result in reduced gene flow between populations (Brown 2001) and increased genetic differentiation. The genetic makeup of isolated populations may differ due to a loss of allele variants as a consequence of genetic drift (Spielman et al. 2004; Willi et al. 2006) and loss of heterozygosity due to the inbreeding effect (Brook et al. 2002; Whitlock 2003).

Two of the described drivers of Alpine chamois (*R. r. rupicapra*) spatial genetic structure are as follows: sex specific dispersal (Loison et al. 1999; Nesti et al. 2010; Unterthiner et al. 2012) and habitat fragmentation caused by anthropogenic and natural landscape features (Soglia et al. 2010; Buzan et al. 2013). Loison et al. (1999) confirmed the hypothesis of philopatry of female chamois and a tendency for dispersal in males using telemetry. Later, Lovari et al. (2006) described two categories of dispersal in adult males: territorial males and seasonally migrant ones. This preference of different habitat use between females and resident and migrant males

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was confirmed by radio tracking chamois in the Italian Alps by Nesti et al. (2010) and Unterthiner et al. (2012). Adapted to mountain habitats, chamois is often unable to disperse across large swaths of dense forest in mountain (alpine) areas (Oravec 2017). On the other hand, spontaneous expansions of chamois populations were recorded throughout the twentieth century, some of which resulted in the formation of new tiny colonies, even in forested regions of Dinaric Mountains (at lower altitudes) between Alps and Mt. Pohorje in Slovenia (Adamic and Jerina 2010). The genetic diversity of these populations is lower than in the Alps due to the patchiness of suitable habitat and low dispersal probability in the predominantly forested landscape, though there is existing gene flow between forest habitats (Buzan et al. 2013; Šprem et al. 2015).

Habitat fragmentation caused by natural and anthropogenic landscape features can have an effect on the genetic structure of ungulates. Soglia et al. (2010) analyzed the genetic structure of Alpine chamois in the Italian Alps and presented that deep river basins between mountain peaks represent strong migration barriers that cause differentiation of populations.

Similar effect of landscape features such as rivers and waterflows on the genetic structure of ungulates was confirmed by Coulon et al. (2004). They described how several landscape features with low permeability can lead to population differentiation of roe deer (*Capreolus capreolus*). Ferreira et al. (2006, 2009) suggest that the two-river system may play a role in the genetic structure of wild boar (*Sus scrofa*) in Portugal.

Reduction of gene flow between fragmented populations can be further increased by anthropogenic landscape elements such as roads or fences. Kuehn et al. (2007) concluded that anthropogenic transportation infrastructure can cause habitat fragmentation, which in turn resulted in genetic fragmentation of a roe deer population. Similarly, Šprem et al. (2013) presented evidence of the contribution of fenced motorways to genetic divergence of a red deer (*Cervus elaphus*) population.

