SHORT COMMUNICATION

Microsatellite based assignment reveals history of extirpated mountain ungulate

Toni Safner[1,2] · Elena Buzan[3,4] · Laura Iacolina[5] · Sandra Potušek[3] · Andrea Rezić[5] · Magda Sindičić[6] · Krešimir Kavčić[5] · Nikica Šprem[5]

Abstract

During the early 1900s, Northern chamois (*Rupicapra rupicapra*) populations in the northern Dinaric Mountains were extirpated. During the 1960s and 1970s there were several reintroductions of individuals from two Northern chamois subspecies (Alpine chamois, *R. r. rupicapra* and Balkan chamois, *R. r. balcanica*) from neighbouring areas in the attempt to re-establish the population. Accurate taxonomic classification, at subspecies level, of the autochthonous extirpated population was not known. To clarify which subspecies was present before reintroduction, we genotyped four male chamois skulls originating from Velebit Mountain, collected around 25 years before the population local extinction. DNA was successfully extracted from middle layer and outer sheath of horns. Assignment based on microsatellite loci, using both Bayesian clustering in STRUCTURE (with q values between 0.55 and 0.73) and DAPC (with individual membership probabilities of 0.99 and 1.00) indicated higher assessed likelihood for the Alpine subspecies.

Keywords *Rupicapra rupicapra* · Genetic origin · Historical samples · Reintroduction

Introduction

Wildlife introduction outside a species' native range, release of captive-reared individuals, co-occurrence of related domestic animals, or the introduction of related exotic

✉ Elena Buzan
  elena.buzan@famnit.upr.si

1 Department of Plant Breeding, Genetics and Biometrics, Faculty of Agriculture, University of Zagreb, Svetošimunska cesta 25, 10000 Zagreb, Croatia

2 Centre of Excellence for Biodiversity and Molecular Plant Breeding (CoE CroP-BioDiv), Svetošimunska cesta 25, 10000 Zagreb, Croatia

3 Department of Biodiversity, Faculty of Mathematics, Natural Sciences and Information Technologies, University of Primorska, Glagoljaška 8, 6000 Koper, Slovenia

4 Environmental Protection College, Trg mladosti 7, 3320 Velenje, Slovenia

5 Department of Fisheries, Apiculture, Wildlife Management and Special Zoology, Faculty of Agriculture, University of Zagreb, Svetošimunska cesta 25, 10000 Zagreb, Croatia

6 Department for Game Biology, Pathology and Breeding, Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, 10000 Zagreb, Croatia

species—all represent possible sources of genetic pollution and alterations in wild ungulate populations (Carpio et al. 2016; Iacolina et al. 2019). Translocation of ungulates as management tool for reinforcement or restoration of game populations for hunting purposes became a widespread practice in the nineteenth century (Apollonio et al. 2014). At that time, the genetic makeup of autochthonous and translocated populations was not considered. Therefore, translocations have inevitably compromised the genetic integrity of some populations of native European ungulates (Linnell and Zachos 2011), like, for example: red deer (*Cervus elaphus*—Senn and Pemberton 2009; Frantz et al. 2017), roe deer (*Capreolus capreolus*—Olano-Marin et al. 2014; Biosa et al. 2015), and chamois (*Rupicapra* spp.—Crestanello et al. 2009; Zemanová et al. 2015; Šprem and Buzan 2016). In the case of the chamois, translocations of individuals for hunting purposes greatly increases the risk of losing differentiated gene pools and can lead to genetic extinction of some taxa (Corlatti et al. 2011).

Contrary to some other ungulates' species chamois was not subject to large scale (re)introduction operations to support full recovery of species historical range. It was mainly managed under some local efforts aiming to restore autochthonous populations on isolated mountain chains

(Apollonio et al. 2014). Historical data and archaeological research confirmed the past great abundance of chamois in the wider Dinaric region (Knežević 1938; Miracle and Sturdy 1991) but, at the beginning of twentieth century, due to predation, natural events and unsustainable hunting, this population was extirpated before its taxonomic classification was assessed (Buzan et al. 2013; Šprem and Buzan 2016). The last recorded sighting of autochthonous chamois in the Velebit Mtn, part of the northern Dinaric Mts, dates back to 1907, when several animals were observed (Skorup 2005). After a few decades from the local extinction, the chamois population was newly established by a couple of reintroduction actions between 1964 and 1978. Individuals used for reintroduction originated from two Northern chamois subspecies (Alpine chamois—*Rupicapra rupicapra rupicapra* and Balkan chamois *Rupicapra rupicapra balcanica*) and diverse neighbouring areas (Šprem et al. 2015). In total, ten animals (5 F, 5 M) of the Balkan subspecies from the Prenj Mtn in Bosnia and Herzegovina and five animals (3 F, 2 M) of the Alpine subspecies from the Kamnik Alps in Slovenia were translocated to the Velebit Mtn (Frković 1981; Skorup 2005; Frković 2008). So, today, historical samples represent the only source of information on the taxonomic status of the autochthonous northern Dinaric chamois population on Velebit Mtn.