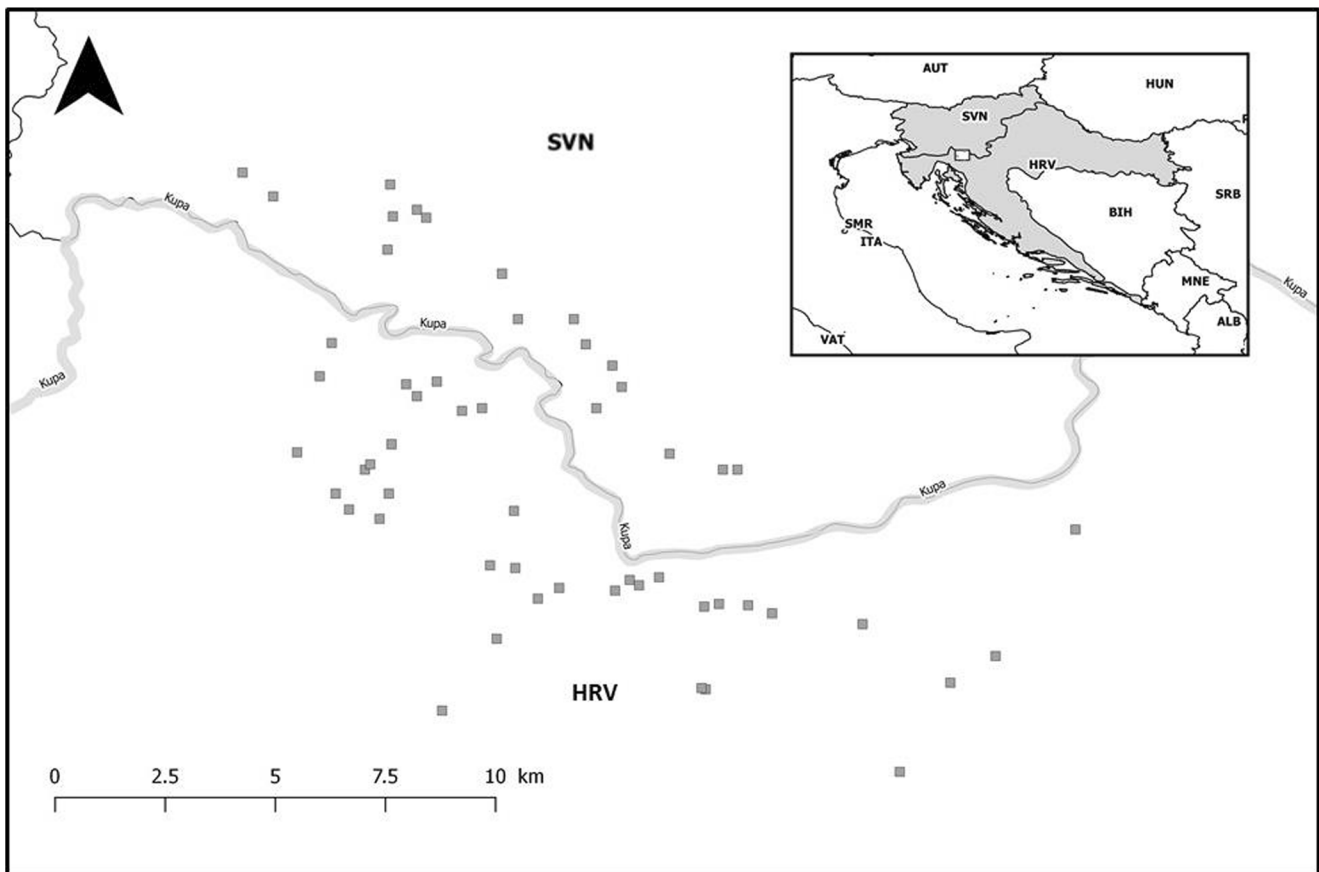
Recently, as response to large migrant influxes, border fences have been erected in border areas in some parts of Europe (Linnell et al. 2016a) and these fences additionally increase habitat fragmentation. In 2015, a razor wire border fence was constructed along the Kupa River (see Supplementary Fig. S1), between Croatia and Slovenia, over a total length of 178 km (Pokorny et al. 2017). The fence has been proven to prevent movements of red deer, roe deer, and wild boar across it: during a 10-month monitoring period on the Slovenian side, Pokorny et al. (2017) reported the presence of 21 ungulate carcasses entangled in or deceased close to the razor wire fence. Since there are no records of chamois carcasses found by the border fence during the monitoring period, the question arose as to whether they crossed the Kupa River before the fence was raised, whether the fence is acting as a barrier to chamois movements, or whether this is due simply to a lack of information.

While the wide-scale population genetic structure of chamois in entire Dinaric region has been previously investigated and described by Buzan et al. (2013) and Šprem and Buzan (2016), in the present study, we aim to investigate small-scale spatial genetic structure of the Northern Dinaric Mountains population around the Kupa valley. The results will serve as a baseline for the future monitoring of the effects of the border fence on the genetic structure of this population. In addition, based on the fact that no chamois were found affected by the border fence, we tested the possible influence of the Kupa River on population isolation and genetic differentiation.