Molecular forensic methods, which include multilocus genotyping of historical samples and statistical tools called assignment tests are being increasingly helpful in wildlife management and conservation (Manel et al. 2005; Frantz et al. 2006). DNA isolated from historical samples can be used for the genetic reconstruction of populations origin or even to determine the source population of a single individual (Wandeler et al. 2007; Lister et al. 2011; Polanc et al. 2012; Dufresnes et al. 2019). Additionally, historic information, based on genetic data, are important resources for the choice of the most suitable individuals in reintroduction or translocation actions (Paplinska et al. 2011; Apollonio et al. 2014). However, genetic analysis of historical samples presents many challenges, including the selection of an appropriate sampling method, which will provide optimal quality and quantity of DNA, but which, at the same time, will not damage the sample/museum piece (Casas-Marce et al. 2010; Burrell et al. 2015). For cervids and bovids, antlers and horns were identified as suitable source of DNA for such analyses (Hoffmann and Griebeler 2013; Buzan et al. in preparation). For bovids, the middle layer between the bone core and outer sheath of the horn can be sampled without significantly damaging the horn (Jing et al. 2015). DNA isolated from these sources can be successfully genotyped and used to determine taxonomic status of sampled animals (Woods et al. 2017; Dufresnes et al. 2019), and to identify impacts of past (re)introductions on genetic variability and its geographic distribution (King and Burke 1999; Apollonio et al. 2014).

Assignment tests attempt to 'assign' multi-locus genotypes of individuals to their population of origin, based on the expected probabilities of that specific genotype occurring in each of the potential sources (Manel et al. 2005). A standard approach is to compute a discriminant function based on the expected genotypic frequency distribution under the assumption of Hardy–Weinberg and linkage equilibrium in each source population and then classify unknowns to the group with the highest discriminant score (Manel et al. 2005). This methodology has shown high efficiency in wildlife forensics (Ogden and Linacre 2015), as for example: to detect a fraud in a fishing competition in Finland (Primmer et al. 2000), to confirm illegal translocation of red deer (Frantz et al. 2006), to confirm poaching of the protected Sardinian mouflon (*Ovis orientalis musimon*) (Lorenzini et al. 2011).

In this study we aim to determine the taxonomic status of chamois in the northern Dinaric Mts prior to their extirpation and reintroductions, using DNA isolated from museum samples and microsatellite markers.

Material and methods

Four skulls of male chamois from northern Dinaric Mts—Velebit Mtn (N 44° 57′ 55″, E 15° 00′ 04″), stored in the trophy collection of the Croatian hunting association's Museum and Hunting club "Jarebica-Senj", were the only available samples with certified origin location and time from the autochthonous northern Dinaric population. Museum samples were collected before reintroductions, in years: 1886, 1893, 1895, and 1939.

Samples were handled in a clean room facility to avoid contamination. From two skulls, the outer sheath of the horn could not be removed, so bones were drilled with a dentist drill at 10,000 rpm to produce up to 400 mg of bone powder. For the other two samples, bone tissue from the middle layer of horns was grounded into powder with a mortar and pestle. DNA was extracted from bone powder using QIAamp DNA micro Kit (Qiagen) and EDTA followed by binding to silica to produce 100 μL of DNA extract (Adler et al. 2011). Detailed protocol is described in Buzan et al. (in preparation). Each sample was isolated at least three times and separately amplified.

Genotyping

We genotyped four museum samples with 20 microsatellites, grouped in three multiplex sets, containing six or seven loci, using the protocol described in Zemanová et al. (2011). If the multiplex was unsuccessful, we repeated the analysis

with single locus PCR. Multiplex and single locus PCR were performed using the Qiagen Multiplex PCR Kit. Each reaction contained 5 µL of Multiplex PCR Master Mix, 1 µL of Q-Solution, primers (forward fluorescently labelled) with concentrations between 0.1 and 0.8 µM, 1 µL of extracted DNA and ddH$_2$O to a volume of 10 µL. Fragment analysis of all samples was performed on an ABI 3130 Genetic Analyser (Life Technologies) using a LIZ500 Size Standard (Life Technologies) and separately to confirm replicability also on SeqStudio genetic analyser. Microsatellite genotypes were examined using GeneMapper software v.3.7 (Life Technologies).