## Material and methods

The study area is a mountainous region in Western Croatia and Southern Slovenia, intersected by the Kupa River that forms the administrative state border between Croatia and Slovenia (Fig. 1). The Kupa River Valley is a Habitats Directive Special Area of Conservation (SAC) and Wild Birds Directive Special Protection Area (SPA) NATURA 2000 sites. The area is covered by coniferous and beech forests (41%), composed mainly of silver fir (*Abies alba*), spruce (*Picea abies*), and beech (*Fagus sylvatica*), at elevations between 200 and 1534 m. A humid boreal climate with a Mediterranean influence (mean annual temperature, 7.7 °C; mean annual rainfall, 2079 l/m<sup>2</sup>) is dominant in the valley (Šprem et al. 2016). The Kupa River springs from the karst landscape beneath the Risnjak Mountains and flows over 296 km before draining into the Sava River (Treer et al. 2014). It contains a range of habitats from a deep pool section (maximum depth about 4.0 m), to rapids and riffles (mean depths 0.3–1.0 m) and a shallow pool section. In the study area, the river is 25 to 45 m wide. Today, the chamois population on the Croatian side is estimated at approximately 130 individuals (Kavčić et al. 2018) while the population on the Slovenian site is estimated at approximately 200 individuals (Buzan et al. 2013).

Tissue samples were collected from 54 chamois (37 from the Croatian side and 17 from the Slovenian side of Kupa River) during regular culls between 2015 and 2017 in compliance with game management plans. Tissue samples were stored in ethyl alcohol at –80 °C. DNA was extracted using commercial Isolate II Genomic DNA Kit 250 (Bioline) following the manufacturer's protocol. All samples were pre-screened for DNA concentration and only samples with > 5 ng/mL were used in the study. Twenty microsatellites were amplified loci in 3 multiplex sets, containing 7, 6, and 7 loci, respectively, using the protocol described in Zemanová et al. (2011). Three multiplex polymerase chain reactions (PCRs) were performed using KAPA2G Fast Multiplex Mix (KAPA Biosystems) according to the protocol described in Buzan et al. (2013). PCR reactions (10 µL) for all three sets contained 1.0 µL extracted DNA, 3.9 µL KAPA2G Fast Multiplex Mix,



**Fig. 1** Map of the study area with Kupa River and state border between Croatia (HRV) and Slovenia (SLO). Gray squares represent locations of samples. Inset shows location of the study area (white square on the

border between Croatia and Slovenia) on a partial map of Europe. Countries are labeled with official three letter ISO 3166-1 alpha-3 code

0.8  $\mu$ L 1 M BSA, 2.8  $\mu$ L ddH<sub>2</sub>O, and 1.5  $\mu$ L primers set (forward fluorescently labeled primers). The amplified PCR products were used to perform fragment analysis with Gene Scan LIZ 500 Size Standard (Applied Biosystems) on an ABI 3130 Genetic Analyzer (Applied Biosystems). Microsatellite genotypes were examined using GeneMapper software v. 4.1 (Applied Biosystems).

The observed and expected heterozygosity ( $H_O$  and  $H_E$ ) and inbreeding coefficient ( $F_{IS}$ ) for each locus were estimated using FSTAT 2.9.3.2 software (Goudet 1995). Departures from Hardy–Weinberg equilibrium (HWE) for each locus were tested by 1000 permutations, at the nominal  $p$  level of 0.05 using same software, with corrections for multiple comparisons using Bonferroni correction.

To test for the presence of population substructure, and if the Kupa River acts as a boundary to gene flow between the Croatian and Slovenian locations, we used Bayesian clustering method STRUCTURE 2.3 (Pritchard et al. 2000). To estimate the number of subpopulations ( $K$ ), ten independent runs of  $K$  from one to five were carried out with  $10^6$  Markov-chain Monte Carlo (MCMC) iterations after a burn-in period of  $10^5$  iterations. An admixture model with uncorrelated allele frequencies was used. Log-likelihood values of all

runs were then compared using the website and program STRUCTURE HARVESTER (Earl and von Holdt 2012) and the optimal  $K$  was chosen, following the STRUCTURE manual, as the one with highest average and smallest standard deviation of estimated  $\ln$  probabilities of data from ten runs. The same software was used to summarize the results of individual assignment from 10 independent runs with the determined optimal value of  $K$ .