Data analysis

Assignment of the four museum samples to a subspecies of origin, based on microsatellite genotypes, was performed using Bayesian model-based clustering implemented in STRUCTURE 2.3 (Pritchard et al. 2000) and discriminant analysis of principal components (DAPC, Jombart et al. 2010) implemented in R package adegenet (Jombart 2008). For both analyses, we used microsatellite genotypes previously published by Šprem and Buzan (2016) as a reference sample group for each subspecies. For the reference populations, we retained only genotypes with estimated membership coefficients (q) to one of the subspecies > 85%. This selection criteria left us with 20 genotypes assigned to the Alpine and 32 to the Balkan subspecies.

STRUCTURE analysis was performed using admixture model with correlated allele frequencies, population information (Alpine, Balkan or unknown) and selected the option to update allele frequencies using only individuals with known subspecies. Ten independent runs with 10^6

Markov-chain Monte Carlo (MCMC) iterations after a burn-in period of 10^5 iterations, and number of populations (K) set to 2. The run with the highest loglikelihood value was selected as the best assignment, and q values for each individual were then plotted using strataG package (Archer et al. 2016) in R 3.3.2 (R Core Team 2016).

In DAPC analysis, we used 52 genotypes of individuals with known origin (previously described reference samples) to estimate the discriminant function. The data were first transformed using principal component analysis, and the discriminant function was estimated from 20 retained principal components. We then used estimated discriminant coefficients to predict the scores of the discriminant functions for the unassigned genotypes (museum samples) and estimate the membership probabilities for each museum sample in each of the subspecies.

Results and discussion

Three out of four museum samples were successfully amplified for all 20 microsatellite loci, while for the fourth sample we were able to obtain the alleles at 16 microsatellite loci. Re-screening with SeqStudio genetic analyser showed lower rates of allelic dropout in longer alleles compared to ABI 3130 Genetic Analyser. Scoring patterns between machines were consistent at all loci.

STRUCTURE analysis detected higher proportions of Alpine chamois genome, with individual membership values ranging from 0.55 to 0.73 in all four samples (Fig. 1). While these proportions suggest assignment of all museum samples to Alpine subspecies rather than to the Balkan one, the support for this result (in terms of q-values) is not conclusive.

Fig. 1 Result from STRUCTURE analysis. Each bar represents one individual. Proportions of grey (*Alpine* subsp.) and black (*Balkan* subsp.) represent the contribution of each subspecies to each individual's genome.
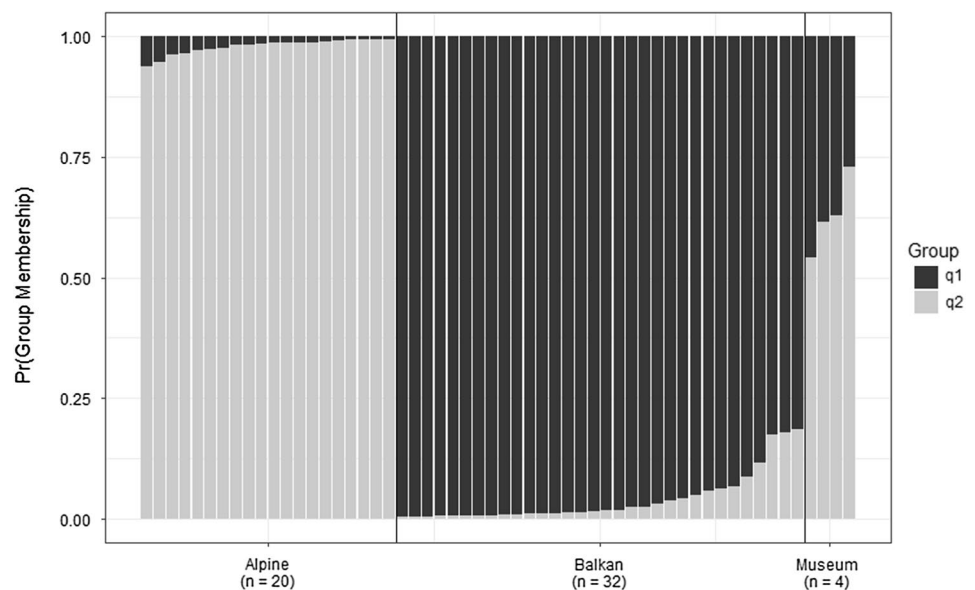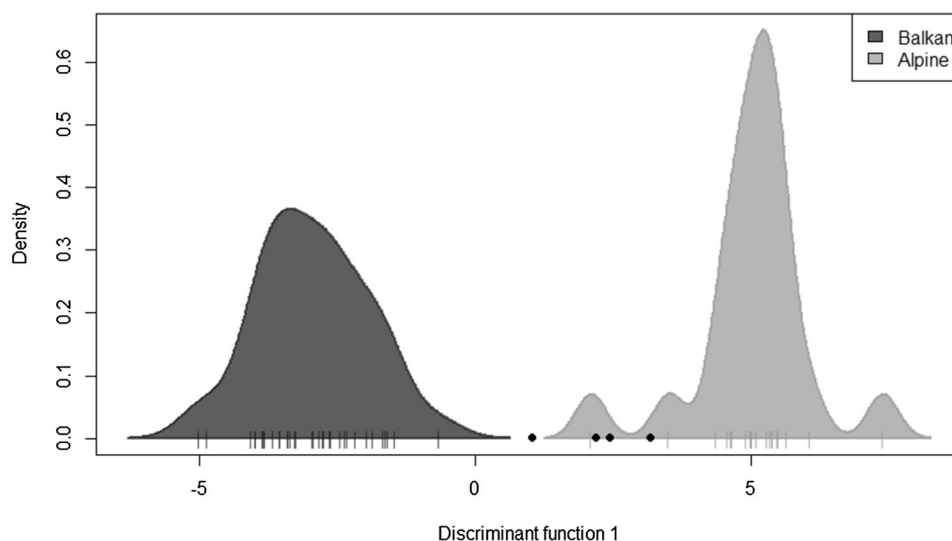
Fig. 2 Discriminant function densities for individuals from reference populations. Grey lines represent discriminant function scores for reference individuals. Circles represent discriminant function scores for the four museum samples.

Membership probabilities obtained by DAPC analysis were 1.00 for the first three museum samples, and 0.99 for the fourth sample, all in favour of belonging to the Alpine subspecies. The discrimination between reference individuals from different subspecies is visible on the plot of the densities of individuals on the single discriminant function, and discriminant function scores for all museum samples illustrate their assignment into Alpine subspecies (Fig. 2). While this result seems more certain than STRUCTURE q-values, it is important to stress that DAPC does not include any estimates of uncertainty for membership probabilities. Discriminant function scores for the three museum samples with complete genotypes were higher (2.44–3.16) than the score of a single reference individual from the Alpine subspecies (2.10), while the fourth museum sample had a score (1.03) lower than all Alpine individuals.

Several factors might have contributed to this lack of resolution in the results of both analyses. In the wide study of the evolutionary history of chamois published by Rodríguez et al. (2010) a Neighbour-Joining tree based on microsatellite allele-sharing distances between 179 individuals from all *Rupicapra* subspecies revealed two main clades corresponding to the Iberian chamois and the Eastern chamois. Most of Balkan and Alpine individuals belonged to different subclades in Eastern lineages without a clear-cut between them. On the other hand, their STRUCTURE results for K = 3 showed that individuals from Alpine and Balkan subspecies grouped in the same genetic cluster, likely sharing the same evolutionary history. Higher orders of structure (K = 7–9) showed a differentiation between populations of the ten currently recognized subspecies, though differences were not always clear-cut. They also indicate that microsatellite differentiation seems to be more closely related to morphological variation than mitochondrial DNA is. This is evidence that microsatellites are better markers for recent

geographical population differentiation, but still in some cases they are not sufficient to detect local rates of gene introgression (low genetic differentiation) which, can then be underestimated (Oliveira et al. 2008). Šprem and Buzan (2016) reported that the average F_{ST} value between all analysed chamois population in the Dinaric Mts was 0.103. This relatively low genetic differentiation between reference subspecies presents an additional problem for the assignment methods we applied (Ogden and Linacre 2015).

Furthermore, on the premise that temporal variance in neutral genetic allele frequencies, and therefore the amount of random genetic drift, is inversely proportional to the effective population size, we would expect direct consequences of past population decline and extirpation on the genetic patterns of historical populations (Waples 2005; Luikart et al. 2010). Our reference and museum samples come from different populations with different effective population sizes and more than 100 years apart, which could be enough for allele frequencies to change due to admixture and drift.

It has to be kept in mind that STRUCTURE attempts to fit the parameters of the model as best as it can to explain the patterns observed in the data. So, even though the "true" population of origin of the museum samples was not sampled, STRUCTURE would still have to show one of the sampled subspecies as the most likely source population (Cornuet et al. 1999) regardless of its biological ancestry.

Finally, even with all described uncertainties, our results still provide a valuable indication on the most likely genetic and taxonomic composition of the autochthonous chamois population. Such information is valuable for the management and conservation implications of the population.

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