To confirm the robustness of STRUCTURE results, we analyzed the same data with GENELAND (Guillot et al. 2005), a Bayesian spatial clustering method that includes geographic locations of sampled individuals. GENELAND was chosen among spatial clustering methods because it can account for the presence of null alleles (Guillot et al. 2008) and provides the most accurate estimates of true genetic structure (see Safner et al. 2011 and Blair et al. 2012 for more details). The number of chamois genetic clusters in the study area was determined by running the algorithm ten times, allowing the estimate of the number of genetic clusters ( $K$ ) to vary, with the following parameters: model with null allele estimation,  $10^6$  MCMC iterations with a thinning of 100, uncertainty attached to the spatial coordinates fixed at 10, minimum  $K = 1$ , maximum  $K = 6$ , maximum number of nuclei in the Poisson–

Voronoi tessellation fixed to 300. The Dirichlet model (uncorrelated allele frequencies) was used as a prior for all allele frequencies. After inferring the number of populations, the algorithm was run a further five times with  $K$  fixed to the inferred number of clusters, using the same parameters as above. The modal cluster membership for each individual for all five runs was selected and plotted on a map. To test the robustness of the clustering results, we performed analyses using models with and without spatial information with the same parameters as described.

## Results and discussion

All 20 loci were successfully amplified and genotyped in all 54 samples. The detected number of alleles per loci varied between 2 (for loci INRA12 and SY434) and 15 (for locus SRCRSP09), with an average of 6.75 alleles per locus (Table 1). This result is slightly higher than reported by Buzan et al. (2013), who detected an average of 4.30 alleles per locus, and Šprem and Buzan (2016), who detected an average of 4.68 alleles per locus using the same markers. The observed heterozygosity varied between loci from 0.129 for locus NRAMP1 to 0.778 for loci ETH10 and ILST03, with

**Table 1** Genetic diversity estimates for 20 microsatellite loci.  $N$ , number of detected alleles;  $H_E$ , expected heterozygosity;  $H_O$ , observed heterozygosity;  $F_{IS}$ , inbreeding coefficient;  $p(F_{IS})$ ,  $p$  value of permutation test for  $F_{IS}$  after Bonferroni correction (nominal level = 0.05), significant values are in boldface

Locus	$N$	$H_E$	$H_O$	$F_{IS}$	$p(F_{IS})$
BM1258	8	0.712	0.611	0.142	0.113
BOBT24	8	0.799	0.722	0.097	0.162
CSSM66	5	0.614	0.444	0.278	<b>0.025</b>
ETH10	6	0.755	0.778	-0.031	0.765
ETH225	3	0.563	0.574	-0.02	0.741
ILST03	12	0.847	0.778	0.082	0.158
INRA12	2	0.471	0.481	-0.023	0.752
MAF214	9	0.779	0.667	0.145	0.113
NRAMP1	3	0.342	0.129	0.623	<b>0.005</b>
OarFCB20	6	0.725	0.63	0.132	0.122
OarFCB304	3	0.522	0.593	-0.137	0.894
SRCR-Sp0	7	0.758	0.593	0.22	<b>0.023</b>
SRCRSP06	12	0.830	0.741	0.109	0.113
SRCRSP09	15	0.837	0.500	0.405	<b>0.005</b>
SRCRSP11	8	0.686	0.648	0.055	0.399
SY259	5	0.702	0.593	0.157	0.113
SY434	2	0.214	0.167	0.222	0.202
SY58	6	0.676	0.574	0.152	0.113
SY84	7	0.519	0.426	0.181	0.113
TGLA53	8	0.741	0.722	0.026	0.528

an average value across all loci of 0.611. After FDR correction, four loci (CSSM66, NRAMP1, SRCR-Sp0, RCRSP09) had significant deviation from HWE due to the lack of heterozygosity. The  $F_{IS}$  estimate for entire population was 0.133, and it was significant after testing using 10,000 permutations ( $p < 0.001$ ). This may indicate the presence of null alleles or possible inbreeding within the population, which leads to the loss of genetic diversity and adaptability. Such results can be expected within isolated populations with limited gene flow.

Crestanello et al. (2009) and Soglia et al. (2010) presented higher estimates of genetic diversity in high-elevation chamois populations from the southern edge of the Alps in Italy, while Markov et al. (2016) reported similar values for three populations from the Austrian Alps. The reasons for those differences could be better connectivity between populations in the Alps, and a different set of markers used in those studies. Buzan et al. (2013) reported lower estimates of genetic diversity for Dinaric populations of chamois in Slovenia in comparison with Alpine ones. They attributed this to the small effective population sizes and a lowered chance of long distance migration through the forest matrix. Šprem and Buzan (2016) analyzed genetic diversity of Alpine and Balkan chamois subspecies in the Northern and Southern Dinarides.  $F_{IS}$  estimates for all populations in that study were lower than estimated here (all  $< 0.106$ ) and not significant, even for the small population of Balkan chamois on Mt. Prenj (Bosnia and Herzegovina). All these parameters indicate that the analyzed population has relatively low genetic variability, and that from the genetic perspective, might be vulnerable to isolation and habitat fragmentation.

Ten STRUCTURE runs with  $K = 1$  had the same estimated Ln probabilities of data (Supplementary Fig. S2), higher than any other runs. Since they had the highest average and smallest standard deviation (0), we chose 1 as the inferred number of ancestral populations in the data. This indicates that there is no sub-structuring within the population, and that there are no recent immigrants from other, genetically distinct, populations. Isolation of the population (low immigration) could also be deduced by the genetic parameters presented in Table 1. When the individual cluster memberships for solutions with  $K = 2$  were examined, none of the individuals had more than 80% of single ancestral cluster membership, indicating that all individuals in the population share the same origin.

All ten independent runs of GENELAND, both for the spatial and non-spatial model, found only one population in the study area, confirming the STRUCTURE result. The presented results support a strong indication that the Kupa River does not present a barrier for chamois movement, and that there is existing gene flow within the population. While this contradicts the results of Soglia et al. (2010), this discrepancy can be explained by landscape differences between the areas of Italian Alps where populations analyzed by Soglia et al. were sampled and Kupa Valley. While in the Alps chamois occupy high-altitude peaks separated by rivers, the population

in the Kupa Valley lives at much lower elevations in forested habitats. In addition, the flow of the Kupa River in the study region is much calmer than the rapid Alpine rivers. Homogeneity of the population might be the consequence of the ability of chamois to swim or cross the river at spots with low and calm water. While there is no indication of an effect of the border fence on chamois, our results confirmed that there was gene flow across the river before the fence construction. Based on the observations of professional game keepers from both sides of the Kupa River, chamois have been seen crossing the river several times (Stane Frbežar and Mladen Mauhar, pers. comm.). The swimming capability of chamois was recently confirmed by the first record of one swimming in the Adriatic Sea, at the foot of Mt. Velebit (44°43'34.2" N 14°53'34.6" E), that was published online (Hodak 2018).

Fragmentation of the chamois population in the Kupa River Valley by the razor wire border fence between Croatia and Slovenia could have strong negative consequences for future population viability and structure. If the fence remains as it is, free gene flow within the population would be stopped, and genetic diversity would diminish due to the effect of drift and reduced effective population size. Such a negative effect of the border fence between Poland and Belarus on the genetic diversity of European bison (*Bison bonasus*) over a period of 30 years was reported by Daleszczyk and Bunevich (2009). In Arizona (USA), 39 protected species, or species proposed for protection under the Endangered Species Act, are already affected by the security fence that was built along more than one-third (1125 km) of the USA-Mexico border (US Public Law 109–367) (Clark et al. 2011). In order to uphold the paradigm of transboundary conservation, more wildlife and nature-friendly solutions should be applied in protecting administrative borders (Fonseca et al. 2014). In addition to the negative biological effects for certain species/population, border fences disrupt the connectivity of the Natura 2000 network in Europe and violate several major wildlife treaties (Linnell et al. 2016a, b), challenging the policies of European Union and creating a dangerous precedent for other activities affecting the connectivity of ecosystems.

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